

Published in final edited form as:

J Biol Chem. 2004 June 25; 279(26): 27246–27256. doi:10.1074/jbc.M311039200.

## Trypanothione S-transferase activity in a trypanosomatid ribosomal elongation factor 1B

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### SUMMARY

Trypanothione is a thiol unique to the Kinetoplastida and has been shown to be a vital component of their antioxidant defences. However, little is known as to the role of trypanothione in xenobiotic metabolism. A trypanothione S-transferase activity was detected in extracts of *Leishmania major*, *L. infantum*, *L. tarentolae*, *Trypanosoma brucei* and *Crithidia fasciculata*, but not *Trypanosoma cruzi*. No glutathione S-transferase activity was detected in any of these parasites. Trypanothione S-transferase was purified from *C. fasciculata* and shown to be a hexadecameric complex of three subunits with a relative molecular mass of 650,000. This enzyme complex was specific for the thiols trypanothione and glutathionylspermidine, and only used 1-chloro-2,4- dinitrobenzene from a range of glutathione S-transferases substrates. Peptide sequencing revealed that the three components were the alpha, beta and gamma subunits of ribosomal eukaryotic elongation factor 1B (eEF1B). Partial dissociation of the complex suggested that the S-transferase activity was associated with the gamma subunit. Moreover, Cibacron blue was found to be a tight-binding inhibitor and reactive blue 4 an irreversible time-dependent inhibitor that covalently modified only the gamma subunit. The rate of inactivation by reactive blue 4 was increased more than 600-fold in the presence of trypanothione and Cibacron blue protected the enzyme from inactivation by 1-chloro-2,4- dinitrobenzene, confirming that these dyes interact with the active site region. Two eEF1B $\gamma$  genes were cloned from *C. fasciculata* but recombinant *C. fasciculata* eEF1B $\gamma$  had no S-transferase activity, suggesting that eEF1B $\gamma$  is unstable in the absence of the other subunits.

### INTRODUCTION

Infections with parasitic protozoa of the order Kinetoplastida are a common cause of serious illness and death in the tropics. *Trypanosoma brucei* spp. cause sleeping sickness in Africa, *Trypanosoma cruzi* is the cause of Chagas' disease in South America and infections with *Leishmania* spp. produce a variety of pathologies termed the leishmaniases in Asia, Africa, South America and Europe. In general, the chemotherapy of these diseases is poor, with the available drugs suffering various drawbacks such as toxicity, limited efficacy and drug resistance (1;2). For instance, the majority of cases of visceral leishmaniasis in India do not respond to antimonials, which are the first-line drugs (3). A promising target for the design of new drugs to treat these illnesses involves thiol metabolism. In these parasites, this depends upon trypanothione (T[SH]<sub>2</sub> or N<sup>1</sup>,N<sup>8</sup>-bis(glutathionyl)- spermidine) (4), in contrast to most other organisms (including their mammalian hosts) which use glutathione as the unmodified tripeptide (Figure 1) (5). The main function of trypanothione is the maintenance

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The nucleotide sequences of the clones reported in this paper have been deposited in the Genbank<sup>TM</sup>/EBI Data Bank under the accession numbers AY545587 (RT-PCR clone of *C. fasciculata* eEF1B $\gamma$  and AY545588, full-length PCR clone of *C. fasciculata* eEF1B $\gamma$ ).

of cellular redox state and consequently, studies have concentrated on its role as an antioxidant (6).

Another major function for cellular thiols is the detoxification of xenobiotics, i.e. chemicals that are foreign to a particular organism. These include naturally occurring compounds, industrial chemicals, drugs, herbicides and pesticides. Hydrophobic xenobiotics can readily diffuse into cells and are usually eliminated by Phase I and Phase II biotransformation reactions. These produce generally less reactive and more polar compounds, which can be actively extruded from the cytosol. An important group of Phase II reactions are catalysed by glutathione *S*-transferases (GSTs) and involve conjugation with glutathione. (7). GSTs have been purified from a wide range of organisms and are almost invariably dimers of subunits with masses of approximately 25 kDa. In any one organism, many different GST isozymes are usually expressed simultaneously, with these isozymes having wide and overlapping substrate specificities. Such a broad specificity system assists an organism in the metabolism of the multitude of different reactive xenobiotics to which it may be exposed. In addition, GSTs are also important in the detoxification of endogenous reactive chemical species produced during oxidative stress, such as lipid hydroperoxides (8) or reactive aldehydes (9). The GSTs therefore have a central role in detoxification metabolism and the overexpression of these enzymes is a common mechanism of drug resistance (10).

Although the classical GSTs have been well characterised, the GST fold is also found in functionally unrelated proteins such as plant stress-induced proteins (11),  $\beta$ -etherases (12), ion channels (13) and the eukaryotic translation elongation factor 1B gamma (eEF1B $\gamma$ ) (14;15). The functions of the GST domain in these proteins remain poorly understood, although the eEF1B $\gamma$  from rice has recently been shown to possess a GST activity (16). This result was surprising, as eEF1B was previously thought only to be involved in protein synthesis and no enzymatic activity had been proposed for the eEF1B $\gamma$  subunit. Previously, the other two subunits (eEF1B $\alpha$  and  $\beta$ ) of eEF1B were shown to function to recycle elongation factor 1A (eEF1A) complexed with GDP back to the active eEF1A.GTP form (17;18). This eEF1A.GTP complex is then able to bind to an amino-acyl tRNA, forming a ternary complex that is able to enter the A-site of the ribosome, with the hydrolysis of GTP.

In contrast to the wealth of information on GSTs and related proteins in other organisms, little is known about thiol-xenobiotic conjugation in trypanosomes. Although there has been one report of low levels of GST activity in *T. cruzi* (19), this has been disputed (20). In addition, no GST activities have been reported in the related *Leishmania spp* or *T. brucei*. However, since the major low molecular mass thiol in these organisms is trypanothione, it has been proposed that these trypanosomatids instead possess a trypanothione *S*-transferase or TST (5). This activity may also be involved in the resistance of *Leishmania spp.* to the metalloid antimony (21), which is currently front-line drug for the treatment of leishmaniasis. It has been found that the acquisition of high-level antimony resistance in *L. tarentolae* requires the overproduction of trypanothione, the overexpression of metal-thiol conjugate transporters and a third unidentified trypanothione-dependent factor (22). This factor was postulated to be a TST activity involved in the formation of an antimony-trypanothione conjugate. Indeed, in an analogous system, the mechanism of resistance of mammalian cells to arsenite involves the efflux of the metalloid, which is thought to be facilitated by the overexpression of a GST-pi (23;24).

Here, we report the detection of TST activities in trypanosomatids and the purification and characterisation of the *Crithidia fasciculata* TST. This is identified as the ribosomal elongation factor eEF1B complex and the subunit containing the TST active site defined as eEF1B $\gamma$ .

## EXPERIMENTAL PROCEDURES

### Materials

All reagents were standard commercial products and of the highest available purity. Trypanothione and glutathionylspermidine were obtained from Bachem. Glutathione affinity agaroses were obtained from Sigma, all other chromatographic resins and columns were from Amersham Pharmacia Biotech. Rat liver GST was purchased as a mixture of isoforms from Sigma.

### Cell culture

*C. fasciculata* choanomastigotes *T. cruzi* epimastigotes *T. brucei* bloodstream form and *L. major*, *L. infantum* and *L. tarentolae* promastigotes were grown as described (25;26).

### Enzyme assays

GST was assayed at 25°C in 100 mM (Na<sup>+</sup>) phosphate pH 6.5 with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM GSH as substrates. The rate of formation of the glutathione *S*-dinitrobenzene conjugate was followed at 340 nm, using the published extinction coefficient of 9.6 mM cm<sup>-1</sup> (27). TST activity was measured under identical conditions with T[SH]<sub>2</sub> and CDNB being added to 400 μM. Assay reactions were initiated with thiol, which was produced immediately before addition to assays by mixing trypanothione disulphide (T[S]<sub>2</sub>) with a 2-fold excess of tris(2-carboxyethyl)phosphine (TCEP) and a five-fold excess of NaOH. The absorbance coefficient for the T[SDNB]<sub>2</sub> conjugate was measured by allowing assays to proceed to completion and found to be 9.2 mM<sup>-1</sup>cm<sup>-1</sup> per mM of sulphhydryl group. One unit of TST activity corresponds to one micromole of sulphhydryl group conjugated per minute.

Where necessary, in TST assays with alternative electrophilic substrates, the pH and substrate concentrations given for the corresponding GST assay (27) were altered to minimise the rate of the spontaneous reaction. The activities with these substrates were calculated using the absorbance coefficients for the corresponding GSH conjugates. In the case of the peroxidase, dehydroascorbate reductase and thioltransferase assays, TCEP was omitted and T[S] was reduced by 10 μg ml<sup>-1</sup> 2 trypanothione reductase and 540 μM NADPH, before addition of the second substrate.

For enzyme purification, aliquots of fractions were screened for TST activity using a Molecular Devices Thermomax plate reader. In this assay, samples were diluted in the plate with water to give a final volume of 50 μl and the reactions initiated by the addition of 200 μl of a mixture containing buffer, dithiothreitol (DTT) and substrates. The final assay mixture contained 800 μM CDNB, 1 mM DTT, 50 μM T[S]<sub>2</sub> and 100 mM (Na<sup>+</sup>) phosphate pH 6.5. The average rate of two blank assays was subtracted from these rates. Protein concentrations were measured using the procedure of Bradford (28), using BSA as the analytical standard. All trypanosomatid extracts were produced as described below for *C. fasciculata* and mouse liver was extracted by disruption in a Dounce homogeniser and then processed as for *C. fasciculata* cells.

### Purification of the *C. fasciculata* TST

Unless otherwise stated, all the steps in the following purification were performed at 4°C. Frozen *C. fasciculata* cell pellets were thawed on ice and resuspended in an equal volume of ice-cold lysis buffer, giving in the final mixture 75 mM (Na<sup>+</sup>) phosphate pH 7.2, 2 mM DTT, 1 mM EDTA, 1 mM benzamidine, 1 mM phenanthroline, 3 μg ml<sup>-1</sup> leupeptin, 250 μM 4-(2-aminoethyl)benzenesulphonyl fluoride and 1 μM pepstatin A. The cells were lysed by sonication, (4 × 30 s pulses at 20 microns amplitude from a 19 mm end-diameter probe)

in a Sanyo Soniprep 150 sonicator with cooling to  $<4^{\circ}\text{C}$  in an ice-salt bath between pulses. After centrifugation for 80 min at  $40,000 \times g$  the resulting supernatant was brought to a final concentration of 4% (w/v) polyethylene glycol 6000 (PEG) over 5 min, by the dropwise addition of PEG from a 50% (w/v) stock, stirred for 30 min and centrifuged for 30 min at  $30,000 \times g$ . The supernatant was adjusted to 9% (w/v) PEG and centrifuged as before. The resulting pellet was resuspended in 150 ml of buffer A (25 mM ( $\text{Na}^+$ ) Bis-tris, 1 mM EDTA, 1 mM DTT, pH 6.5) and then centrifuged to remove insoluble material.

The clarified supernatant was applied at  $2 \text{ ml min}^{-1}$  to a 100 ml ( $19 \times 2.6 \text{ cm}$ ) Pharmacia Q-sepharose anion exchange column equilibrated in buffer A. After washing with 2 column volumes of buffer A, bound proteins were eluted at  $1 \text{ ml min}^{-1}$  with a linear gradient of 200-500 mM NaCl in the same buffer and active fractions pooled.

Following overnight dialysis against two litres of 550 mM  $(\text{NH}_4)_2\text{SO}_4$  in buffer B (25 mM ( $\text{Na}^+$ ) HEPES, 1 mM EDTA, 1 mM DTT, pH 7), the sample was applied at  $2 \text{ ml min}^{-1}$  to a 85 ml ( $16 \times 2.6 \text{ cm}$ ) Pharmacia phenyl sepharose (low substitution) column equilibrated with 550 mM  $(\text{NH}_4)_2\text{SO}_4$  in buffer B. The column was washed with 5 column volumes of the same buffer and TST activity eluted with 250 ml buffer B.

Active fractions were pooled and concentrated to 1 ml by vacuum ultrafiltration in a Sartorius collodion bag. The concentrated TST was applied to a 319 ml ( $2.6 \times 60 \text{ cm}$ ) Superdex 200 26/60 size-exclusion column equilibrated with buffer C (50 mM ( $\text{Na}^+$ ) HEPES, 300 mM NaCl, 0.01% (w/v) sodium azide, pH 7.5) and eluted at a flow rate of  $2 \text{ ml min}^{-1}$ . Fractions with TST activity were pooled and concentrated as before.

### Analytical chromatography

Analytical size-exclusion chromatography was carried out using a 24 ml ( $1 \times 30 \text{ cm}$ ) Superdex 200 HR 10/30 size-exclusion column equilibrated with buffer C, with separations performed at a flow rate of  $0.5 \text{ ml min}^{-1}$ . This procedure was also used to calculate the relative molecular mass ( $M_r$ ) of proteins, using carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa),  $\beta$ -amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa) as analytical standards.

Analytical anion-exchange chromatography was carried out using a 6 ml ( $1.6 \times 3 \text{ cm}$ ) Pharmacia Resource Q column under the same buffer conditions as the Q-sepharose step in the TST purification scheme. The sample was applied at  $1 \text{ ml min}^{-1}$  and the column washed with 2 column volumes of buffer. The bound proteins were then eluted with a complex gradient which went from 0- 200 mM NaCl in 6 ml, 200-500 mM NaCl in 60 ml and then 500-1000 mM NaCl in 6 ml, all in buffer A.

### Analysis of products of TST reaction

Assays for mass-spectrometric analysis were performed in 200 mM ammonium acetate pH 6.5 with the other conditions as before. The reactions were followed at 340 nm and after 30 min 2-hydroxyethyl disulphide was added to 10 mM, oxidising the  $\text{T}[\text{SH}]_2$  by disulphide exchange and thus quenching the trypanothione reaction. The products were then lyophilised in glass vials, re-dissolved in water and diluted 1:500 with 1:1 acetonitrile and water. Samples were analysed on a Micromass Ultima electrospray mass spectrometer in positive mode. Several scans were combined, background subtracted and smoothed to produce the final spectra.

## Cross-linking analysis

All samples and controls were dialysed overnight against 55 mM (Na<sup>+</sup>) phosphate pH 7.5 reaction buffer, protein samples were then diluted to 1 mg ml<sup>-1</sup> in reaction buffer. The amine-reactive homobifunctional reagent bis(sulphosuccinimidyl)suberate (BS<sup>3</sup>) was added, from a freshly prepared 10 mM stock in 5 mM sodium succinate pH 5, to the required final concentration. The reactions were incubated at room temperature for 40 min and then quenched by the addition of ethanolamine to a final concentration of 5 mM. Aldolase was used as a positive control and BSA and carbonic anhydrase as negative controls. Samples from the reactions were then analysed by using either 10 % SDS-PAGE gels (29), to examine complexes less than 150 kDa or 6 % Weber SDS-PAGE (30) to examine complexes greater than 150 kDa.

## Dissociation of complex

For thiocyanate disassociation, samples of TST in buffer C were incubated on ice with 2 mM DTT for 3 h in the presence or absence of 1.5 M NaSCN. The proteins were then applied to a Superdex 200 HR 10/30 column equilibrated with either buffer C or this buffer plus 500 mM NaSCN, and eluted at 0.5 ml min<sup>-1</sup>. Since NaSCN inhibits the TST assay, 200 µl samples from each column fraction were dialysed against TST assay buffer (100 mM (Na<sup>+</sup>) phosphate, pH 6.5), before analysis by enzyme assay and SDS-PAGE.

## Inhibition and chemical modification of TST

Modification with *N*-ethylmaleimide (NEM) was carried out in a 80 µl volume at room temperature in buffer C. Samples of TST (16 µg, 61 mU) were incubated with 100 µM DTT for 5 min and then NEM added to a final concentration of 400 µM. After a 15-min incubation the reactions were quenched by the addition of DTT to 625 µM.

Inhibition of TST by Cibacron blue F3G-A was determined under standard assay conditions, in the presence or absence of 0.5% (w/v) BSA. Routinely, TST was preincubated with the inhibitor but no decrease in inhibition was observed when assays were initiated with enzyme. Dye concentrations were determined spectrophotometrically, using the absorbance coefficients of  $\epsilon_{\text{mM}} = 11.6$  (622 nm) for Cibacron blue (31) and  $\epsilon_{\text{mM}} = 4.2$  (610 nm) for reactive blue 4 (32).

The rate of time-dependent inhibition of TST by chlorotriazine dyes was measured in reaction mixtures of 100 µl containing 70 mU (18 µg) samples of TST and varying concentrations of dye in 20 mM (Na<sup>+</sup>) phosphate pH 7.5. The reactions were allowed to proceed at room temperature, with 5-µl samples being removed at intervals to determine residual TST activity. These samples were assayed by 100-fold dilution into assay mixtures containing 0.5% (w/v) BSA, to prevent inhibition by any unreacted dye. Incubations with CDNB were performed under identical conditions and samples assayed in standard assay conditions. T[SH]<sub>2</sub> and GSH were generated in incubations by the addition of 600 µM NADPH, 1 mU TryR or glutathione reductase and 500 µM T[S]<sub>2</sub> or GSSG. The resulting data were fitted by non-linear regression analysis to the single exponential decay function and expressed as a percentage of the activity at zero time.

Samples of proteins for mass spectrometry were desalted by dialysis against one litre of water for 3 h at 4°C, before analysis by MALDI (matrix-assisted laser desorption and ionisation) mass spectrometry on a PerSeptive Biosystems Voyager-DE STR mass spectrometer.

## Cloning of eEF1B $\gamma$ genes from *C. fasciculata*

A partial clone of the *C. fasciculata* eEF1B $\gamma$  was obtained by RT-PCR from cDNA prepared using the Cells-to-cDNA kit (Ambion). The sense primer (5'-CGCTATATAAGTATCAGTTTCTGTA-3') contained 25 nucleotides of the mini-exon sequence that is trans-spliced onto the 5' end of all trypanosomatid mRNA transcripts (33;34). The degenerate antisense primer (5'-CCYTCCCANGCNAGGTAKTC-3') was designed to the tryptic peptide ITDYLA(F/W)EGPTIPLPV. PCR was performed in 50  $\mu$ l volume reactions containing template cDNA, 20 ng ml<sup>-1</sup> sense and antisense primers, 250  $\mu$ M dNTPs, 5 U *Taq* polymerase (Promega) and *Taq* buffer plus 1.5 mM MgCl<sub>2</sub>. The reactions were placed in a thermocycler that had been preheated to 95°C and subjected to the following: 95°C for 5 min 30 cycles of 95°C for 1 min 55°C for 1 min 72°C for 2 min and finally the reactions were heated to 72°C for 10 min. PCR products were then cloned into the pCR-Blunt II-TOPO plasmid using the Zero Blunt TOPO PCR cloning kit (Invitrogen).

Southern blotting was carried out by the standard capillary transfer method (35), using the RT-PCR clone of eEF1B $\gamma$  as a probe. The probe was labelled using the Gene Images labelling kit and hybridisation detected with the Gene Images dioxetane detection module (Amersham Biosciences).

PCR amplification of *C. fasciculata* eEF1B $\gamma$  intergenic regions was carried out using sense (5'-GGAGCTGTTTGACTGGGAGGAGAT-3') and antisense primers (5'-GAAGTCCGCCGTCTC-GTTGTC-3') as described above, with the substitution of *Pfu* polymerase and *Pfu* buffer (Promega) for *Taq* polymerase. Four intergenic regions were amplified, cloned and sequenced. Multiple primers were then designed to their 5' and 3' regions. One pair of these primers (sense 5'-GCACCGGCGTACCTGATGACTT-3' and antisense 5'-TTAGTGGCCACTGATGCGACAGC-3') produced a product that was cloned, sequenced and identified as an eEF1B $\gamma$  gene. This gene was then cloned into the expression vector pET15b (Novagen) and recombinant protein expressed and purified according to the manufacturer's instructions.

## Nomenclature

The terminology of eukaryotic elongation factors is somewhat confusing. In this paper we have used the IUBMB nomenclature recommendations (<http://www.chem.qmul.ac.uk/iubmb/misc/trans.html>), with the elongation factor 1B complex, previously named eEF-1 $\beta\gamma\delta$  being referred to as eEF1B. The complex's subunits are, in order of increasing size, elongation factor 1B alpha (previously eEF-1 $\beta$ ), now referred to as eEF1B $\alpha$  elongation factor 1B beta (previously eEF-1 $\delta$ ) referred to as eEF1B $\beta$  and elongation factor 1B gamma (previously eEF-1 $\gamma$ ) referred to as eEF1B $\gamma$ . The SWISS-PROT identifier is used when referring to previously characterised elongation factors.

## RESULTS

### Detection and initial characterisation of trypanothione S-transferase activities in trypanosomatids

Clarified extracts of various organisms and cell types were assayed for S-transferase activities (Table 1). *L. tarentolae* (a lizard parasite) was included since this organism has been frequently used in studies on *Leishmania* antimony resistance. Mouse liver extract was assayed as a positive control for the GST assay and trypanothione reductase activity was used to confirm adequate extraction of the parasites. No GST activity was detected in the trypanosomatids. Instead a trypanothione S-transferase (TST) activity was detected in *C. fasciculata*, all the *Leishmania* spp. and *T. brucei*. The TST activity in mouse liver extracts is probably due to the ability of GSTs to use T[SH]<sub>2</sub> as an alternative substrate, although with

a specific activity 100-fold less than that given with GSH. Since TST activity was highest in *C. fasciculata* and *L. major*, these activities were further characterised. Activity in these extracts was proportional to the amount of protein added, heat labile, and, when analysed by size-exclusion chromatography the activity eluted close to the void volume of the column, showing  $M_r$  values greater than 400,000 (data not shown). This is in contrast to the GST activity in the mouse liver extract, which eluted with an  $M_r$  of 45,000 (this value being identical to the reported  $M_r$  of the three major mouse glutathione *S*-transferases) (36).

A pilot study showed that *C. fasciculata* TST activity was not retained on S-hexylglutathione, glutathione disulfide or glutathione coupled to epoxy-activated agarose in contrast to the mouse liver GSTs, which were completely retained on S-hexylglutathione agarose (data not shown).

### Purification of *C. fasciculata* TST

Since *C. fasciculata* had the highest TST activity, it was used in further studies. The *C. fasciculata* TST was concentrated by PEG 6000 precipitation and then purified to constant specific activity by a combination of anion-exchange, hydrophobic-interaction and size-exclusion chromatography (Table 2). Typically, TST activity can be purified 200-fold with about 10% recovery. Surprisingly, when the purification fractions were analysed by SDS-PAGE, three major polypeptides of mass 31, 38 and 45 kDa and one minor polypeptide of 65 kDa were visible. These proteins did not alter in relative abundance in the last two purification steps (Figure 2). In order to determine if these polypeptides were associated through disulphide bonds, samples of the final preparation were separated in the presence and absence of DTT. No association between the four polypeptides was seen in the oxidised sample, showing that any complex is formed by non-covalent interactions.

As a further test of purity, samples of purified TST were applied to high-resolution anion-exchange and size-exclusion columns. The TST activity again eluted as a single peak from both columns, with no increase in the specific activity of the recovered enzyme. The chromatogram of the size-exclusion separation (Figure 3) shows a single major peak making up >95% of the total protein, with minor amounts of higher and lower molecular mass contaminants. The TST activity co-elutes with the main peak, which contains the four polypeptides that were visible in the last stage of the purification procedure in an unaltered stoichiometry. This stoichiometry was investigated by image analysis of multiple preparations of TST, showing a constant ratio between the 31 ( $\alpha$ ), 36 ( $\beta$ ) and 45 kDa ( $\gamma$ ) subunits of 1:1:2, respectively. In addition, the  $M_r$  value of 650,000 calculated for this species is consistent with the high molecular mass activities seen in the *C. fasciculata* and *L. major* crude extracts.

### Peptide sequencing and identification of TST subunits

The proteins in a sample of purified TST were separated by SDS-PAGE, digested in the gel with trypsin and the resulting peptides separated by reversed-phase HPLC. Selected peptides were Edman sequenced and the sequences used in database searches. These identified p31 (subunit  $\alpha$ ) to be similar to the 25 kDa ribosomal elongation factor eEF1Ba from *T. cruzi* (SWISS-PROT P34827). Peptides from p38 (subunit  $\beta$ ) were likewise similar to the *T. cruzi* 30 kDa elongation factor eEF1B $\beta$  (Q26914) and peptides from p45 (subunit  $\gamma$ ) were similar to the 47 kDa eEF1B $\gamma$  from *T. cruzi* (P34715) and *L. infantum* (Q9BHZ6). Peptides from the protein of approximately 65 kDa, which was present in low and variable amounts between different TST preparations, were similar to the sequence of the predicted product of a *L. major* gene ID:LmjF15.0230. This is a 67 kDa protein that shows a high level of identity to amino-acyl tRNA synthetases (49% identity with a hamster lysyl-tRNA synthetase (P37879)). In combination, these data suggest that the *C. fasciculata* TST is an

eEF1B complex, as the mammalian form of this assembly contains the eEF1B $\alpha$ ,  $\beta$  and  $\gamma$  subunits and has also been shown to associate with amino-acyl tRNA synthetases (37).

### Quaternary structure of TST complex

The purified TST preparation contained three major polypeptides and the relative intensities of the protein bands were not altered in the last three purification steps, suggesting that these three proteins associate to form the high molecular mass TST complex. The association of these proteins was therefore investigated by chemical cross-linking. As seen in Figure 4, addition of BS<sup>3</sup> to purified TST results in cross-linking of the eEF1B subunits, producing high molecular mass species (Panel A, lanes 2-4). In the positive control, the tetrameric enzyme aldolase is cross-linked into dimers, trimers and tetramers (Panel A, lane 7) whilst in the negative control, the monomeric proteins, BSA (68 kDa) and carbonic anhydrase (29 kDa) are unaffected (Panel A, lane 6). Significantly, the product formed in the TST cross-linking reaction (Panel B, lane 4) has a mass between 150 and 200 kDa, which is in the range of the 160 kDa mass given by the sum of the SDS-PAGE masses of the  $\alpha$ ,  $\beta$  and  $\gamma$  subunits in a 1:1:2 ratio obtained by image analysis. In addition, 170 kDa is also one quarter of the  $M_r$  given by gel filtration. These results suggest that the three subunits associate in a  $\alpha\beta\gamma_2$  tetramer and then this tetramer itself tetramerises to form a hexadecamer. Indeed the  $(\alpha\beta\gamma_2)_4$  hexadecameric species may have been formed in the cross-linking reaction, because, despite equal amounts of protein being loaded in each lane, the total amount of protein visible in the gel decreases with increasing degrees of cross-linking. This effect may be due to species of more than 400 kDa being unable to enter these gels. These results are in good agreement with previous studies on the mammalian eEF1B, which was shown to be a  $M_r$  670,000 complex containing the eEF1B $\alpha$ ,  $\beta$  and  $\gamma$  subunits in a 1:1:2 ratio (38).

### Catalytic activities of the *C. fasciculata* TST

The enzyme is highly specific for the glutathione conjugates, trypanothione and glutathionylspermidine (GspdSH). It showed only low activity with glutathione ethyl ester (H-Glu(Cys-Gly-OEt)-OH) and no detectable activity with glutathione. In contrast, when the thiol specificity of a purified mixture of rat liver GST isoforms was tested, activity was detected with T[SH]<sub>2</sub> and GSH (data not shown). Kinetic parameters for TST with the three active thiol substrates with a fixed concentration of 400  $\mu$ M CDNB were determined (Table 3). Interestingly, the  $K_m$ (app) for GspdSH is twice that of trypanothione, suggesting that T[SH]<sub>2</sub> is recognised as two independent GspdSH moieties, giving an effective concentration twice its actual concentration. TST showed no conjugation activity ( $<0.01 \mu\text{moles min}^{-1} \text{mg}^{-1}$ ) with *t*-butyl hydroperoxide, cumene hydroperoxide, ethacrynic acid, 1,2-dichloro-4-nitrobenzene, *p*-nitrophenyl acetate, 1,2-epoxy-3-(4-nitrophenoxy)propane, *trans*-2-nonenal, benzyl isothiocyanate, 4-androstene-3,17-dione, butyl nitrate or *trans*-4-phenyl-3-buten-2-one. In addition, no thiol-transferase activity was found with GSSG or 2-hydroxyethyl disulphide as substrates and no dehydroascorbate reductase activity was detected. The only good electrophilic substrate for TST is CDNB (3300 mU  $\text{mg}^{-1}$ ), with the structurally related *p*-nitrobenzyl chloride showing marginal activity (70 mU  $\text{mg}^{-1}$ ).

In order to confirm that the spectrophotometric TST assay is actually measuring substrate conjugation, the products formed by TST in the CDNB/trypanothione assay were compared with both a T[SDNB]<sub>2</sub> standard and the products of a control assay with no added TST. The UV spectrum of the TST product was identical to that of a T[SDNB]<sub>2</sub> standard. Assay reactions performed in volatile buffer in the presence and absence of TST were analysed by electrospray mass spectrometry. This showed that significant amounts of both the bis-( $M + H^+ = 1056.1 \text{ Da}$ ) and the mono-( $M + H^+ = 966 \text{ Da}$ ) adducts with CDNB are only formed in the presence of TST (data not shown).



## Denaturation of the TST complex and separation of subunits

In order to identify which subunit is responsible for the TST activity in the eEF1B complex, samples of TST were partially denatured and the subunits separated and assayed for activity. This was achieved by treatment with the chaotropic agent sodium thiocyanate (NaSCN). TST was incubated on ice in the presence or absence of NaSCN and the proteins then separated by size exclusion chromatography, again in the presence or absence of thiocyanate. As expected, a sample of the eEF1B complex that had not been exposed to NaSCN eluted as a single species (Figure 5, Panel A), with a protein peak in fraction 14 and all the subunits present in a 1:1:2 ( $\alpha$ ,  $\beta$  and  $\gamma$  respectively) ratio (Figure 5, Panel C). In contrast, when the TST was preincubated for 3 h with NaSCN and then chromatographed in the presence of NaSCN, two partially resolved protein species were observed to elute from the column (Figure 5, Panel A). Due to the incomplete separation of these complexes, their subunit compositions cannot be clearly defined. However, when the amounts of each subunit present in these fractions (Figure 5, panel D) is compared to the level of enzyme activity, a clear correlation between the levels of eEF1B $\gamma$  and TST activity can be observed. Significant TST activity is present in fractions 13-17, which corresponds well with the elution profile of eEF1B $\gamma$ , but not that of eEF1B $\alpha$  or  $\beta$ , which elute mainly in fractions 16 and 17.

## Inhibition and affinity labelling of TST

The effects of the modification of protein thiol groups on TST activity was investigated by alkylation of a sample of reduced TST with NEM. TST was inactivated by  $81 \pm 2\%$  upon exposure to  $400 \mu\text{M}$  NEM for 15 min, compared to no detectable loss of activity in a parallel negative control. Mass spectrometry of the NEM-treated TST showed the modification of 1 cysteine residue in the eEF1B $\beta$  subunit and 5 residues in the eEF1B $\gamma$  subunit (data not shown). This result demonstrates that the eEF1B $\alpha$  subunit is not responsible per se for the TST activity of eEF1B and suggests the possible involvement of protein thiol groups in the TST catalytic mechanism. Interestingly, the actual molecular masses of the eEF1B $\alpha$  and  $\beta$  subunits, determined by mass spectrometry, are significantly different from their apparent molecular weights by SDS-PAGE, with the 23 kDa eEF1B $\alpha$  migrating as a 31 kDa protein and the 27 kDa eEF1B $\beta$  migrating with an apparent mass of 38 kDa. These effects have previously been noted for the equivalent mammalian eEF1B subunits, with for example the 25 kDa rabbit eEF1B $\alpha$  (previously designated eEF-1 $\beta$ , P34826) migrating as a 32 kDa protein on SDS-PAGE. This anomalous migration is probably the result of the highly acidic nature of the eEF1B $\alpha$  and  $\beta$  proteins (39).

Cibacron blue F3G-A, a monochloro triazine dye, was found to be an extremely potent inhibitor of the *C. fasciculata* TST, with an  $\text{IC}_{50}$  of  $13 \pm 0.8 \text{ nM}$  (Figure 6). Inhibition constants in this range can indicate an irreversible active-site titration, and indeed no recovery of activity was observed upon prolonged dialysis of Cibacron blue-inhibited TST. However, this result was found to be due to the inability of Cibacron blue to pass through dialysis membranes, possibly as a result of dye aggregation (40). In light of the known affinity of this dye for serum albumin (41), TST inhibition was therefore determined in the presence of 0.5% (w/v) BSA. The addition of this protein caused a large shift in the  $\text{IC}_{50}$  to  $12 \pm 0.9 \mu\text{M}$ , most probably due to the sequestration of the inhibitor from the assay solution (Figure 6). This modified assay allows the accurate determination of TST activity in the presence of micromolar concentrations of Cibacron blue. Using this procedure, samples of TST were incubated with  $100 \mu\text{M}$  Cibacron blue and then samples diluted into assays containing 0.5% (w/v) BSA. No change in TST activity was observed over a 30 min period, indicating that Cibacron blue does not irreversibly inactivate the enzyme (Figure 7, Panel A, inverted triangles).

However, when these experiments were repeated using reactive blue 4, a more reactive dichloro triazine dye, irreversible time-dependent inhibition was observed (Figure 7, Panel A). The inactivation reaction was saturable, with a pseudo-first order rate constant of ( $k_{\text{obs}}$ ) of  $0.08 \pm 0.01 \text{ min}^{-1}$  and a calculated dissociation constant ( $K_{\text{i(app)}}$ ) of  $49 \pm 17 \mu\text{M}$  (Figure 7, Panel B). This indicates that a reversible EI complex is formed as a reaction intermediate and therefore suggests that the inhibitor is binding at or near the TST active site. Duplicate 18  $\mu\text{g}$  samples of TST were incubated at room temperature in the presence and absence of 50  $\mu\text{M}$  reactive blue 4. As expected, the dye completely inactivated the enzyme ( $98 \pm 2 \%$ ). Mass spectrometry of these samples showed no modification of the  $\alpha$  or  $\beta$  subunits, whereas the eEF1B $\gamma$  subunit underwent considerable peak broadening, with a peak shift of  $1,330 \pm 480 \text{ Da}$  (Figure 8). Within the precision of the method, this mass shift would be consistent with the covalent addition of two dye molecules to the eEF1B $\gamma$  subunit.

More evidence as to the location of the triazine dyes' binding site was provided by the interactions between these inhibitors and the substrates T[SH]<sub>2</sub> and CDNB. Incubation of TST with high concentrations of the xenobiotic substrate CDNB, in the absence of T[SH]<sub>2</sub>, causes an irreversible time-dependent inactivation of the enzyme (Figure 9, Panel A). This inactivation can be prevented by addition of either the co-substrate T[SH]<sub>2</sub> or the reversible inhibitor Cibacron blue. The protection produced by Cibacron blue suggests that the association of this inhibitor with the TST active site prevents the binding of CDNB. However, the effect of substrate on the inhibition of TST by reactive blue 4 is strikingly different. When the enzyme is incubated with 50  $\mu\text{M}$  reactive blue 4 and 500  $\mu\text{M}$  T[SH]<sub>2</sub> the rate of inactivation is increased from  $0.045 \pm 0.003 \text{ min}^{-1}$  to  $29 \pm 5 \text{ min}^{-1}$  (Figure 9, Panel B). This corresponds to an over 600-fold increase in rate, with 80% of TST activity being lost in the first 3 seconds of the reaction. This effect is specific to T[SH]<sub>2</sub>, since addition of equal amounts of sulphhydryl group in the form of the dithiol DTT or the monothiol GSH had no significant effect on the rate of inactivation, with  $k_{\text{obs}}$  of  $0.037 \pm 0.005 \text{ min}^{-1}$  and  $0.047 \pm 0.003 \text{ min}^{-1}$ , respectively. The specificity of this effect suggests that T[SH]<sub>2</sub> may be causing a conformational change that increases either the affinity of the enzyme for the inhibitor or the reactivity of an active site residue. In combination, these data therefore strongly suggest that the triazine dyes bind in the active site region of the TST and that this is located within the eEF1B $\gamma$  subunit.

### Cloning of *C. fasciculata* eEF1B $\gamma$ genes

The tryptic peptides produced from the digestion of purified *C. fasciculata* eEF1B $\gamma$  were aligned with the sequence of the *L. infantum* eEF1B $\gamma$  (Q9BHZ6). A peptide with the sequence ITDYLA(F/W)EGPTIPLPV aligned with the C-terminus of this protein and was therefore used to design a degenerate 3'-primer. This allowed the cloning of a partial *C. fasciculata* eEF1B $\gamma$  gene from cDNA by RT-PCR, using the spliced leader sequence as the 5' primer (Figure 10, *C. f.* RT-PCR, and data not shown †). This clone was then used as a probe in a Southern blot analysis of *C. fasciculata* gDNA, which indicated that multiple copies of the eEF1B $\gamma$  gene were present as tandem repeats (data not shown †). Primers were therefore designed to sequences in the 5' and 3' regions of the *L. infantum* eEF1B $\gamma$  that were conserved between the known trypanosomatid eEF1B $\gamma$  genes. This allowed the PCR cloning of four intergenic regions that were flanked in the *C. fasciculata* genome by copies of eEF1B $\gamma$  genes, confirming that the *C. fasciculata* eEF1B $\gamma$  genes are organised in a non-identical tandem array. Primers were then designed to these sequences and a full-length eEF1B $\gamma$  sequence cloned (Figure 10, *C. f.* full, and data not shown †). Of the thirteen peptide sequences isolated from the purified *C. fasciculata* eEF1B $\gamma$  protein, seven can be aligned with the predicted sequence of the full-length gene, confirming the identity of the

†Vickers, T.J. (2003) 'Trypanothione *S*-transferase in the Trypanosomatidae', PhD thesis, University of Dundee.

clone. The predicted products of the two *C. fasciculata* eEF1B $\gamma$  genes are 88% identical to each other and the full-length *C. fasciculata* eEF1B $\gamma$  is 32%, 68%, 77% and 63% identical to the human, *T. brucei*, *L. infantum* and *T. cruzi* eEF1B $\gamma$  proteins, respectively. The full-length and RT-PCR *C. fasciculata* eEF1B $\gamma$  proteins contain eight and six cysteine residues, respectively. These values are in agreement with the five NEM-accessible cysteine residues detected in the purified *C. fasciculata* eEF1B $\gamma$  by mass-spectrometry. Interestingly, only one of these residues (Cys53 in the full-length eEF1B $\gamma$ ) is conserved in all the trypanosomatid proteins and this residue may therefore be the site where alkylation by NEM inactivates the enzyme. The full-length *C. fasciculata* eEF1B $\gamma$  was expressed and the recombinant protein purified, however this protein showed no TST activity (data not shown<sup>‡</sup>).

## DISCUSSION

The novel *S*-transferase activity detected in trypanosomatids has a unique substrate specificity for trypanothione and glutathionylspermidine. The *C. fasciculata* enzyme appears to recognise the GspdSH moiety as a substrate, with trypanothione probably being used as two independent GspdSH substrate molecules. This requirement for a glutathione amide is in complete contrast with the substrate requirements of classical GSTs. Indeed, rat liver GSTs' glutathione-binding sites have been probed with a large number of glutathione derivatives and it was found that the least important part of the substrate for enzyme recognition is the glycyl moiety (42). Our finding that rat liver GSTs can utilise T[SH]<sub>2</sub> is in agreement with these results. As discussed later, the specificity of the *C. fasciculata* TST for CDNB and the comparatively low specific activity with this substrate appears to be a common feature of the *S*-transferase activities of eEF1B $\gamma$  subunits.

The assignment of the TST activity of the *C. fasciculata* eEF1B to eEF1B $\gamma$  is supported by several independent studies. From sequence analysis and homology modelling Koonin *et al.* predicted that the N-terminal domain found in eEF1B $\gamma$  proteins would form a GST-like fold, which was expected to possess GST activity (15). Subsequent structural studies on the *Saccharomyces cerevisiae* eEF1B gamma-1 (P29547) N-terminal domain confirmed that this domain's fold is remarkably similar to that of the GSTs, but no GST activity was detected in this truncated protein (14). However, an unusual 50 kDa GST from the yeast *Yarrowia lipolytica* has been purified (43) and we note that the published N-terminal sequence shows high similarity to the *S. cerevisiae* eEF1B gamma-2 (P36008 61% identity and 80% similarity in 29 residues). In common with the *C. fasciculata* TST, this unusual GST was highly specific for CDNB as an electrophilic substrate, and did not bind to glutathione-affinity resins. These data suggest that native forms of yeast eEF1B $\gamma$  proteins should be examined for *S*-transferase activities.

More recently, GST activity was detected in recombinant rice eEF1B $\gamma$  (Q9ZRI7) and preparations of the native eEF1 $\beta\beta'$  $\gamma$  complex (16). The eEF1B $\gamma$  had a low specific activity, with a  $k_{\text{cat}}$  value of approximately a fiftieth of a classical rice GST. Moreover, unlike classical GSTs from rice, this eEF1B $\gamma$  lacked glutathione peroxidase activity. The *S*-transferase activity of the *C. fasciculata* eEF1B $\gamma$  therefore appears to be similar to that of the rice subunit, with no activity towards organic peroxides and a comparatively low specific activity with CDNB. Interestingly, in common with the *T. cruzi* and *Bombyx mori* (Q9BPS3) eEF1B $\gamma$  subunits (44;45), and in contrast to the *C. fasciculata* protein, the rice eEF1B can be purified by glutathione-affinity chromatography. This indicates that the eEF1B $\gamma$  of these organisms can bind GSH and may indicate that the *T. cruzi* eEF1B $\gamma$  has a different substrate specificity to those of the other kinetoplastids.

We were unable to detect TST or GST activity in crude extracts of *T. cruzi*, in agreement with another report (20). However, another study did detect trace amounts of GST activity

(70 mU mg<sup>-1</sup>) in *T. cruzi* proteins purified on a glutathione-affinity resin (46). Interestingly, the major components of this preparation were later identified as the three subunits of the *T. cruzi* eEF1B complex (47;48). Purified recombinant *T. cruzi* eEF1B $\gamma$  was found to be devoid of GST activity (49) and activity with trypanothione was not examined in either study (46;49). However, the authors proposed that the GST activity of the  $\gamma$  subunit may require association with the other subunits of the eEF1B complex. This hypothesis is consistent with the instability of the isolated rice eEF1B $\gamma$  protein, in comparison to the native complex (16) and our failure to detect TST activity in the recombinant *C. fasciculata* eEF1B $\gamma$ . Indeed, further studies in our laboratory have shown that no *S*-transferase activity is detectable in the recombinant *L. major* eEF1B $\gamma$  protein either, whereas the *L. major* eEF1B holocomplex possesses TST activity. These findings will be described in a subsequent paper.

The diseases caused by *Leishmania spp.* are usually treated by antimonial compounds and Grondin *et al.* have hypothesised that a TST activity may be important in the development of drug resistance (22). This was suggested by their finding that the overexpression of thiol-metal conjugate transporters and increased T[SH]<sub>2</sub> levels are necessary, but not sufficient for high-level metalloid resistance in *L. tarentolae*. They therefore proposed that overexpressed TST was the missing trypanothione-dependent factor in the resistance phenotype. Indeed, the mechanism of resistance in *Leishmania* may be similar to that of mammalian cells, where increased GSH levels and transporter expression are coupled to the overexpression of GSTs (23;24). Interestingly, *L. tarentolae* is hypersensitive to metals in comparison with pathogenic species (50;51) and this correlates with a low level of extractable TST activity, compared to the pathogens *L. major* and *L. infantum* (Table 1). However, many other factors such as thiol levels differ between these species and more work is required to investigate if the TST activity in leishmania is involved in metal resistance.

The trypanosomatids appear to be unusual amongst eukaryotes in lacking a classical low-molecular mass *S*-transferase activity (52). However, as Moutiez *et al.* (53) suggested, at physiological pH the increased reactivity of T[SH]<sub>2</sub> over GSH may make the non-enzymatic conjugation of xenobiotics far more efficient in the trypanosomatids than other organisms. Thus, high cytosolic T[SH]<sub>2</sub> concentrations may efficiently substitute for a high enzymatic TST activity and at cytosolic pH the eEF1B TST activity may represent only a small fraction of the non-enzymatic *S*-transferase activity in the cell. A minor contribution of eEF1B to total cellular *S*-transferase activity is also consistent with the low specific activity of the rice eEF1B (16) and the failure to detect significant *S*-transferase activity in *T. cruzi*, without partial purification of the eEF1B complex (46). This suggests that either the appropriate xenobiotic substrate of eEF1B $\gamma$  has not yet been identified, or that this activity has a distinct physiological role unrelated to the conjugation and detoxification of xenobiotics. Importantly, the evolution of thiol substrate specificity of the trypanosomatid eEF1B $\gamma$  proteins towards trypanothione appears to have paralleled the evolution of trypanothione as these organisms' major thiol metabolite. This demonstrates that a specific interaction with cellular thiols is required for the eEF1B complex's function.

An alternative activity of GSTs is the binding of ligands. These ligands include hydrophobic compounds such as haemin and glutathione *S*-conjugates (54). In addition, glutathione can form a mixed disulphide with a cystine residue in the active site of the omega class of GSTs (55). It is therefore possible that the eEF1B complex may be involved in regulating protein synthesis in response to oxidative or toxic stress. Tight control of protein synthesis by the redox state of the GSH/GSSG couple has been observed by Kosower *et al.* (56), and *in vivo* studies have indicated that it is the elongation step of protein synthesis which is most sensitive to oxidative stress (57). Significantly, translational control may be especially

important in trypanosomes as they lack RNA polymerase II promoters and are thus unusually dependent on post-transcriptional regulation, for review see (58).

One mechanism for this response is suggested by the finding that the valyl-tRNA synthetase activity (ValRS) in yeast is controlled by an uncharacterised high molecular weight oxidoreductive regulatory apparatus which is activated by reduced glutathione (59-61). ValRS has since been found to be present in mammalian cells exclusively as a complex with eEF1B (62) and to associate with the elongation factor complex through an N-terminal extension similar in sequence to eEF1B $\gamma$  (63). Furthermore, several other aminoacyl-tRNA synthetases (ARSs) have recently been shown to associate with eEF1B *in vivo* (64). Our finding that the *C. fasciculata* eEF1B co-purifies with a probable ARS indicates that this interaction is conserved in the trypanosomatids. The binding of eEF1B to ARS is a functional interaction, as the ValRS activity of the ValRS.eEF1B complex, but not the free ValRS is activated by eEF1A and GTP (62). This activation could be reversed by the addition of anti-eEF1A and eEF1B $\gamma$  antibodies (64). Translational control by eEF1B could therefore be due to either effects upon tRNA synthetases, or by ligands directly modulating the complex's GDP/GTP exchange activity.

The identification of this unique activity within the trypanosomatid protein synthesis machinery has revealed a potential drug target in what was thought to be a highly conserved part of the eukaryotic cell. Furthermore, the investigation of the role of the eEF1B complex in drug resistance, thiol metabolism and translational control promises to yield important new insights into these parasites' biochemistry.

## Acknowledgments

Tim Vickers was funded by a research studentship from the Medical Research Council and Alan Fairlamb is funded by the Wellcome Trust. We thank Dr Nick Morrice and Dr Douglas Lamont for protein sequencing and Dr Angela Mehlert for mass-spectrometry.

## Abbreviations

<b>T[SH]<sub>2</sub> and T[S]<sub>2</sub></b>	trypanothione and trypanothione disulphide, respectively
<b>GSH and GSSG</b>	glutathione and glutathione disulphide, respectively
<b>GspdSH</b>	glutathionylspermidine
<b>CDNB</b>	1-Chloro-2,4-dinitrobenzene
<b>T[SDNB]<sub>2</sub></b>	trypanothione <i>bis</i> -dinitrobenzene
<b>TCEP</b>	tris(2- carboxyethyl)phosphine
<b>BS<sup>3</sup></b>	bis(sulfosuccinimidyl)suberate
<b>GST</b>	glutathione <i>S</i> -transferase
<b>TST</b>	trypanothione <i>S</i> -transferase
<b>eEF1A</b>	eukaryotic elongation factor 1A (formerly eEF-1 $\alpha$ )
<b>eEF1B</b>	the eukaryotic elongation factor 1B complex (formerly eEF-1 $\beta\gamma\delta$ )
<b>eEF1B<math>\alpha</math></b>	was formerly eEF-1 $\beta$
<b>eEF1B<math>\beta</math></b>	was formerly eEF-1 $\delta$
<b>eEF1B<math>\gamma</math></b>	was formerly eEF-1 $\gamma$
<b>ARS</b>	amino-acyl tRNA synthetase

## PCR

## polymerase chain reaction

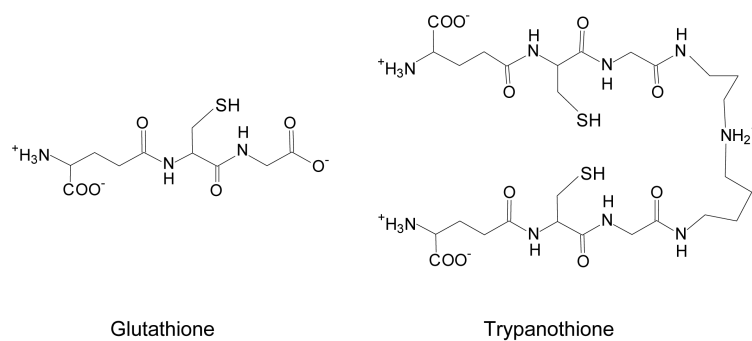
## REFERENCES

1. Fairlamb AH. Trends Parasitol. 2003; 19:488–494. [PubMed: 14580959]
2. Croft SL, Coombs GH. Trends Parasitol. 2003; 19:502–508. [PubMed: 14580961]
3. Zilberstein, D.; Ephros, M. Clinical and laboratory aspects of *Leishmania* chemotherapy in the era of drug resistance. In: Black, SJ.; Seed, JR., editors. World Class Parasites. Vol. Vol. 4. Leishmania Kluwer Academic Press; London: 2002.
4. Fairlamb AH, Blackburn P, Ulrich P, Chait BT, Cerami A. Science. 1985; 227:1485–1487. [PubMed: 3883489]
5. Fairlamb AH, Cerami A. Annu.Rev.Microbiol. 1992; 46:695–729. [PubMed: 1444271]
6. Flohé L, Hecht HJ, Steinert P. Free Radic.Biol.Med. 1999; 27:966–984. [PubMed: 10569629]
7. Sies, H.; Ketterer, B., editors. Glutathione Conjugation: Mechanisms and Biological Significance. Academic Press; London: 1988.
8. Hurst R, Bao YP, Jemth P, Mannervik B, Williamson G. Biochem.J. 1998; 332:97–100. [PubMed: 9576856]
9. Berhane K, Widersten M, Engstrom A, Kozarich JW, Mannervik B. Proc.Natl.Acad.Sci.U.S.A. 1994; 91:1480–1484. [PubMed: 8108434]
10. Hayes, JD.; Wolf, CR. Role of glutathione transferase in drug resistance. In: Sies, H.; Ketterer, B., editors. Glutathione Conjugation: Mechanisms and Biological Significance. Academic Press Limited; London: 1988.
11. Czarnecka E, Nagao RT, Key JL, Gurley WB. Mol.Cell.Biol. 1988; 8:1113–1122. [PubMed: 2835661]
12. Masai E, Katayama Y, Kubota S, Kawai S, Yamasaki M, Morohoshi N. FEBS Lett. 1993; 323:135–140. [PubMed: 8495726]
13. Dulhunty A, Gage P, Curtis S, Chelvanayagam G, Board P. J.Biol.Chem. 2001; 276:3319–3323. [PubMed: 11035031]
14. Jeppesen MG, Ortiz P, Shepard W, Kinzy TG, Nyborg J, Andersen GR. J.Biol.Chem. 2003; 278:47190–47198. [PubMed: 12972429]
15. Koonin EV, Mushegian AR, Tatusov RL, Altschul SF, Bryant SH, Bork P, Valencia A. Protein Sci. 1994; 3:2045–2054. [PubMed: 7703850]
16. Kobayashi S, Kidou S, Ejiri S. Biochem.Biophys.Res.Communic. 2001; 288:509–514. [PubMed: 11676472]
17. van Damme HT, Amons R, Karssies R, Timmers CJ, Janssen GM, Moller W. Biochim.Biophys.Acta. 1990; 1050:241–247. [PubMed: 2207149]
18. Janssen GM, Moller W. Eur.J.Biochem. 1988; 171:119–129. [PubMed: 3276514]
19. Yawetz A, Agosin M. Comp.Biochem.Physiol. 1981; 68B:237–243.
20. Moutiez M, Meziani-Cherif D, Aumercier M, Sergheraert C, Tartar A. Chem.Pharm.Bull.(Tokyo). 1994:2641–2644.
21. Legare D, Richard D, Mukhopadhyay R, Stierhof YD, Rosen BP, Haimeur A, Papadopoulou B, Ouellette M. J.Biol.Chem. 2001; 276:26301–26307. [PubMed: 11306588]
22. Grondin K, Haimeur A, Mukhopadhyay R, Rosen BP, Ouellette M. EMBO J. 1997; 16:3057–3065. [PubMed: 9214623]
23. Liu J, Chen H, Miller DS, Saavedra JE, Keefer LK, Johnson DR, Klaassen CD, Waalkes MP. Mol.Pharmacol. 2001; 60:302–309. [PubMed: 11455017]
24. Wang HF, Lee TC. Biochem.Biophys.Res.Communic. 1993; 192:1093–1099. [PubMed: 8099479]
25. Ariyanayagam MR, Fairlamb AH. Mol.Biochem.Parasitol. 2001; 115:189–198. [PubMed: 11420105]
26. Haimeur A, Brochu C, Genest PA, Papadopoulou B, Ouellette M. Mol.Biochem.Parasitol. 2000; 108:131–135. [PubMed: 10802326]

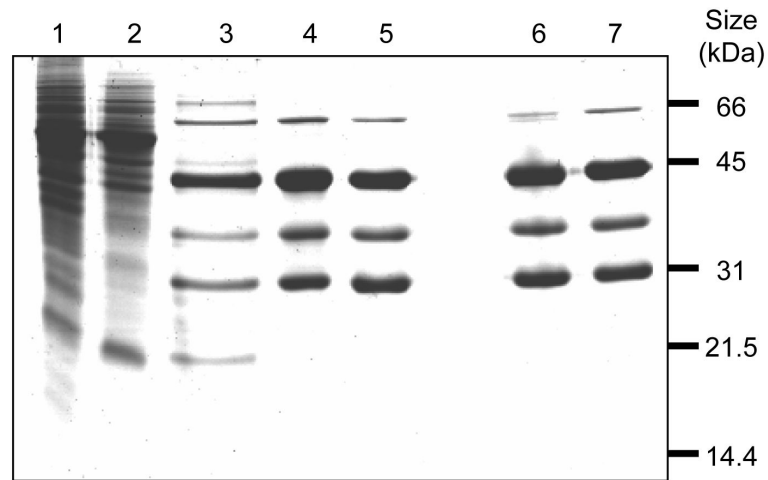
27. Habig WH, Jakoby WB. *Methods Enzymol.* 1981; 77:398–405. [PubMed: 7329316]
28. Bradford MM. *Anal.Biochem.* 1976; 72:248–254. [PubMed: 942051]
29. Laemmli UK. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
30. Davies GE, Stark GR. *Proc.Natl.Acad.Sci.U.S.A.* 1970; 66:651–656. [PubMed: 4913206]
31. Ashton AR, Gideon GM. *Biochem.J.* 1978; 175:501–506. [PubMed: 217344]
32. Small DAP, Lowe CR, Atkinson T, Bruton CJ. *Eur.J.Biochem.* 1982; 128:119–123. [PubMed: 6756917]
33. Perry KL, Watkins KP, Agabian N. *Proc.Natl.Acad.Sci.U.S.A.* 1987; 84:8190–8194. [PubMed: 3120186]
34. Gabriel A, Sisodia SS, Cleveland DW. *J.Biol.Chem.* 1987; 262:16192–16199. [PubMed: 3680249]
35. Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular Cloning: a Laboratory Manual.* 2 Ed. Cold Spring Harbor Laboratory Press; Cold Spring Harbor: 1989.
36. Warholm M, Jensson H, Tahir MK, Mannervik B. *Biochemistry.* 1986; 25:4119–4125. [PubMed: 3091071]
37. Bec G, Kerjan P, Zha XD, Waller JP. *J.Biol.Chem.* 1989; 264:21131–21137. [PubMed: 2556394]
38. Sheu GT, Traugh JA. *J.Biol.Chem.* 1997; 272:33290–33297. [PubMed: 9407120]
39. Graceffa P, Jancso A, Mabuchi K. *Arch.Biochem.Biophys.* 1992; 297:46–51. [PubMed: 1637182]
40. Datta J, Samanta TB. *Mol.Cell.Biochem.* 1992; 118:31–38. [PubMed: 1488053]
41. Naval J, Calvo M, Lampreave F, Pineiro A. *Comp.Biochem.Physiol.B.* 1982; 71:403–407. [PubMed: 7067402]
42. Adang AE, Brussee J, Vandergen A, Mulder GJ. *Biochem.J.* 1990; 269:47–54. [PubMed: 2375757]
43. Foley V, Sheehan D. *Biochem.J.* 1998; 333:839–845. [PubMed: 9677348]
44. Plumas-Marty B, Taibi A, Pessoa H, Verwaerde C, Loyens M, Pommier V, Velge P, Capron A, Ouaisi A. *Res.Immunol.* 1993; 144:553–563. [PubMed: 8303073]
45. Kamiie K, Nomura Y, Kobayashi S, Taira H, Kobayashi K, Yamashita T, Kidou S, Ejiri S. *Biosci.Biotechnol.Biochem.* 2002; 66:558–565. [PubMed: 12005049]
46. Plumas-Marty B, Verwaerde C, Loyens M, Velge P, Taibi A, Cesbron M-F, Capron A, Ouaisi MA. *Parasitol.* 1992; 104:87–98.
47. Billaut-Mulot O, Pommier V, Schoneck R, Plumas-Marty B, Taibi A, Loyens M, Capron A, Ouaisi MA. *Nucleic Acids Res.* 1993; 21:3901. [PubMed: 8367313]
48. Plumas-Marty B, Schoneck R, Billaut-Mulot O, Taibi A, Capron A, Ouaisi MA. *Parasitol.Res.* 1994; 80:626–628. [PubMed: 7855131]
49. Billaut-Mulot O, Fernandez-Gomez R, Ouaisi A. *Gene.* 1997; 198:259–267. [PubMed: 9370290]
50. Légaré D, Papadopoulou B, Roy G, Mukhopadhyay R, Haimeur A, Dey S, Grondin K, Brochu C, Rosen BP, Ouellette M. *Exp.Parasitol.* 1997; 87:275–282. [PubMed: 9371094]
51. Callahan HL, Beverley SM. *J.Biol.Chem.* 1991; 266:18427–18430. [PubMed: 1680861]
52. Fahey RC, Sundquist AR. *Adv.Enzymol.Relat.Areas Mol.Biol.* 1991; 64:1–53. [PubMed: 1675828]
53. Moutiez M, Aumercier M, Parmentier B, Tartar A, Sergheraert C. *Biochim.Biophys.Acta.* 1995; 1245:161–166. [PubMed: 7492572]
54. Mannervik B, Danielson UH. *C.R.C. Crit.Rev.Biochem.* 1988; 23:283–337.
55. Board PG, Coggan M, Chelvanayagam G, Easteal S, Jermin LS, Schulte GK, Danley DE, Hoth LR, Griffor MC, Kamath AV, Rosner MH, Chrnyk BA, Perregaux DE, Gabel CA, Geoghegan KF, Pandit J. *J.Biol.Chem.* 2000; 275:24798–24806. [PubMed: 10783391]
56. Kosower NS, Vanderhoff GA, Benerofe B, Hunt T, Kosower EM. *Biochem.Biophys.Res.Commun.* 1971; 45:816–821. [PubMed: 5128185]
57. Ayala A, Parrado J, Bougria M, Machado A. *J.Biol.Chem.* 1996; 271:23105–23110. [PubMed: 8798501]
58. Clayton CE. *EMBO J.* 2002; 21:1881–1888. [PubMed: 11953307]
59. Black S. *Biochem.Biophys.Res.Commun.* 1993; 191:95–102. [PubMed: 8447838]

60. Black S. *Science*. 1986; 234:1111–1114. [PubMed: 3535073]
61. Black S. *J.Biol.Chem.* 1983; 258:2112–2114. [PubMed: 6337152]
62. Negrutskii BS, Shalak VF, Kerjan P, El'skaya AV, Mirande M. *J.Biol.Chem.* 1999; 274:4545–4550. [PubMed: 9988688]
63. Yang DCH. *Curr.Top.Cell.Regul.* 1996; 34:101–136. [PubMed: 8646845]
64. Sang LJ, Gyu PS, Park H, Seol W, Lee S, Kim S. *Biochem.Biophys.Res.Commun.* 2002; 291:158–164. [PubMed: 11829477]



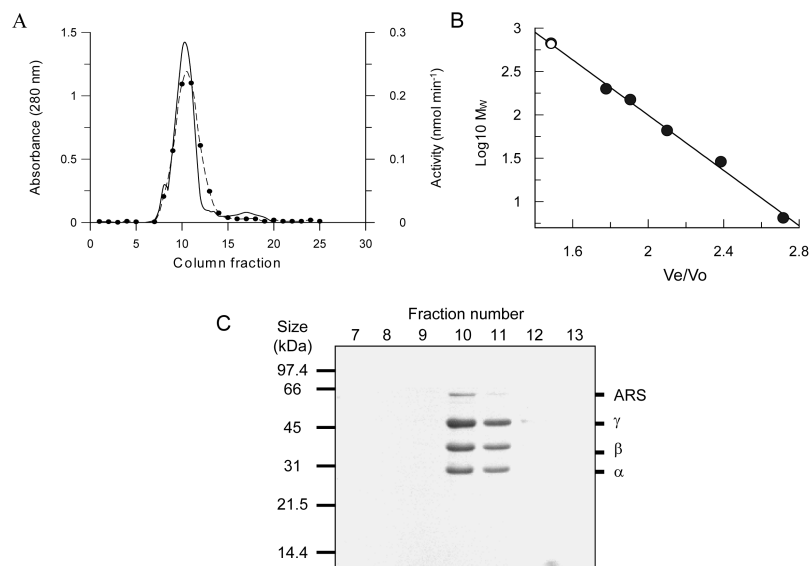


**Figure 1.** Structures of glutathione ( $\gamma$ -L-glutamyl-L-cysteinylglycine) and trypanothione ( $N^1, N^8$ -bis(glutathionyl)spermidine)

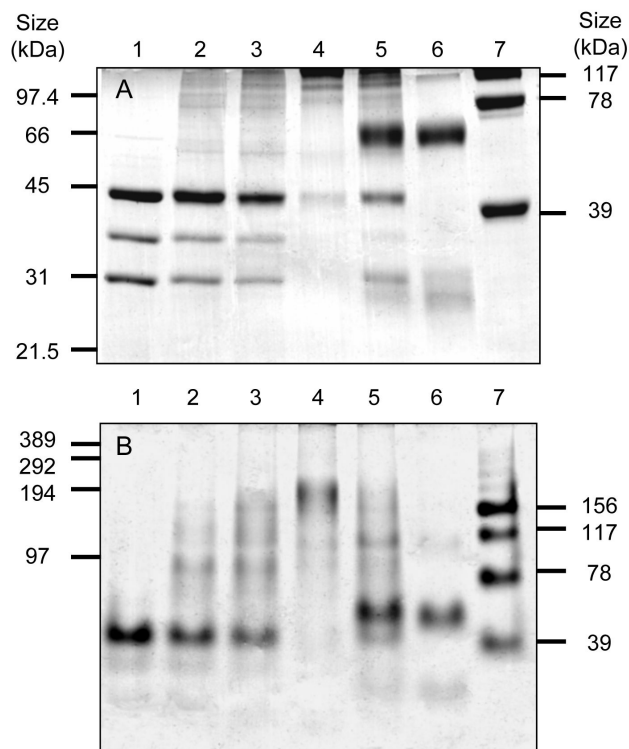


**Figure 2. SDS-PAGE of *C. fasciculata* TST purification fractions**

Samples (10  $\mu$ g protein in each lane) from the purification were separated in a 10% SDS-PAGE gel. Lane 1, clarified cell extract lane 2, 2-10% redissolved PEG 6000 precipitate lane 3, Q-sepharose eluant lane 4, Phenyl sepharose eluant lane 5, Superdex 200 eluant. Lanes 6 and 7 were additional samples of the Superdex 200 pool with 1mM DTT being added to the sample in lane 6 and the sample in lane 7 containing no reducing agent.

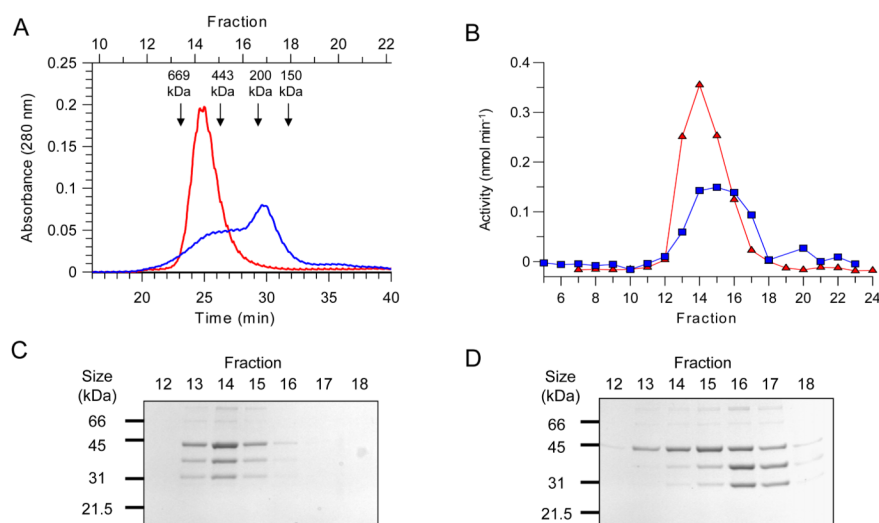


**Figure 3. Analytical size-exclusion chromatography of purified *C. fasciculata* TST.** (A) Elution profiles of TST activity and protein from a Superdex 200 HR analytical size exclusion column. The absorbance at 280 nm is shown with a solid line, the fraction numbers are shown above this trace and are corrected for the delay volume between the fraction collector and the UV flowcell. The TST activity of fractions was measured using the microtitre plate assay and shown in filled circles joined with a dotted line. (B) Determination of the relative molecular mass of this species, with the sample (○) shown relative to a set of analytical standards (●). (C) SDS-PAGE analysis of 10  $\mu$ l-samples taken from the 0.5 ml fractions collected during this separation. The four visible bands, subsequently identified as the eEF1B $\alpha$ ,  $\beta$  and  $\gamma$  subunits of the eEF1B complex and an aminoacyl-tRNA synthetase are labelled at the right-hand side of the gel.

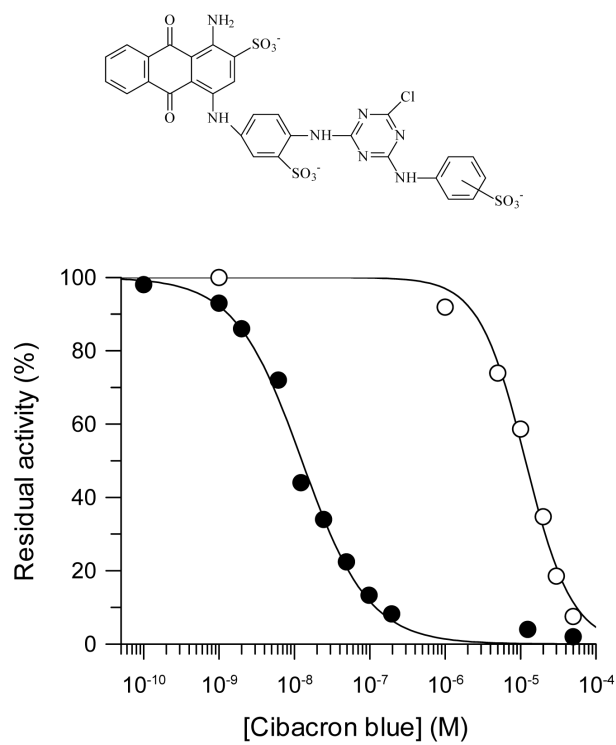


**Figure 4. Cross-linking of the TST complex**

(A) 10% Laemmli SDS-PAGE analysis of the products of the reaction between increasing concentrations of  $\text{BS}^3$  and TST (lane 1 - no  $\text{BS}^3$  lane 2 - 75  $\mu\text{M}$  lane 3 - 200  $\mu\text{M}$  and lane 4 - 1 mM). The negative control used the monomeric proteins BSA (66 kDa) and carbonic anhydrase (29 kDa), which were treated with 1 mM  $\text{BS}^3$  in the presence (lane 5) or absence (lane 6) of TST. The positive control in lane 7 shows the products from treatment of the tetrameric protein aldolase with 75  $\mu\text{M}$   $\text{BS}^3$ , with the masses of the products formed indicated on the right-hand side of the gel. (B) The same samples were analysed in a 6% Weber SDS-PAGE gel. The masses on the left of the gel are from a cross-linked phosphorylase b marker and again the masses of the aldolase products are indicated on the right-hand side of the gel. Samples as described in panel A.

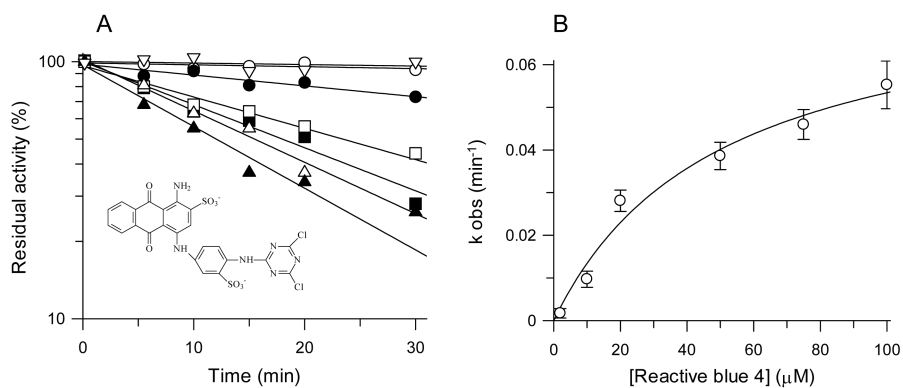


**Figure 5. Separation of the subunits of the TST complex using thiocyanate**  
 Samples of TST were incubated in the presence and absence of 1.5 M NaSCN and then applied to a size-exclusion column. **(A)** Elution profiles of these samples, the untreated control is shown in red and the NaSCN-treated sample in blue. The elution positions and masses of protein standards are indicated with arrows. **(B)** TST activity profiles of the fractions collected from this separation, the untreated control is indicated with red triangles and the NaSCN-treated sample with blue squares. **(C and D)** SDS-PAGE analysis of these column fractions, with Panel C corresponding to the untreated control and Panel D to the NaSCN-treated sample.



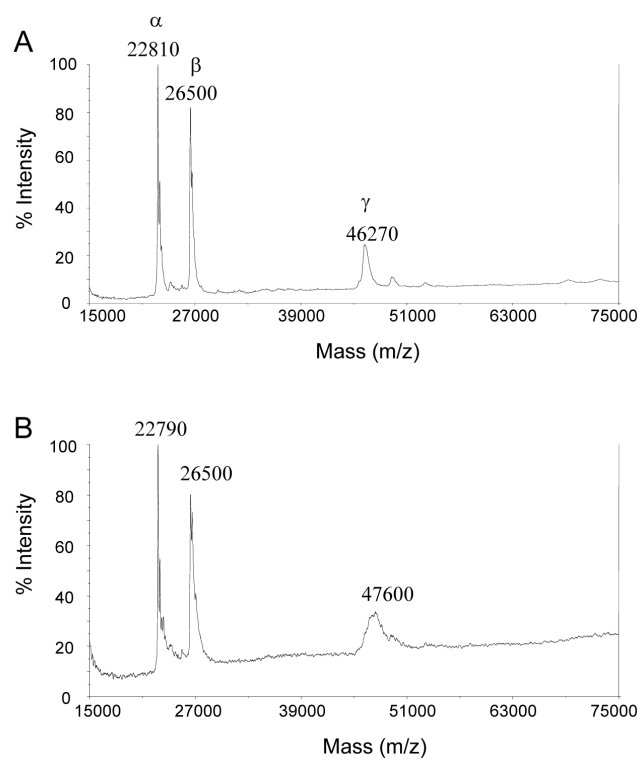
**Figure 6. Inhibition of TST by Cibacron blue**

Inhibition of TST by Cibacron blue in the presence (○) and absence (●) of 0.5% (w/v) BSA in the assay buffer.



**Figure 7. Time-dependent inhibition of TST by reactive blue 4**

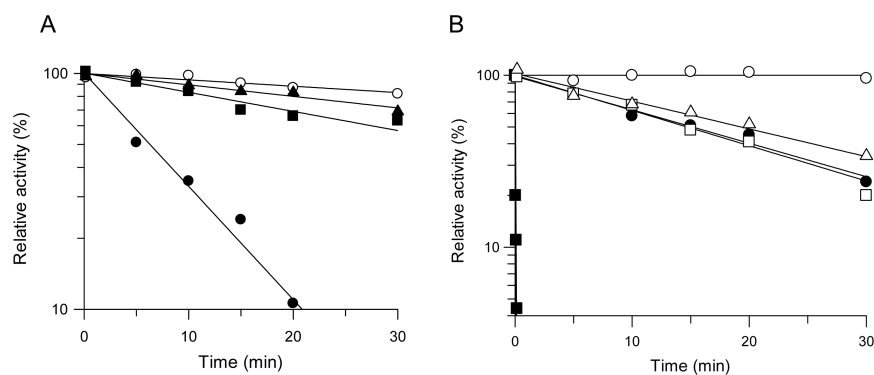
(A) Time-dependent inactivation of TST by reactive blue 4. TST was incubated with the inhibitors in 20 mM (Na<sup>+</sup>) phosphate pH 7.5 at 25°C and the residual activity determined at intervals, as described in the methods. The incubations contained either 100 μM Cibacron blue (▽) or reactive blue 4 at 2 μM (○), 10 μM (●), 20 μM (□), 50 μM (■), 75 μM (△) and 100 μM (▲). (B) Observed rates of TST inactivation by reactive blue 4 as a function of the concentration of inhibitor.



**Figure 8. Covalent modification of eEF1B $\gamma$  by reactive blue 4**

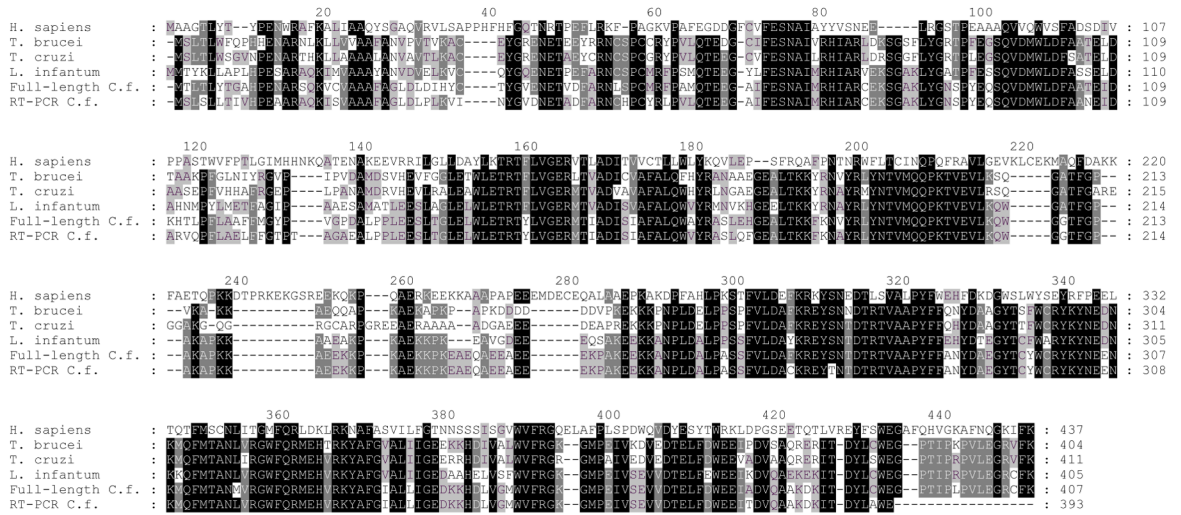
Reactive blue 4-inactivated TST was analysed by MALDI-TOF mass spectrometry. The two samples were prepared as described in the text, with the TST sample shown in panel A being an untreated negative control and the TST sample shown in panel B being inactivated by incubation with 50  $\mu$ M reactive blue 4 for 1 h.





**Figure 9. Interactions between TST substrates and triazine dyes**

Incubations were performed in 20 mM (Na<sup>+</sup>) phosphate pH 7.5 at 25°C and the residual activity determined at intervals, as described in the methods. **(A)** Time-dependent inactivation of TST by CDNB. The incubations contained no CDNB (○), 500 μM CDNB (●), 500 μM CDNB and 500 μM T[SH]<sub>2</sub> (■) and 500 μM CDNB and 100 μM Cibacron blue (▲). **(B)** Effects of thiols on the inactivation of TST by reactive blue 4. The incubations contained no reactive blue 4 (○), 50 μM reactive blue 4 (●), 50 μM reactive blue 4 and 1 mM GSH (□), 50 μM reactive blue 4 and 500 μM T[SH]<sub>2</sub> (■) and 50 μM reactive blue 4 and 500 μM DTT (Δ).



**Figure 10. Alignment of the sequences of trypanosomatid eEF1B $\gamma$  proteins**  
 The predicted amino-acid sequences of the *C. fasciculata* eEF1B $\gamma$  genes isolated by RT-PCR (RT-PCR *C.f.*) and intergenic PCR (Full-length *C.f.*) are shown aligned with the sequences of the *Homo sapiens* (P26641), *T. brucei* (temporary name - TRYPTp\_ends-17b11.p1k\_188), *L. infantum* (Q9BHZ6) and *T. cruzi* (P34827) proteins. Gaps are indicated with dashes and the alignment shaded according to similarity, the colours indicate black background, white text - over 80% identity dark grey background, white text - over 60% identity and light grey background, black text – over 40% identity.

**Table 1**  
**Specific activities of TST, GST and trypanothione reductase in soluble extracts of trypanosomatids**

All enzyme activities are assayed as described in the relevant methods section and corrected for non-enzymatic background rates.

Organism	Cell type	TST (mU mg <sup>-1</sup> )	GST (mU mg <sup>-1</sup> )	Trypanothione reductase (mU mg <sup>-1</sup> )
<i>C. fasciculata</i>	choanomastigotes	16.7	<0.1 <sup>a</sup>	920
<i>L. major</i>	promastigotes	6.4	<0.1	580
<i>L. infantum</i>	promastigotes	2.9	<0.1	80
<i>L. tarentolae</i>	promastigotes	0.4	<0.1	430
<i>T. cruzi</i>	epimastigotes	<0.1 <sup>a</sup>	<0.1	510
<i>T. brucei</i>	bloodstream form	2.8	<0.1	30
<i>M. musculus</i>	liver	23	1,970	N.D.

<sup>a</sup>) less than limit of detection

N.D. – not determined

Table 2

Purification of *C. fasciculata* TST

Fraction	Activity (U/ml)	Total Protein (mg)	Total Units (mU)	Specific Activity (mU mg <sup>-1</sup> )	Purification factor	Recovery (%)
Crude extract	510	19,880	145,410	7.3	-	100
4-9% PEG	640	2,010	63,110	31.4	4.3	43
Q-sepharose	770	72	58,239	815	112	40
Phenyl sepharose	2,080	9.1	13,960	1,530	210	9.6
Superdex 200	4,140	5	8,290	1,658	227	5.7

**Table 3**Kinetic parameters of the *C. fasciculata* TST

Substrate <sup>a)</sup>	$K_{m(\text{app})}$ ( $\mu\text{M}$ )	$V_{\text{max}}$ ( $\text{nmol min}^{-1}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ ) <sup>f)</sup>	$k_{\text{cat}}/K_m$ ( $\text{M}^{-1} \text{s}^{-1}$ )
CDNB <sup>b)</sup>	190 $\pm$ 27	3.45 $\pm$ 0.14	236	1.2 $\times$ 10 <sup>6</sup>
T(SH) <sub>2</sub> <sup>c)</sup>	159 $\pm$ 19	2.77 $\pm$ 0.12	190	1.2 $\times$ 10 <sup>6</sup>
GspdSH <sup>d)</sup>	310 $\pm$ 31	2.81 $\pm$ 0.13	193	0.62 $\times$ 10 <sup>6</sup>
GSH monoethyl ester <sup>e)</sup>	6,100 $\pm$ 590	0.59 $\pm$ 0.02	17.4	0.003 $\times$ 10 <sup>6</sup>

<sup>a)</sup> The absorbance coefficient for the T[DNB]<sub>2</sub> conjugate of 9.2 mM cm<sup>-1</sup> was used for both T[SH]<sub>2</sub> and GspdSH, whilst the published value for GS-DNB of 9.6 mM cm<sup>-1</sup> was used for GSH ester.

<sup>b)</sup> Measured with 400  $\mu\text{M}$  T[SH]<sub>2</sub> as the fixed substrate

<sup>c)</sup> Measured with 400  $\mu\text{M}$  CDNB as the fixed substrate

<sup>d)</sup> Measured with 400  $\mu\text{M}$  CDNB as the fixed substrate

<sup>e)</sup> Measured with 400  $\mu\text{M}$  CDNB as the fixed substrate

<sup>f)</sup> Calculated using the calculated concentration of the  $[(\alpha\beta\gamma\delta)]_4$  complex