Expression of *Trypanosoma congolense* Trypanothione Reductase in *Escherichia coli*: Overproduction, Purification, and Characterization†

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Received November 30, 1988; Revised Manuscript Received March 13, 1989

ABSTRACT: The cloned trypanothione reductase gene from *Trypanosoma congolense* has been expressed in *Escherichia coli* to a level of 1% of the soluble protein. This has allowed facile purification and initial characterization of the reductase, and it appears by all criteria to be a representative member of the trypanothione reductase family. Most importantly, it shows the same exclusive substrate specificity for trypanothione over glutathione characteristic of other trypanothione reductases examined to date. The availability of the pure, cloned, sequenced reductase from *T. congolense* makes this enzyme a good target for structure/function studies and trypanocidal inhibitor design.

Trypanothione reductase catalyzes the NADPH-dependent reduction of trypanothione (Figure 1), N^1,N^8-diglutathionylspermidine, a glutathione analogue unique to the kinetoplastid parasites (Fairlamb et al., 1985; Shames et al., 1986). The reductase has been purified recently from the insect trypanosomatid *Crithidia fasciculata* (Shames et al., 1986) and the human parasite *Trypanosoma cruzi* (Krauth-Siegel et al., 1987). Characterization of the enzyme from these two sources revealed that trypanothione reductase and its host analogue glutathione reductase display mutually exclusive substrate specificities; trypanothione reductase will not reduce glutathione, and glutathione reductase will not reduce trypanothione. Thus the enzyme appears to be an excellent target for the development of more effective and less toxic antitrypanosomal agents. Such an approach is further supported by the parasites' inherent sensitivity to oxidative stress (Boveris et al., 1980; Docampo & Moveno, 1984) and by the discovery that trypanosomes lack the iron-dependent peroxidases found in most mammalian systems. This leaves the parasites heavily dependent on the trypanothione reductase/trypanothione-dependent peroxidase redox couple for protection from oxidative stress (Penketh & Klein, 1986; Henderson et al., 1987a). Given the widespread occurrence of diseases caused by the trypanosomes and leishmanias, and the lack of satisfactory treatment for these diseases, such work is well motivated.

To date, research on trypanothione reductase has been hampered by the inability to obtain sufficient quantities of the reductase. This is in a large part due to the difficulty in culturing the pathogenic parasites as well as the low levels of reductase found in these parasites. Since heterologous expression of trypanothione reductase might provide greater quantities of enzyme than those available from natural sources, and might allow greater experimental flexibility for structure/function studies, we recently undertook the cloning and sequencing of the gene encoding trypanothione reductase from *Trypanosoma congolense* (Shames et al., 1988). Here we report the successful expression of the gene in *Escherichia coli*, leading to its overproduction and purification in tens of milligram amounts which has permitted its characterization. The enzyme appears to be a representative member of the trypantohione reductase family, and its ease of production makes it the enzyme of choice for mechanistic and structural studies on trypanothione reductase.

Materials and Methods

Materials. Deoxyadenosine 5′-α-[^{35}S]thiotriphosphate and Klenow polymerase were obtained from Amersham. Restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were obtained from New England Biolabs or International Biotechnologies Inc. (IBI). Calf intestinal alkaline phosphatase was from Boehringer-Mannheim or Stratagene. Diisopropyl (β-cyanoethyl)phosphoramidites, DEAE-Sephacel, and 2′,5′-ADP–Sepharose were from Pharmacia. Sequenase DNA sequencing kit was from U.S. Biochemicals. Trypanothione and deazatrypanothione (Henderson et al., 1987b) were kind gifts of Dr. G. Henderson (The Rockefeller University, New York, NY). All other biochemical reagents were obtained from Sigma Chemical Co. unless otherwise indicated.

Vectors and Bacterial Strains. M13mp18 and M13mp19 were from Amersham. pKK233-2 and pUC13 were from Pharmacia. pBS+ was purchased from Stratagene. pGP1-2 (Tabor & Richardson, 1985), a plasmid containing the T7 RNA polymerase gene under control of the temperature-sensitive cI857 λ repressor, was a gift of Dr. S. Tabor (Harvard Medical School, Boston, MA). Plasmid pCGTR-2 contains the *T. congolense* trypanothione reductase gene on a 3.3-kb *Bam*HI insert (Shames et al., 1988). Plasmid pSD8SP that contains a *tac II* promoter and strong ribosome binding site (Shindeling et al., submitted for publication) was a gift from Dr. L. Gold (University of Colorado, Boulder, CO). *E. coli* strains JM101 and JM105 were from New England Biolabs. *E. coli* strain SG5 (Greer & Perham, 1986), a glutathione reductase deletion mutant, was a gift of Dr. R. Perham (Cambridge University, Cambridge, England). All bacterial strains were grown on 2XTY media that contained 16 g of Bactotryptone, 10 g of Bacto yeast extract, and 5 g of NaCl per liter. Antibiotics were included when appropriate. For enzyme preparations, bacterial strains transformed with either

† This work supported in part by a grant from the National Institutes of Health, GM 21643, a postdoctoral fellowship from the Damon Runyon–Walter Winchell Cancer Fund (F.X.S.), and a postdoctoral fellowship from the National Institutes of Health (S.L.S.).

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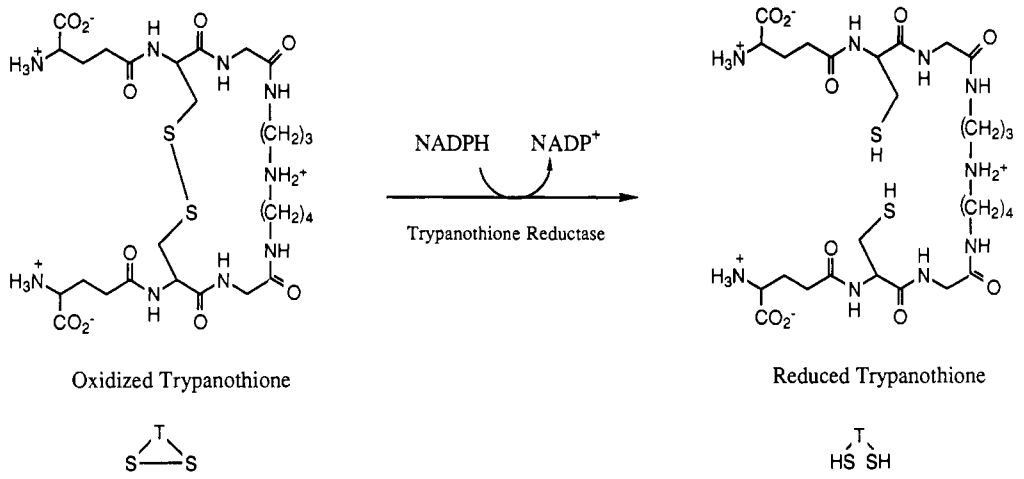

FIGURE 1: NADPH-dependent reduction of trypanothione by trypanothione reductase.

pKKTR-2 or pTacTR-1 were grown to an optical density of 0.75 at which time IPTG[1] was added to a final concentration of 2 mM. Following a 5-h induction period, the cells were harvested by centrifugation. Bacterial strains transformed with pT7TR-3+ and pGP1-2 were grown at 30 °C to an optical density of 1.2 at which time the temperature was raised to 42 °C for 1 h, and then the incubation was continued for 1.5 h at 37 °C. The cells were harvested by centrifugation.

Preparation of Oligonucleotide Linkers. Oligonucleotide linkers were synthesized on a Pharmacia Gene Assembler and purified by preparative polyacrylamide gel electrophoresis followed by chromatography on SepPak C-18 columns (Waters Associates) as described by Shames et al. (1988). When desired, oligonucleotides were phosphorylated on the 5′ end with T4 polynucleotide kinase prior to annealing. Oligonucleotides were annealed in 77 mM Tris buffer, pH 7.5, containing 11 mM $MgCl_2$ and 1 mM dithiothreitol by heating to 65 °C for 10 min followed by slow cooling to room temperature.

DNA Manipulations and Sequencing. All manipulations of DNA were by standard procedures as described by Maniatis et al. (1982) or by manufacturers' instructions. Sequencing was performed by the dideoxy chain termination method of Sanger (1977) according to the suppliers' directions for the Amersham (Klenow) or U.S. Biochemicals (Sequenase) kits. Plasmids were propagated in *E. coli* strain JM105 or DH5α and were purified on CsCl gradients.

Enzyme Assays and Kinetic Analysis. Trypanothione reductase activity was assayed as described previously (Shames et al., 1986) except that deazatrypanothione was substituted for trypanothione in routine assays. The reaction mixture contained 100 mM Hepes pH 7.8, 1 mM EDTA, 200 μM NADPH, and 40 μM deazatrypanothione. One unit of activity is defined as that amount of enzyme required to convert 1 μmol of NADPH to $NADP^+$ per minute at 25 °C. Protein was assayed according to the method of Bradford (1976) as supplied by Bio-Rad with bovine serum albumin as a standard. Activities of purified enzyme are expressed per enzyme-bound flavin equivalent with $\epsilon_{464} = 10.6\ mM^{-1}\ cm^{-1}$. The kinetic data were analyzed by the nonlinear method of Cleland (1979) with the program HYPER. The calculated constants are presented along with their standard errors.

Purification of T. congolense Trypanothione Reductase. Trypanothione reductase was purified 107-fold to homogeneity from *E. coli* in an overall yield of 60%. The following buffers were used: (buffer A) 20 mM potassium phosphate (pH 7.2), 5 mM β-mercaptoethanol, 1 mM EDTA; (buffer B) 20 mM Tris (pH 7.2), 5 mM β-mercaptoethanol, 1 mM EDTA. A brief description of a typical preparation follows.

The cell pellet obtained from a 1-L culture of induced cells was resuspended in 25 mL of buffer A containing 0.1 mM PMSF, and the cells were lysed by sonication with six 1-min pulses with intermittent 1-min cooling periods. Following centrifugation to remove the cellular debris, the nucleic acids were precipitated by adjusting the supernatant to a final concentration of 0.4% protamine sulfate. The cloudy solution was then brought to 40% saturating ammonium sulfate, and the precipitated nucleic acids and proteins were removed by centrifugation. The supernatant was then adjusted to 60% ammonium sulfate and the trypanothione reductase containing 40–60% ammonium sulfate fraction was isolated by centrifugation. The resulting pellet was dissolved in 30 mL of buffer B and dialyzed extensively against the same buffer. The dialyzed solution was applied to a DEAE-Sephacel column (1.5 × 8 cm) that had been preequilibrated in buffer B, and the column was washed with 150 mL of buffer B. The proteins were eluted with a liner salt gradient (0.0–0.3 M KCl, in buffer B, 150 mL of each), and 5-mL fractions were collected. *T. congolense* trypanothione reductase eluted at 0.1 M KCl as a yellow protein. The fractions containing activity were pooled and dialyzed extensively against buffer A. The yellow protein solution was applied to a 2′,5′-ADP–Sepharose column (1.5 × 11 cm) that had been preequilibrated in buffer A. After the column was washed with 100 mL of buffer A, the trypanothione reductase was eluted with buffer A containing 5 mM $NADP^+$, yielding homogeneous protein. The enzyme was dialyzed extensively against buffer A and concentrated on an Amicon concentrator (PM10 membrane) and stored at 4 °C.

T. congolense trypanothione reductase was also prepared from 18-L fermentations. When the method was scaled up, the cells were lysed in a French pressure cell, and the volume of the DEAE-Sephacel column was increased 4-fold. All other conditions were identical with those described.

Protein Sequencing and Amino Acid Analysis. Amino-terminal sequence determination and amino acid analysis were performed at the Harvard Microchemistry facilities, Cambridge, MA. Amino acid composition was determined by Dr.

[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; kb, kilobase pair; bp, base pair; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

David Andrews as follows: the protein was hydrolyzed and then derivatized with phenyl isothiocyanate (PITC), and the resulting PTC-amino acids were resolved on a Hewlet-Packard 108B HPLC using an Altex-ODS column according to Ebert (1986). Amino-terminal sequence analysis was performed by Dr. William Lane on an Applied Biosystems 470A protein sequencer equipped with an on-line 120A phenylthiohydantoin (PTH) analyzer.

Molecular Weight Determination. Subunit molecular weight was determined by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) using BRL high molecular weight protein markers as standards: myosin, 200 000; phosphorylase *b*, 97 400; bovine serum albumin, 68 000; ovalbumin, 43 000; α-chymotrypsinogen, 25 700; β-lactoglobulin, 18 400; lysozyme, 14 300. Native molecular weight was determined by chromatography on a Pharmacia FPLC Superose 12 column run in buffer A using catalase (232 000), aldolase (158 000), bovine serum albumin (68 000), and ovalbumin (43 000) as standards.

Flavin Identification. Enzyme-bound flavin was liberated by heat denaturation at 100 °C for 10 min. The denatured protein was removed by centrifugation. The free flavin was analyzed by HPLC on a Waters Associates C-18 column using an isocratic buffer system containing the ion pair reagent tetra-*n*-butylammonium sulfate (Shames et al., 1986). To enhance separation of the flavin standards, the methanol concentration as reduced to 25%.

Reductive Titration with NADPH. Trypanothione reductase was reduced under anaerobic conditions with NADPH. Titrations were carried out under argon in cuvettes modified with gas outlet tubes and a gas-tight Hamilton syringe (Williams et al., 1979). The reactions were performed in 100 mM Hepes, pH 7.8, buffer containing 1 mM EDTA. The spectra were recorded on an HP 8452A spectrophotometer.

Results

Expression of T. congolense Trypanothione Reductase in E. coli. Expression of the eukaryotic *T. congolense* trypanothione reductase gene in *E. coli* required engineering of the isolated gene fragment immediately downstream of an *E. coli* RNA polymerase promoter and ribosome binding site. The expression vector pKK233-2, containing a *trc* promoter and ribosome binding, was chosen for the initial attempt at reductase expression. First, the gene encoding *T. congolense* trypanothione reductase was excised from vector pCGTR-2 as a 2-kb fragment by digestion with *Xba*I and *Pst*I and ligated into pUC13 that had been digested with *Xba*I and *Pst*I and treated with calf intestinal alkaline phosphatase. This removed 1 kb of noncoding DNA and yielded pCGTR-3, a plasmid containing the reductase gene with a convenient *Pst*I site at the 3′ end of the gene. The following steps of vector construction are outlined in Figure 2. Digestion of pCGTR-3 with *Pst*I released the trypanothione reductase gene on a 2-kb fragment. A partial digestion of this fragment with *Xmn*I gave a 1.5-kb fragment that contained all but the first 10 bp of the trypanothione reductase gene. To restore the 5′ end of the gene, the expression vector pKK233-2 was engineered. pKK233-2 was digested with *Nco*I, filled by use of Klenow polymerase, and ligated to the palindromic linker AGCAAGGCCTTGCT that contained an appropriately placed *Stu*I restriction site. This yielded plasmid pKKSTU-8 and provided the mechanism for restoring the lost ATG initiation codon and subsequent 7 bp of the *Xmn*I partially digested trypanothione reductase gene. Ligation of the *Xmn*I–*Pst*I-treated trypanothione reductase gene fragment into *Stu*I–*Pst*I-digested, alkaline phosphatase treated pKKSTU-8 yielded pKKTR-1, an expression vector containing the *trc*

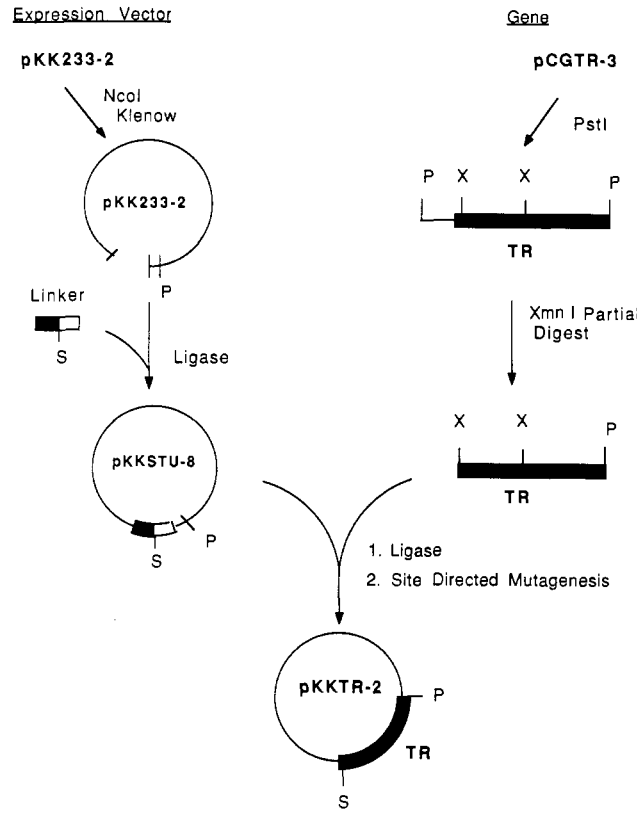

FIGURE 2: Construction of trypanothione reductase expression vector pKKTR-2. Schematic of steps in construction of pKKTR-2 showing isolation of trypanothione reductase gene and its trimming with *Xmn*I, engineering of expression vector pKK233-2 by addition of a palindromic linker immediately downstream of the initiating ATG, and reconstruction of the entire trypanothione reductase gene, now adjacent to a bacterial promoter and ribosome binding site. pCGTR-3 is a pUC 13 derivative containing the trypanothione reductase gene from *T. congolense* as a 2-kb insert. pKK233-2 is an expression vector containing the *trc* promoter, a ribosome binding site, and a transcriptional terminator. Solid lines indicate trypanothione reductase gene sequence. Restriction sites are indicated by single letters: X, *Xba*I; P; *Pst*I; S, *Stu*I.

promoter, a ribosome binding site, and the fully restored *T. congolense* trypanothione reductase gene. Sequence analysis of pKKTR-1 revealed that the G-C bp of the initiation codon had been deleted during construction. Therefore, site-directed mutagenesis was performed to restore the initiation codon and to reconstruct the *Stu*I restriction site that had been destroyed during vector construction. This yielded plasmid pKKTR-2, which now contained an intact ATG start codon and a unique *Stu*I restriction site 10 bp from the 5′ end of the gene. A summary of the construction is shown in Table I.

To test the level of trypanothione reductase expression from pKKTR-2, *E. coli* JM105 transformed with pKKTR-2 were grown, and expression was induced with 2 mM ITPG for 5 h. Assays of the crude extracts from these inductions showed low levels of trypanothione reductase activity. To improve the level of trypanothione reductase expression, we replaced the *trc* promoter of pKK233-3 with a *tac II* promoter (de Boer et al., 1983) and substituted a longer ribosome binding site that contained a stretch of eight deoxyadenosine residues immediately preceding the initiation codon. Initially, the *tac II* promoter from pSD8SP was isolated on a 0.3-kb *Eco*RI–*Hin*dIII restriction fragment. The desired ribosome binding site and first 10 bp of the trypanothione reductase gene were synthesized and introduced as a 31-bp linker having *Hin*dIII- and *Stu*I-compatible ends. As shown in Figure 3, a new expression vector, pTacTR-1, was constructed in a single

Table I: Construction of Trypanothione Reductase Expression Vector pKKTR-2

step	sequence								
TR protein	Met	Ser	Lys	Ala	Phe	···	*	···	
TR gene in pCGTR-3	ATG	TCG	AAG	GCG	TTC	···	TAG	···	*Pst*I
TR gene in *Pst*I–*Xmn*I fragment				CG	TTC	···	TAG	···	*Pst*I
pKK233-3 *Nco*I–Klenow	ATG								*Pst*I
palindromic linker		AGC	AAG	GCC	TTG	CT			
pKKSTU-8	AT-	AGC	AAG	GCC	TTG	CT	···		*Pst*I
pKKSTU-8 *Stu*I–*Pst*I	AT-	AGC	AAG	G					*Pst*I
pKKTR-1	AT-	AGC	AAG	GCG	TTC	···	TAG	···	*Pst*I
pKKTR-2	ATG	AGC	AAG	GCC	TTC	···	TAG	···	*Pst*I

Table II: Comparison of Trypanothione Reductase Expression Levels

vector	promoter	sequence	expression level (mg/L)[a]
pKKTR-2	*trc*	TAACAATTTCACACAGGAAACAGACCATG AGC AAG GCC TTC	1.0
pTacTR-1	*tac II*	AAGCTTAAATAAGGAGGAAAAAAAAATG AGC AAG GCC TTC	5.2
pT7TR-3+	T7	AAGCTTAAAATAAGGAGGAAAAAAAAATG AGC AAG GCC TTC	5.2

[a] Expression levels were determined by assay of crude extracts with the substrate analogue deazatrypanothione.

three-piece ligation containing *Eco*RI–*Stu*I-digested pKKTR-2, the 0.3-kb *Eco*RI–*Hin*dIII *tac II* promtoer fragment from pSD8SP, and the 31-bp ribosome binding site containing linker with *Hin*dIII- and *Stu*I-compatible ends. To minimize unwanted ligation products, pKKTR-2 was treated with alkaline phosphatase following digestion with *Eco*RI, and the 31-bp linker was not phosphorylated. Sequence analysis of pTacTR-1 confirmed the construction as shown in Table II.

Expression of trypanothione reductase from pTacTR-1 was assayed as described for the initial expression vector pKKTR-2. To preclude the possibility of background contamination of trypanothione reductase with *E. coli* glutathione reductasee, a glutathione reductase deficient strain, SG5, was used to assay for reductase expression. As shown in Table II, the expression vector pTacTR-1 gave a 5-fold increase in trypanothione reductase expression levels compared with pKKTR-2 and was thus used for large-scale preparation of *T. congolense* trypanothione reductase.

In an attempt to further increase the level of trypanothione reductase expression, the reductase gene and the strong ribosome binding site of pTacTR-1 were placed downstream of a T7 RNA polymerase promoter. This was accomplished by removing the reductase gene and the ribosome binding site from pTacTR-1 as a 1.5-kb *Hin*dIII fragment and ligating it to *Hin*dIII-digested pBS+ to create pT7TR-3+. *E. coli* transformed with pT7TR-3+ and pGP1-2 were grown and induced by raising the temperature to 42 °C. The level of trypanothione reductase in crude extracts of these cells was identical with that from *E. coli* transformed with pTacTR-1 (Table II).

Purification of T. congolense Trypanothione Reductase. As shown in Table III *T. congolense* trypanothione reductase was purified 107-fold to apparent homogeneity from *E. coli* as judged by Coomassie blue staining of a 8% SDS–polyacrylamide gel (data not shown). The protocol employed (Materials and Methods) was similar to that described for the purification of the crithidial enzyme from *C. fasciculata* (Shames et al., 1986). The only change was that chromatography on hydroxyapatite proved to be unnecessary and was omitted. *E. coli* strain JM105 transformed with pTacTR-1 was used for large-scale preparation of trypanothione reductase (Figure 4). Since *E. coli* strain JM105 contains an active glutathione reductase gene, there was the possibility of contamination of the vector-encoded trypanothione reductase with the chromosomally encoded glutathione reductase. However, the two disulfide reductases were well resolved by chromatography on

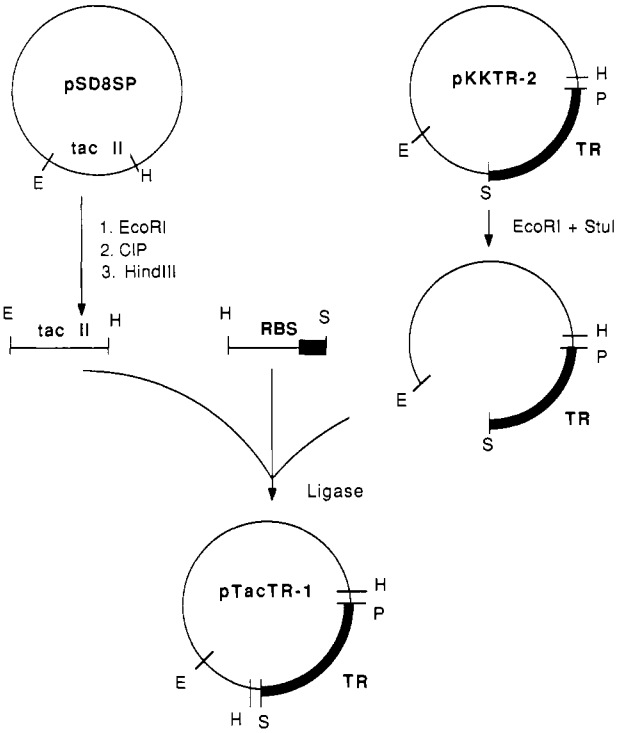

FIGURE 3: Construction of trypanothione reductase expression vector pTacTR-1. Schematic of steps in construction of pTacTR-1 showing isolation of *tac II* promoter from pSD8SP and the introduction of a strong ribosome binding site on a short synthetic linker. These two fragments are used to replace the existing *trc* promoter and ribosome binding site of pKKTR-2. The solid line indicates trypanothione reductase gene sequence. Restriction sites are indicated by single-letter codes: E, *Eco*RI; H, *Hin*dIII; P, *Pss*tI; S, *Stu*I; RBS, ribosome binding site.

Table III: Purification of *T. congolense* Trypanothione Reductase from *E. coli* SG5

step	vol (mL)	total protein (mg)[a]	total act. (units)[b]	sp act. (units/mg)	purification (*x*-fold)	yield (%)
crude	25	575	153	0.27		
40–60% ammonium sulfate	37	370	122	0.33	1.2	80
DEAE-Sephacel	27	6.7	98	14.6	54	64
2′,5′-ADP–Sepharose	16	3.2	93	28.9	107	61

[a] Protein was determined by the method of Bradford (1976). [b] Activity was measured with the substrate analogue deazatrypanothione; units are micromoles of NADPH consumed per minute.

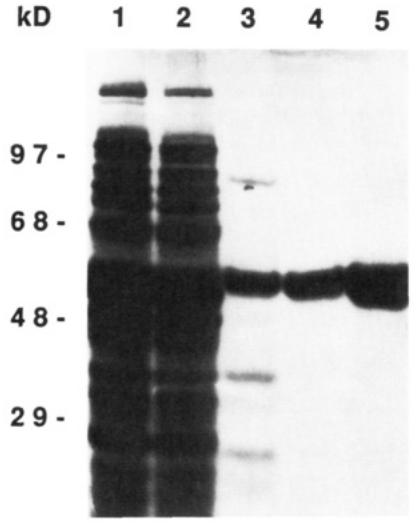

FIGURE 4: SDS–PAGE analysis of large-scale trypanothione reductase purification. An 8% SDS–polyacrylamide gel stained with Coomassie blue is shown: (lane 1) protein in crude extract; (lane 2) protein after 40–60% ammonium sulfate cut; (lane 3) protein following chromatography on DEAE-Sephacel; (lane 4) protein following chromatography on 2′,5′-ADP–Sepharose; (lane 5) same as lane 4 but overloaded to show purity.

Table IV: Amino-Terminal Analysis of Purified Trypanothione Reductase

purified protein	Ser-Lys-Ala-Phe-Asp-Phe-Asp-Leu-Val-Ile-Ile-Gly-Ala-Gly
expected	Met-Ser-Lys-Ala-Phe-Asp-Phe-Asp-Leu-Val-Ile-Ile-Gly-Ala-Gly

DEAE-Sephacel, where trypanothione reductase eluted at 0.1 M KCl and glutathione reductase eluted above 0.2 M KCl. Furthermore, trypanothione reductase purified from strain JM105 showed exclusive substrate specificity for trypanothione versus glutathione, indicating the lack of any contaminating glutathione reductase.

Physical and Spectroscopic Characterization of T. congolense Trypanothione Reductase. T. congolense trypanothione reductase, as purified from *E. coli*, migrated on SDS–polyacrylamide gel electrophoresis with a subunit molecular weight of 52 000. This value is similar to the molecular weight of 53 443 anticipated from the predicted primary sequence (Shames et al., 1988). Amino-terminal sequencing of the purified reductase revealed that translation initiated at the expected codon although the initiator methionine was post-translationally deleted (Table IV). The amino acid composition was as expected from the gene sequence (data not shown). Together these data support that the protein isolated was indeed fully intact *T. congolense* trypanothione reductase. The molecular weight of the native enzyme was determined to be 110 000. This value is indicative of a dimer structure consistent with that found with the enzymes from *C. fasciculata* (Shames et al., 1986) and *T. cruzi* (Krauth-Siegel et al., 1987).

The trypanothione reductase from *T. congolense* showed an electronic spectrum with absorption maxima at 268, 378, and 464 nm (see Figure 5). Titration of the enzyme with 1 equiv of NADPH under anaerobic conditions gave the characteristic absorption maximum at 530 nm expected for the formation of a charge-transfer complex between the oxidized flavin and now reduced active site dithiol. The extended absorption from 600 to 800 nm may reflect the charge-transfer complex of reduced flavin and enzyme-bound $NADP^+$. Taken together, the spectra are similar to that observed with trypanothione reductases from *C. fasciculata* (Shames et al., 1986) and *T. cruzi* (Krauth-Siegel et al., 1987) and are typical for other

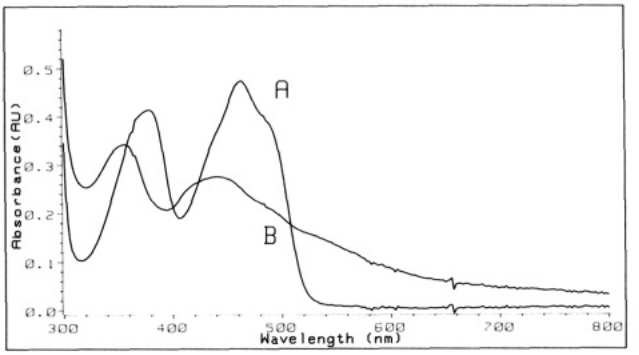

FIGURE 5: Anaerobic titration of trypanothione reductase with NADPH. Spectra of (A) oxidized trypanothione reductase and (B) enzyme after reduction with 1 equiv of NADPH.

redox disulfide-containing flavoprotein reductases (Fox & Walsh, 1982; Williams, 1976).

To identify the flavin at the active site of *T. congolense* trypanothione reductase, the flavin was liberated by thermal denaturation of the protein at 100 °C for 10 min. The resulting free flavin showed absorption maxima at 370 and 450 nm, and the ratio of A_{464} of the enzyme-bound flavin to A_{450} of the free flavin yielded an extinction coefficient for the reductase of $\epsilon_{464} = 10.6\ mM^{-1}\ cm^{-1}$. This value is slightly lower than the extinction coefficient determined for trypanothione reductase from *C. fasciculata* (Shames et al., 1986) and *T. cruzi* (Krauth-Siegel et al., 1987) which have $\epsilon = 11.3\ mM^{-1}\ cm^{-1}$, the same as free flavin. The liberated flavin was identified by HPLC to be FAD as described under Materials and Methods. It eluted with a capacity ratio[2] of 4.11, very close to that of FAD, 4.12, and well resolved from those of FMN, 3.35, and riboflavin, 2.69. Furthermore, coinjection of the enzyme-derived flavin with FAD yielded a single peak.

Kinetic Characterization. T. congolense trypanothione reductase displayed high specificity for trypanothione and NADPH as substrates. At pH 7.8, both substrates showed Michaelis–Menten kinetics. The K_m for trypanothione at saturating NADPH was 31 ± 6 μM, and the apparent K_M for NADPH at 26 μM trypanothione was 5 ± 2 μM. The K_M for the substrate analogue deazatrypanothione at saturating NADPH was 120 ± 7 μM. Glutathione was an extremely poor substrate of *T. congolense* trypanothione reductase with 100 mM glutathione eliciting less than 0.01% the rate of reduction as 50 μM trypanothione. The k_{cat} for trypanothione at saturating NADPH was determined to be 9600 ± 900 min^{-1}, and that for deazatrypanothione was 5000 ± 100 min^{-1}. *T. congolense* trypanothione reductase also utilized NADH as a source of reducing equivalents, albeit at a much-reduced rate. The apparent K_M for NADH at 26 μM trypanothione was 96 ± 12 μM or 19 times greater than the apparent K_M for NADPH. Reduction of trypanothione was also much less efficient with NADH; the apparent k_{cat} for NADH at 26 μM trypanothione was only 7% of that for NADPH, giving a ratio of k_{cat} to K_M of 310 ± 90 $\mu M^{-1}\ min^{-1}$ for NADPH versus 7 ± 1 $\mu M^{-1}\ min^{-1}$ for NADH.

Discussion

The ability to express trypanothione reductase in a heterologous system at levels high enough to allow for adequate physical and kinetic characterization represents a clear advance in research on the reductase given the difficulty in culturing the parasites, the inherent low level of trypanothione reductase

[2] Capacity ratio = elution time of sample/elution time of void.

Table V: *T. congolense* Trypanothione Reductase Is a Representative Trypanothione Reductase

	trypanothione reductase			glutathione reductase
source	*T. congolense*	*T. cruzi*[a]	*C. fasciculata*[b]	human erythrocyte[c]
flavin	FAD	FAD	FAD	FAD
pyridine dinucleotide	NAPDH	NAPDH	NAPDH	NAPDH
M_r of monomer	54000	50000	54000	50000
oligomeric structure	dimer	dimer	dimer	dimer
E_{Ox}, λ_{max} (nm)	464	460	464	460
ϵ_0 at λ_{max} (mM^{-1} cm^{-1})	10.6	ND	11.3	11.3
charge transfer in EH_2				
λ_{max} (nm)	540	550	530	530
ϵ_0 at λ_{max} (nM^{-1} cm^{-1})	3.7	ND	3.6	3.6
K_M (μM)				
trypanothione	31	45	51	
glutathione				65
k_{cat} (min^{-1})				
trypanothione	9600	14200	31000	
glutathione				12000
k_{cat}/K_M (min^{-1} μM^{-1})	310	316	608	185
K_M^{app}(NADPH)	5[d]	5	7[e]	9

[a] Data from Krauth-Siegel et al. (1987). [b] Data from Shames et al. (1986). [c] Data from Pai et al. (1978). [d] At 26 μM trypanothione. [e] At 200 μM trypanothione.

present in the organism, and their pathogenicity. Furthermore, the ability to express *T. congolense* trypanothione reductase in *E. coli* confirms that the gene cloned from *T. congolense* and sequenced recently by Shames et al. (1988) does indeed encode trypanothione reductase. Although this was predicted from the degree of similarity that the gene and its expected gene product displayed to the trypanothione reductases from *C. fasciculata* and *T. cruzi* and to glutathione reductase, heterologous expression of active enzyme is the most convincing evidence. To our knowledge this is the third trypanosomatid enzyme expressed in a heterologous system, the first being thymidylate synthase–dihydrofolate reductase from *Leishmania major* (Groumont et al., 1988) and the second being ornithine decarboxylase from *Trypanosoma brucei* (Phillips et al., 1989). The level of trypanothione reductase expressed in *E. coli*, 1% of the soluble protein, is comparable to the expression levels of the other trypanosomatid enzymes. For example, ornithine decarboxylase was expressed at 0.2% of the soluble protein in *E. coli*, and thymidylate synthase–dihydrofolate reductase was expressed at a level of 2% of the soluble protein in *E. coli*.

The low levels of trypanothione reductase expressed from our original construct, pKKTR-2, prompted us to attempt to increase the expression level. To this end we replaced the promoter and ribosome binding site of pKKTR-2 to yield pTacTR-1. The extended ribosome binding site and stretch of eight adenine residues before the initiation codon present in pTacTR-1 were chosen for their demonstrated efficiency in translational initiation (Shindeling et al., submitted for publication). This led to a 5-fold increase in trypanothione reductase expression. Given the similar strengths of the *trc* and *tac II* promoter, we attribute most of this increased expression to an increase in translational initiation.

In an effort to increase expression levels further, we cloned the reductase gene with the ribosome binding site from pTacTR-1 into pBS+ to create pT7TR-3+, an expression vector with a highly efficient ribosome binding site, having RNA synthesis driven by a T7 promoter. Our initial attempts to express trypanothione reductase from this vector yielded levels identical with those from pTacTR-1. The level of trypanothione reductase expression in *E. coli* transformed pTacTR-1 and pT7TR-1 was about 1% of the soluble protein, as determined by activity assays in crude extracts. This level of expression was consistent with SDS–PAGE analysis of the whole cell lysates (data not shown). Comparison of the whole cell lysates to the soluble and insoluble fraction of these lysates indicated that trypanothione reductase did not form inclusion bodies when expressed in *E. coli*. *T. congolense* trypanothione reductase expressed in *E. coli* reacted weakly with polyclonal antisera raised against the crithidial enzyme.

The 10–20-fold increase in levels of trypanothione reductase present in the recombinant *E. coli* versus the crithidial parasite enabled us to simplify the already straight-forward purification scheme of Shames et al. (1986) to three steps. The success of the purification is due primarily to the early elution of the reductase from the DEAE-Sephacel column, at 0.1 M KCl, resolving the reductase from other NADPH-utilizing enzymes in *E. coli*-, and to the reductase's high affinity for 2′,5′-ADP–Sepharose.

As shown in Table V, *T. congolense* trypanothione reductase is very similar to the trypanothione reductases from *C. fasciculata* and *T. cruzi* as well as human erythrocyte glutathione reductase in its physical and kinetic properties including subunit molecular weight, dimeric structure, optical spectrum, FAD cofactor, and preference for NADPH. While the k_{cat} for *T. congolense* trypanothione reductase is lower than that of the other two trypanothione reductases examined so far, its K_M for trypanothione is also lower. Thus, the ratio of k_{cat} to K_M is about equal for the reductases from *T. congolense* and *T. cruzi*. Most importantly, *T. congolense* trypanothione reductase shows the same strict substrate specificity for trypanothione over glutathione that is characteristic of the other trypanothione reductases. As a representative member of the family of trypanothione reductases, *T. congolense* trypanothione reductase is currently the enzyme of choice for investigation due to its ease of production and the availability of its gene sequence that may allow in vitro mutagenesis experiments.

A crystal structure of trypanothione reductase, which could be compared to the structure of human erythrocyte glutathione reductase, would do much to shed light on the mechanism by which trypanothione reductase discriminates between trypanothione and glutathione. Krauth-Siegel et al. (1987) reported crystals of *T. cruzi* trypanothione reductase but were deterred from proceeding by the limiting amounts of the enzyme available from natural sources. Expression of trypanothione reductase in *E. coli* should solve the problem of enzyme availability.

The ability to express trypanothione reductase in *E. coli* also now affords the opportunity to use site-directed mutagenesis

Table VI: Comparison of Key Residues in Human Erythrocyte Glutathione Reductase to Those in *T. congolense* Trypanothione Reductase[a]

glutathione reductase	function from X-ray structure	trypanothione reductase
$GGGSGGL_{35}$	FAD binding site	$GAGSGGL_{47}$
Cys-58	redox-active disulfide	Cys-52
Cys-63	redox-active disulfide	Cys-57
Tyr-114	stacks between GS moieties in glutathione	Tyr-110
His-467	active site base	His-461
Glu-472	H bonds to active site His	Glu-466
Arg-37	binds carboxylate of GS1 glycine via H bonds	Trp-21

[a] From Shames et al. (1988).

to probe the residues on the reductase involved in discriminating between trypanothione and glutathione. As shown in Table VI many of the residues identified from the X-ray structure as playing a role in catalysis or substrate binding in glutathione reductase (Pai & Schulz, 1983; Wierenga et al., 1986) are present in trypanothione reductase (Shames et al., 1988). Of particular interest to substrate binding is an arginine in glutathione reductase, arginine-37, which in the crystal structure is close enough to bind to the glycyl carboxylate of the glutathione GS_1 moiety (Karplus et al., 1989). In trypanothione reductase this positively charged residue is replaced by tryptophan-21. The substitution of this positively charged residue on the reductase for an uncharged residue corresponds to a change in negative charge on the disulfide substrate as the carboxylate on glutathione becomes an amide in trypanothione (see Figure 1). Also, as discussed in Shames et al. (1988) the presence of a 20 amino acid tail on the C-terminal of trypanothione reductase and a conserved nonapeptide insertion just prior to the active site cysteine residues may also contribute to substrate discrimination.

Acknowledgments

We gratefully acknowledge the contributions of Dr. O. Peoples in constructing pKKTR-2, Dr. S. Tabor for his knowledgeable assistance with the T7 expression system, and Dr. L. Gold for suggesting the ribosome binding site used in constructs pTacTR-1 and pT7TR-3+. We thank Dr. L. Gold, Dr. G. Henderson, Dr. R. Perham, and Dr. S. Tabor for their generous gifts of materials used in these experiments.

Registry No. Trypanothione reductase, 102210-35-5.

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