Purification and Characterization of Trypanothione Reductase from *Crithidia fasciculata*, a Newly Discovered Member of the Family of Disulfide-Containing Flavoprotein Reductases†

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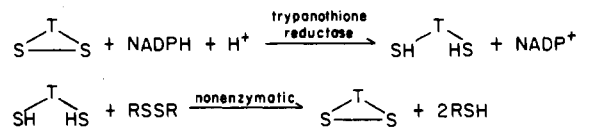
ABSTRACT: Trypanothione reductase from *Crithidia fasciculata* has been purified ca. 1400-fold to homogeneity in an overall yield of 60%. The pure enzyme showed a pH optimum of 7.5–8.0 and was highly specific for its physiological substrates NADPH and trypanothione that had K_m values of 7 and 53 μM, respectively. Trypanothione reductase was found to be a dimer of identical subunits with M_r 53 800 each. The enzyme displayed a visible absorption spectrum that was indicative of a flavoprotein with a λ_{max} at 464 nm. The flavin was liberated by thermal denaturation of the protein and identified, both by high-performance liquid chromatography (HPLC) and by fluorescence studies, as FAD. The extinction coefficient of pure enzyme at 464 nm was determined to be 11.3 mM^{-1} cm^{-1}. Upon titration with 5,5′-dithiobis(2-nitrobenzoic acid), oxidized enzyme was found to contain 2.2 (±0.1) free thiols, whereas NADPH-reduced enzyme showed 3.9 (±0.3). Furthermore, whereas oxidized enzyme was stable toward inactivating alkylation by 2.0 mM iodoacetamide, NADPH-reduced enzyme was inactivated with a half-life of 14 min. These data suggested that a redox-active cystine residue was present at the enzyme active site. Upon reduction of the enzyme with 2 electron equiv of dithionite, a new peak in the absorption spectrum was observed at 530 nm, thus indicating that a charge-transfer complex between one of the newly reduced thiols and the oxidized FAD had formed. The active-site peptide of trypanothione reductase was isolated by alkylation of NADPH-reduced enzyme with iodo[1-^{14}C]acetamide followed by trypsinization of the protein and HPLC purification of the labeled peptide. The labeled peptide was sequenced 24 residues and found to contain 2 cysteine moieties. A comparison of the amino acid sequence of the purified peptide with that of the active-site peptide of glutathione reductase from human erythrocytes revealed homology through 14 residues. However, these two enzymes showed mutually exclusive substrate specificities for their respective disulfide-containing substrates. Thus, trypanothione reductase is a new member of the family of disulfide-containing flavoproteins that includes glutathione reductase, lipoamide dehydrogenase, and mercuric reductase.

Glutathione reductase is a ubiquitous enzyme that catalyzes the NADPH-dependent reduction of intracellular oxidized glutathione. This enzyme has been isolated from several sources including yeast (Racker, 1955), *Escherichia coli* (Pigiet & Conley, 1977), and human erythrocytes (Worthington & Rosemeyer, 1974). All glutathione reductases studied to date have been shown to be dimeric flavoproteins that have a redox-active disulfide in each of their active sites. Furthermore, these enzymes have identical substrate specificities and similar molecular weights and amino acid compositions, and, upon two-electron reduction with NADPH or dithionite, all form a charge-transfer complex between one of the newly generated active-site thiols and the oxidized FAD (Williams, 1976; Pai et al., 1978).

Recently, Fairlamb and Cerami (1985) demonstrated that the NADPH-dependent reduction of oxidized glutathione, glutathione–coenzyme A mixed disulfide, or cystine by dialyzed crude cell extracts from a variety of trypanosomatids required the presence of an unidentified low molecular weight disulfide-containing compound that underwent an enzyme-catalyzed reduction. This compound was later isolated from the insect trypanosomatid *Crithidia fasciculata* by Fairlamb et al. (1985), who identified it to be a novel glutathione analogue, N^1,N^8-bis(glutathionyl)spermidine. This unique 24-membered macrocyclic disulfide, that was also found in several other trypanosomatids including *Trypanosoma brucei*, *Leishmania mexicana*, and *Trypanosoma cruzi*, was given the trivial name trypanothione. The structures of oxidized and reduced trypanothione are shown in Scheme I. The various trypanosomatids examined that contained trypanothione were found to be devoid of any detectable trypanothione-independent glutathione reductase activity in dialyzed crude cell extracts. Thus, the trypanothione-dependent reduction of glutathione by crude extracts from trypanosomatids is likely due to a nonenzymatic disulfide exchange reaction between enzymatically reduced trypanothione and oxidized glutathione.

In the present paper, we show that the enzyme trypanothione reductase catalyzes the NADPH-dependent reduction of trypanothione, but not glutathione. Ultimately, reduced trypanothione is capable of undergoing a rapid nonenzymatic disulfide exchange reaction with intracellular disulfides, among them oxidized glutathione and cystine. The reaction is summarized as



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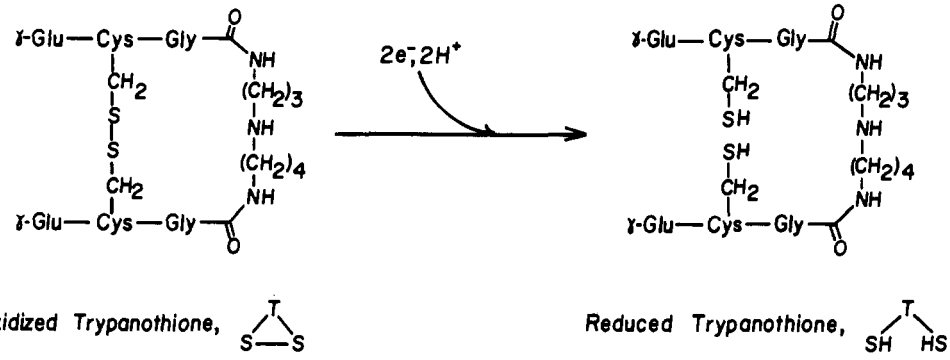
Scheme I



The fact that trypanothione reductase and its substrate are unique to trypanosomatids (Fairlamb & Cerami, 1985) suggests a viable target for the design of new chemotherapeutic agents against these parasitic infections. As a first step in analysis of this novel parasitic enzyme, we report here the purification and characterization of trypanothione reductase from the insect trypanosomatid *Crithidia fasciculata*.

Materials and Methods

Materials. Chromatography resins were from the following sources: DEAE-Sephacel and 2′,5′-ADP–Sepharose were from Pharmacia, hydroxylapatite was from Bio-Rad, and Ultrogel AcA-44 was from LKB. Iodo[1-^{14}C]acetamide (25 mCi mM^{-1}) was from New England Nuclear. Protein molecular weight standards were obtained from Sigma. Trypanothione was prepared by Dr. Graeme Henderson (The Rockefeller University). Details of the synthetic procedure will be published elsewhere (Henderson et al., 1986). Human erythrocyte glutathione reductase was a kind gift of Dr. Heiner Schirmer (Biochemistry Institute, University of Heidelberg). All other reagents and chemicals were of the highest grade commercially available.

Growth of C. fasciculata. Crithidia fasciculata (wild type) were grown in 20-L carboys at 24 °C with vigorous aeration. The medium contained 0.5% yeast extract, 0.4% Bacto-Tryptone, 2% sucrose, 0.02% streptomycin sulfate, 0.0002% hemin, and 2.5 mL L^{-1} triethanolamine (pH 8.0). The growth medium was supplemented with the following vitamins (given as milligrams per liter): nicotinamide, 4; calcium pantothenate, 4; FMN, 2.4; pyridoxamine, 1.2; thiamin, 1.2; folic acid, 4; biotin, 0.2. The yield of cells was ~6 g L^{-1}.

Enzyme Assays. Trypanothione reductase activity was routinely assayed by monitoring the trypanothione-dependent oxidation of NADPH at 340 nm. One unit of activity is defined as the amount of enzyme required to convert 1 μmol of NADPH to NADP$^+$ per minute at 27 °C. The standard assay mixture contained 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)[1] (pH 7.8), 0.5 mM EDTA, 0.25 mM NADPH, and 0.05 mM trypanothione. Absorbance changes were monitored with a Perkin-Elmer 554 recording spectrophotometer with a thermostated cuvette chamber. The pH–activity profile covering the range pH 6.1 to pH 9.0 was obtained by using an equimolar mixture of Pipes, Hepes, and Bicine buffers (33 mM each).

Protein concentrations were determined by using a microscale version of the biuret assay described by Layne (1957) or by the method of Bradford (1976) as supplied by Bio-Rad. Bovine serum albumin was used as a standard for both assays.

Purification of Trypanothione Reductase. Trypanothione reductase was purified ca. 1400-fold to homogeneity in an overall yield of 60%. The buffer solutions utilized were buffer A [20 mM potassium phosphate (pH 7.2), 1 mM EDTA, and 1 mM DTT], buffer B [20 mM Bis-Tris-propane (pH 7.4), 1 mM EDTA, and 1 mM DTT], and buffer C [10 mM potassium phosphate (pH 7.2), 0.5 mM EDTA, and 1 mM DTT]. All operations were carried out at 4 °C.

Ammonium Sulfate Fractionation. C. fasciculata (250 g of frozen paste) were suspended in 220 mL of buffer A and sonicated with 10 1-min pulses with intermittent 1-min cooling periods. After the cellular debris was removed by centrifugation, the supernatant was adjusted to 3% (w/v) with streptomycin sulfate and then to 40% saturation with $(NH_4)_2SO_4$. After the mixture was gently stirred for 0.5 h, the nucleic acids and a fraction of the protein were pelleted by centrifugation. The supernatant was then adjusted to 60% saturation with $(NH_4)_2SO_4$, and after centrifugation, the resulting pellet was dissolved in a minimal quantity of buffer B. The enzyme solution containing trypanothione reductase was then dialyzed extensively against buffer B.

DEAE-Sephacel Chromatography. The dialyzed protein solution was applied to a DEAE-Sephacel column (4 × 36 cm) that had been equilibrated in buffer B. After the column was washed with 1.3 L of buffer B, the remaining proteins were eluted with a linear salt gradient (0.0–0.4 M KCl in buffer B, 1.5 L of each), and 15-mL fractions were collected. Trypanothione reductase eluted at 0.13 M KCl. The fractions containing trypanothione reductase activity were pooled and concentrated on an Amicon concentrator (PM 10 membrane) to 70 mL. The concentrated enzyme solution was dialyzed extensively against buffer C.

Hydroxylapatite Chromatography. The dialyzed solution was applied to a hydroxylapatite column (3.3 × 16 cm) that had been equilibrated in buffer C. The column was washed with 500 mL of buffer C and eluted with a linear phosphate gradient (0.01–0.15 M phosphate in buffer C, 400 mL of each). Fractions (8 mL each) were collected. Two peaks of trypanothione reductase were found. The first eluted in the latter 200 mL of the column wash, and the second eluted with the gradient at 20 mM phosphate. Since the specific activities of these two peaks were identical, the fractions were combined and diluted to 10 mM phosphate by concentration on an Amicon (PM 10) membrane and equilibration with buffer C. The final protein concentration was 1 mg mL^{-1}.

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Bis-Tris-propane, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; PTH, phenylthiohydantoin; Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

2',5'-ADP–Sepharose Chromatography. The combined fractions from the hydroxylapatite column were applied to a 2',5'-ADP–Sepharose column (1.5 × 11 cm) that had been equilibrated in buffer A. Because of the low binding capacity of this resin for trypanothione reductase, only 50% of the sample could be applied at a time. The column was washed with 4 column volumes of buffer A, and the trypanothione reductase activity was eluted with 6 mM $NADP^+$ in buffer A. The fractions that contained trypanothione reductase activity were pooled. At this point, the enzyme preparation showed only one minor contaminant on SDS–polyacrylamide gel electrophoresis and was judged to be >95% pure (Figure 1).

Ultrogel AcA-44 Chromatography. A portion of the pooled fractions containing the trypanothione reductase was purified further by chromatography on Ultrogel AcA-44. The column (2.5 × 110 cm) was equilibrated and eluted with buffer A, and 8-mL fractions were collected. The fractions containing the trypanothione reductase were pooled and adjusted to 90% saturating $(NH_4)_2SO_4$. The specific activity of pure enzyme, stored as an $(NH_4)_2SO_4$ precipitate at 4 °C, decreased less than 10% over 2 months.

Molecular Weight Determination. The subunit molecular weight was determined by using SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) with the following molecular weight standards: bovine serum albumin, 66 000; egg albumin, 45 000; rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, 36 000; carbonic anhydrase, 29 000; trypsinogen, 24 000; trypsin inhibitor, 20 100; α-lactalbumin, 14 200.

The native molecular weight was determined by gel permeation chromatography on an Ultrogel AcA-34 column (1.5 × 82 cm). The column was equilibrated in 25 mM Hepes (pH 7.4) buffer that contained 1 mM EDTA and 1 mM DTT. The molecular weight standards utilized were the following: Blue dextran, void volume; leucine aminopeptidase, 255 000; catalase, 232 000; aldolase, 160 000; glutathione reductase, 120 000; equine liver alcohol dehydrogenase, 80 000; egg albumin, 43 000.

Cross-linking studies of native protein were performed with dimethyl suberimidate as described by Davies and Stark (1970). The trypanothione reductase concentration was 0.5 mg mL^{-1}, and the dimethyl suberimidate concentration was varied between 10 and 40 mM. The reaction was allowed to proceed for 2 h and the cross-linked protein analyzed by SDS–polyacrylamide gel electrophoresis on 5% polyacrylamide gels. The molecular weight standards chosen were β-galactosidase (114 000), egg albumin (43 000), and cross-linked alcohol dehydrogenase that gave species with molecular weights of 40 000, 80 000, 120 000, and 160 000.

Amino Acid Analysis and Sequence. Amino acid analysis and sequencing were performed at the Harvard Microchemistry Facility (Cambridge, MA). The amino acid composition was performed by David Andrews by HPLC of the phenyl isothiocyanate derivatives on a Hewlet-Packard 1084B HPLC that was equipped with an Altex-ODS column. Sequencing was carried out by William Lane on a Beckman 890M microsequencer. The PTH-amino acids were analyzed by HPLC on a Hewlett-Packard 1090 HPLC.

Flavin Identification. The enzyme-bound flavin was liberated by thermal denaturation of the protein at 100 °C for 10 min. The denatured protein was removed by centrifugation, and the free flavin was examined both by high-performance liquid chromatography and by fluorescence spectroscopy.

High-Performance Liquid Chromatography. Riboflavin, FAD, and FMN were separated isocratically by HPLC on an Alltech Associates reversed-phase C-18 column. The isocratic buffer system used, which utilized an ion-pair reagent, was similar to that described by Hoffman and Liao (1977) for the separation of nucleotides. The buffer contained 60 mM monopotassium phosphate (pH 3.5), 15 mM tetra-*n*-butylammonium hydrogen sulfate, 30 mM ammonium chloride, and 40% methanol.

Fluorescence Spectroscopy. The fluorescence of the liberated flavin was examined on a Perkin-Elmer MPF fluorometer (λ^{abs} = 450 nm, λ^{emis} = 535 nm). The flavin sample was subsequently treated with snake venom phosphodiesterase at 37 °C for 10 min and the change in fluorescence monitored.

Thiol Titrations. The number of DTNB-titratable thiols in both oxidized and NADPH-reduced trypanothione reductase was determined under denaturing conditions. For the titration of oxidized enzyme, the reaction mixture contained 3.75 μM trypanothione reductase, 5.5 M guanidine hydrochloride, 0.1 M Tris (pH 8.2), and 1.0 mM EDTA. After 10 min, DTNB was added to a final concentration of 0.1 mM, and the increase in absorbance at 412 nm was monitored. The titration of thiols in reduced enzyme was monitored in a similar fashion except the trypanothione reductase was initially treated with 0.3 mM NADPH for 5–10 min before denaturation with guanidine hydrochloride. For stoichiometric determinations, an extinction coefficient of 13.6 mM^{-1} cm^{-1} (Ellman, 1959) was used for the resulting thionitrobenzoate anion.

The sensitivity of both oxidized and NADPH-reduced trypanothione reductase to inactivating alkylation by iodoacetamide was examined by incubating the enzyme with 2.0 mM iodoacetamide in both the absence and presence of 0.25 mM NADPH. The reaction mixtures contained 50 mM Hepes (pH 7.8) and 0.5 mM EDTA. Aliquots (10 μL) were removed at various times and assayed by using the standard assay mixture described above.

Reductive Dithionite Titration. Trypanothione reductase was reduced with sodium dithionite under anaerobic conditions. The spectra were recorded on a Perkin-Elmer 554 spectrophotometer at 25 °C. The titrations were carried out in anaerobic cuvettes (Williams et al., 1979) under an argon atmosphere. Dithionite was added with a Hamilton gas-tight syringe that was fitted with a screw-thread plunger. The buffer utilized was 50 mM potassium phosphate (pH 7.6) that contained 0.5 mM EDTA. The concentration of the dithionite titrant was determined by anaerobic titration of riboflavin using an extinction coefficient at 450 nm of 12.2 mM^{-1} cm^{-1}.

Isolation of the Active-Site Peptide. The active-site peptide of trypanothione reductase was labeled by inactivating alkylation of the NADPH-reduced enzyme with iodo[1-^{14}C]-acetamide. Following a complete trypsin digest, the labeled peptide was purified by high-performance liquid chromatography as described below.

Alkylation of Trypanothione Reductase with Iodo[1-^{14}C]acetamide. Trypanothione reductase was alkylated with iodo[1-^{14}C]acetamide under conditions that gave a half-life for alkylative inactivation of 14 min. The reaction mixture contained 14.5 nmol of trypanothione reductase, 50 mM Hepes (pH 7.8), 0.5 mM EDTA, and 0.5 mM NADPH. After 10 min, iodo[1-^{14}C]acetamide (specific activity of 12.2 mCi $mmol^{-1}$) was added to a final concentration of 2.0 mM, and the reaction was allowed to proceed 1 h. The reaction was quenched by the addition of mercaptoethanol to a final concentration of 4.0 mM. To determine the stoichiometry of alkylation, two aliquots of the reaction mixture (30 and 40 μL) were removed prior to quenching and added to 20 μL of 0.1 M mercaptoethanol. The protein was precipitated with

Table I: Purification of Trypanothione Reductase from *C. fasciculata*

step	vol (mL)	total protein (mg)	total units	sp act. (units/mg)	purification (*x*-fold)
crude	450	29970	6390	0.21	
3% streptomycin sulfate, 40% $(NH_4)_2SO_4$	395	7426	5649	0.76	3.6
40–60% $(NH_4)_2SO_4$	214	4708	5671	1.20	5.6
DEAE-Sephacel	282	620	5753	9.27	44
hydroxylapatite	70	115	4136	36.0	169
2′,5′-ADP–Sepharose	16.4	14.4	4120	286	1362
Ultrogel[a] AcA-44	12.4	9.8	2889	295	1404

[a] Only 3100 units of activity were applied to the column.

10% trichloroacetic acid and the resulting pellet washed with five portions of acetone. The pellets were dissolved in 0.5 mL of 0.5 N KOH, and the radioactivity was determined on a Beckman LS 1800 scintillation counter. The samples yielded a labeling stoichiometry of 0.85 (±0.01) mol of label/mol of FAD.

Exhaustive Reduction and Alkylation. The quenched reaction mixture was concentrated to 0.15 mL on a collodion bag apparatus (25000 molecular weight cutoff, Schleicher & Schuell), and 1 mL of denaturation buffer, that contained 50 mM Tris (pH 8.1), 6.0 M guanidine hydrochloride, and 3 mM mercaptoethanol, was added. This concentration procedure was repeated 6 times to remove unbound radioactivity. After the protein was allowed to stand in denaturation buffer overnight, unlabeled iodoacetamide was added to a final concentration of 7.0 mM. After 1.5 h, the reaction mixture was quenched by the addition of 6 mM mercaptoethanol. The labeled protein was then washed 3 times by sequential concentration to 0.15 mL and dilution to 1 mL with denaturation buffer.

Proteolysis of Denatured Protein. The labeled protein was concentrated to 0.15 mL, and a mixture of 0.15 mL of denaturation buffer in 0.7 mL of ammonium bicarbonate (0.5 M) was added. Following the addition of 0.2 mg of TPCK-treated trypsin, the reaction was allowed to proceed 21 h. The reaction mixture was then concentrated to dryness in a collodion bag apparatus. Since less than 5% of the total radioactivity was found in the filtrate, it was apparent that the peptides had bound to the collodion bag. The peptides were recovered in 55% yield by incubating the bag in 4 mL of hexafluoroisopropyl alcohol for 1 h. The solvent was removed at room temperature by evaporation under a stream of nitrogen, and the peptides were redissolved in 1 mL of 0.2 M bicarbonate buffer.

Purification of Labeled Peptide. Purification of the labeled peptide was achieved in two steps by HPLC on a Waters μBondapak phenyl column. The solvents employed were solvent A (0.08% TFA in acetonitrile) and solvent B (0.08% TFA in isopropyl alcohol). The elution of the peptides was monitored at 214 nm, and 4-mL fractions were collected. First, the mixture of peptides was chromatographed at 2 mL min^{-1} with a 70-min linear gradient of 10–60% solvent A in 0.15% aqueous TFA. The radioactivity coeluted with a single absorption peak. The fraction containing the labeled peptide was evaporated to dryness and the crude peptide dissolved in 1 mL of 0.15% aqueous TFA. The labeled peptide was then rechromatographed at 2 mL min^{-1} with a 60-min linear gradient of 10–60% solvent B in 0.15% aqueous TFA. Only a single absorbance peak at 214 nm was observed that contained 100% of the radiolabeled peptide.

Results

Purification of Trypanothione Reductase from C. fasciculata. An NADPH-dependent trypanothione reductase from

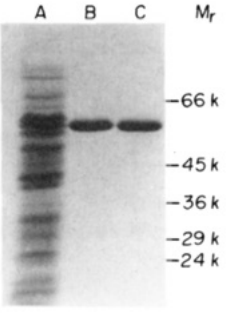


FIGURE 1: Analysis of trypanothione reductase purification by SDS–polyacrylamide gel electrophoresis. (A) 150 μg of crude cell extract; (B) 10 μg of trypanothione reductase following affinity chromatography on 2′,5′-ADP–Sepharose; (C) 10 μg of trypanothione reductase following chromatography on Ultrogel AcA-44.

C. fasciculata was purified ca. 1400-fold in an overall yield of 60% (Table I). Following ammonium sulfate fractionation and column chromatography on DEAE-Sephacel, hydroxylapatite, and 2′,5′-ADP–Sepharose resins, the enzyme showed only a single minor contaminant on SDS–polyacrylamide gel electrophoresis (Figure 1). The contaminant, with M_r 27000 was readily removed by gel permeation chromatography with Ultrogel AcA-44. The purified enzyme showed a single band on SDS–polyacrylamide gel electrophoresis (Figure 1). Enzyme purified through this final step was used in all studies reported in this paper. Trypanothione reductase did not bind to either Orange A Matrex or Reactive Red affinity resins, as do several analogous reductases (Serrano et al., 1984; Fox & Walsh, 1982; Watson et al., 1978); thus, these resins could not be used for purification. However, the enzyme did bind to 2′,5′-ADP–Sepharose, a resin that has been used previously to purify glutathione reductase from several sources (Le Trang et al., 1983; Carlberg & Mannervik, 1977; Mannervik et al., 1976). As expected, no trypanothione-independent glutathione reductase activity could be detected during the purification scheme.

Physical and Kinetic Characterization. Trypanothione reductase was found to have a subunit molecular weight of 53800 by SDS–polyacrylamide gel electrophoresis (average of five separate determinations, data not shown). The molecular weight of native enzyme was determined by gel permeation chromatography on Ultrogel AcA-34 to be 180000 (data not shown). Since these data were suggestive of a unique trimeric structure for trypanothione reductase, a second method was used to examine the molecular weight. The native enzyme was cross-linked with dimethyl suberimidate and the cross-linked protein examined by SDS-polyacrylamide gel electrophoresis. The resulting gel showed two major bands that corresponded to monomeric (M_r 53700) and dimeric (M_r 110000) protein (data not shown). There was no evidence for a trimeric species. On this basis, we conclude that the enzyme may migrate anomalously on Ultrogel AcA-34 and is likely to be a dimer in analogy to other disulfide-containing

Table II: Comparison of Amino Acid Compositions of Trypanothione Reductase and Human Erythrocyte Glutathione Reductase

amino acid	trypanothione reductase[a]	glutathione reductase[b]
Asx	43	38
Thr	29	31
Ser	23	31
Glx	58	40
Pro	13	24
Gly	47	43
Ala	63	42
Val	51	44
Met	10	15
Ile	28	29
Leu	36	34
Tyr	20	13
Phe	18	14
Lys	14	34
His	16	16
Arg	17	17
Cys	12[c]	10
Trp	ND	3
total amino acids	498	478
subunit M_r	53800	50000

[a] The protein was hydrolyzed 24 h. [b] Data from Untucht-Grau et al. (1982). [c] Determined by performic acid oxidation of native enzyme.

flavoprotein reductases; however, this must be resolved in future studies.

Trypanothione reductase was found to be highly specific for trypanothione and NADPH as substrates. Due to minimal quantities of trypanothione, only a limited number of kinetic experiments could be performed. At pH 7.8, both physiological substrates displayed normal Michaelis–Menten saturation kinetics. Whereas the K_m determined for NADPH, with 0.2 mM trypanothione, was 7 μM, the K_m determined for trypanothione with saturating NADPH was 53 μM. When 0.25 mM NADH was substituted for NADPH, the rate of trypanothione reduction was ca. 0.3% of the rate supported by NADPH. With fixed concentrations of 0.1 mM trypanothione and 0.25 mM NADPH, the enzyme displayed a pH optimum of 7.5–8.0. Larger quantities of trypanothione will be necessary to determine the effects of pH on the kinetic parameters V_{max} and V_{max}/K_m. Oxidized glutathione was found to be a very poor substrate of trypanothione reductase. For example, at a concentration of 50 mM glutathione, the rate of NADPH oxidation was ca. 0.01% of the rate observed with 50 μM trypanothione under identical conditions. Furthermore, neither lipoamide, lipoic acid, or DTNB, examined at concentrations as high as 5.0 mM, displayed any detectable substrate activity. Interestingly, in similar fashion to the rigid substrate specificity of trypanothione reductase, glutathione reductase from human erythrocytes was found to reduce 0.28 mM trypanothione at a rate less than 0.08% of the rate of glutathione reduction. Thus, these two enzymes show mutually exclusive substrate specificity.

The specific activity determined for pure trypanothione reductase of 284 units mg^{-1} was determined at a trypanothione concentration of 50 μM. Thus, extrapolating to conditions of saturating trypanothione, a specific activity of ca. 582 μmol min^{-1} mg^{-1} is expected. This corrected value yields a turnover number of ca. 31 000 min^{-1}. This turnover number is comparable to the values of 33 000 and 12 600 min^{-1} determined previously for lipoamide dehydrogenase (Massey et al., 1960) and glutathione reductase (Worthington & Rosemeyer, 1976), respectively.

Amino Acid Composition. The amino acid composition determined for trypanothione reductase is shown in Table II.

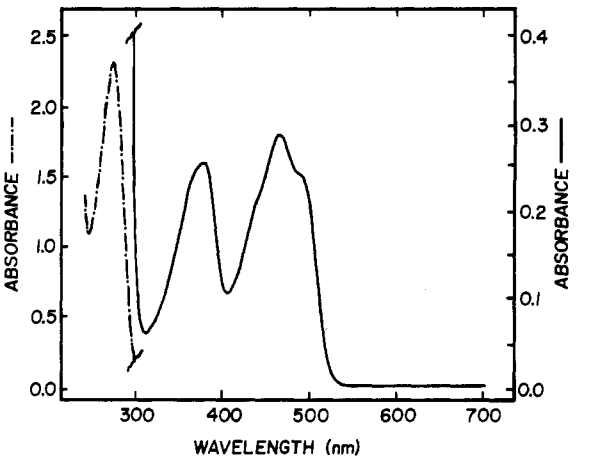


FIGURE 2: Absorbance spectrum of purified trypanothione reductase. Two scales are drawn to include the absorbance at 276 nm.

As shown in Table II, the overall composition is quite similar to that of human erythrocyte glutathione reductase. A major difference between the two proteins appears to be that trypanothione reductase is a more acidic protein. This observation is based on the relative amounts of Glx, Asx, and Lys residues. Attempts to sequence the N-terminus were unsuccessful, suggesting that the terminal amino acid is blocked. Because of a limited quantity of enzyme, no attempts were made to identify the blocking group.

Spectroscopic Properties. Trypanothione reductase has an absorption spectrum similar to that found for glutathione reductase from yeast (Massey & Williams, 1965) and human erythrocytes (Worthington & Rosemeyer, 1974). The spectrum showed absorption maxima at 276, 375, and 464 nm that are indicative of a flavoprotein (Figure 2). The A_{276}/A_{464} ratio of native enzyme was determined to be 10.4 (±0.4). However, enzyme that had been stored as its ammonium sulfate precipitate subsequently gave a lower ratio of 8.2 (±0.2), a value indicative of NADP(H)-free enzyme. Thus, in similar fashion to mercuric reductase isolated from *Pseudomonas aeruginosa* (Fox & Walsh, 1982), trypanothione reductase may bind $NADP^+$ very tightly.

The enzyme-bound flavin was readily liberated by thermal denaturation of the protein at 100 °C for 10 min. The resulting free flavin, now with an absorption maximum at 450 nm, had an extinction coefficient equal to the enzyme-bound species (at 464 nm). When examined by HPLC (see Materials and Methods), the flavin was found to elute with a capacity ratio[2] of 3.23. The capacity ratio is nearly identical with the ratio of 3.33 determined for FAD. Riboflavin and FMN, that were also examined as standards, had capacity ratios of 1.52 and 2.40, respectively. Furthermore, when the flavin and FAD were coinjected, a single elution peak at 254 nm was observed. To confirm the identity of the enzyme-bound flavin as FAD, the flavin was treated with snake venom phosphodiesterase, and the change in fluorescence (λ^{emis} = 535 nm) was monitored. The observed 9.3-fold increase in fluorescence was similar to the value of 10-fold reported for the conversion of FAD to FMN by Bessey et al. (1949). Taken together, these results suggest that the enzyme-bound flavin is FAD and that the extinction coefficient of native enzyme at 464 nm is identical with that of free FAD with E_{450} = 11.3 mM^{-1} cm^{-1}.

Thiol Titrations. If the catalytic mechanism of trypanothione reductase is similar to that of glutathione reductase,

[2] The capacity ratio is defined as compound elution time/elution time of void.

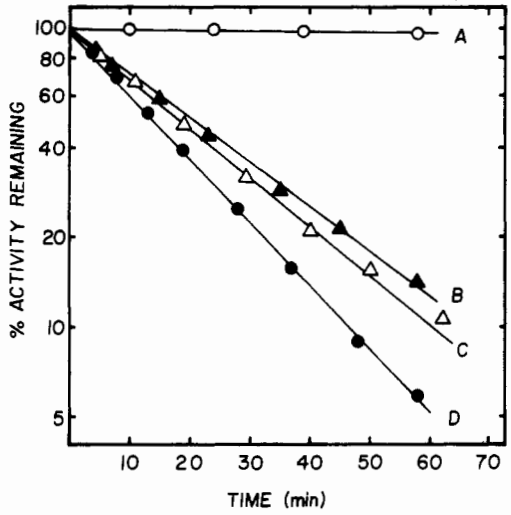


FIGURE 3: Inactivating alkylation of trypanothione reductase by iodoacetamide. The reaction conditions are provided under Materials and Methods. (A) Oxidized enzyme; (B) NADPH-reduced enzyme plus 50 mM oxidized glutathione; (C) NADPH-reduced enzyme plus 25 mM oxidized glutathione; (D) NADPH-reduced enzyme.

a redox-active cystine residue should be present in the enzyme active site. Thus, enzyme that is reduced with 2 electron equiv is expected to have two additional free thiols as compared to the oxidized species. To examine this, both oxidized enzyme and NADPH-reduced enzyme were denatured with guanidine hydrochloride and reacted with DTNB (see Materials and Methods). Using an extinction coefficient of 11.3 mM^{-1} cm^{-1} for native trypanothione reductase, the number of thiols calculated for oxidized enzyme was 2.2 (±0.1) whereas the reduced enzyme gave a value of 3.9 (±0.3). Thus, the increase of ca. 1.7 thiols upon NADPH reduction is consistent with the presence of a redox-active cystine residue in the active site of trypanothione reductase. To examine the chemical reactivity of the redox-active cystine moiety, the susceptibility of oxidized and NADPH-reduced enzyme to inactivating alkylation with 2.0 mM iodoacetamide was examined. In similar fashion to glutathione reductase (Arscott et al., 1981), lipoamide dehydrogenase (Thorpe & Williams, 1976), and mercuric reductase (Fox & Walsh, 1983), oxidized trypanothione reductase was found to be stable to inactivating alkylation (line A of Figure 3), whereas the NADPH-reduced enzyme was inactivated with a half-life of 14 min (line D of Figure 3). Taken together, these data are suggestive of a catalytic mechanism for trypanothione reductase where electron flow proceeds from NADPH to a redox-active cysteine residue via a noncovalently bound FAD moiety.

Initial kinetic experiments showed that the rate of glutathione reduction by trypanothione reductase was at least 10000-fold slower than that for trypanothione. To determine if oxidized glutathione could bind at the enzyme active site, its ability to protect NADPH-reduced enzyme against inactivating alkylation by 2.0 mM iodoacetamide was examined. If a simple mechanism is considered where oxidized glutathione binds only to NADPH-reduced enzyme, and the resulting complex is protected from reaction with iodoacetamide, the observed rate constant for inactivating alkylation (k^{obsd}) is derived to be

$$k^{obsd} = \frac{k^{inact}[TR][IA]}{1 + [GSSG]/K_d}$$

where k^{inact} is the second-order rate constant for inactivation, [TR], [IA], and [GSSG] are the concentrations of trypanothione reductase, iodoacetamide, and oxidized glutathione,

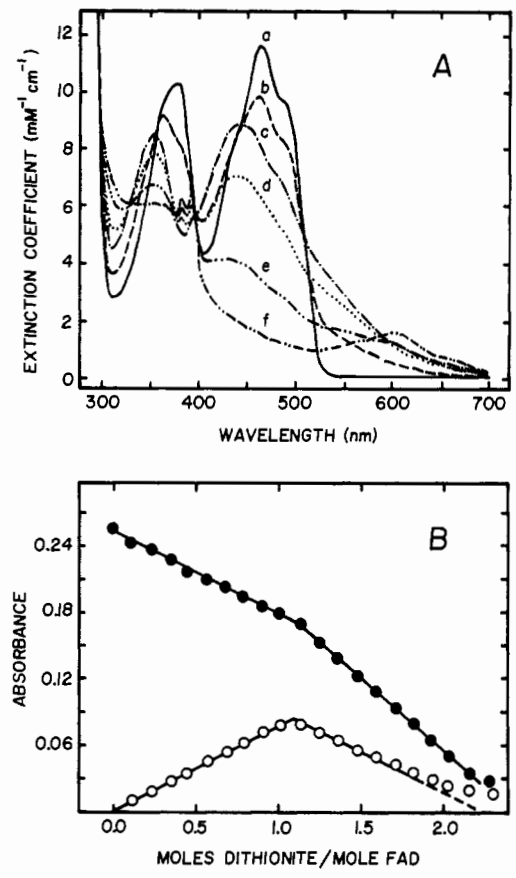


FIGURE 4: Anaerobic reductive dithionite titration of 22.4 μM (14.8 nmol) trypanothione reductase. (A) Spectral changes observed upon the addition of the following number of dithionite equivalents: a, 0.00; b, 0.46; c, 1.02;, d, 1.36; e, 1.82; f, 2.16. (B) Plot showing spectral changes at (●) 464 nm and (○) 530 nm as a function of the stoichiometry of reducing equivalents added.

respectively, and K_d is the dissociation constant for the GSSG–reduced enzyme complex. The results from this experiment, shown in Figure 3, demonstrate that oxidized glutathione provides only limited protection against inactivation. The pseudo-first-order rate constants determined for inactivating alkylation in the presence of 25 and 50 mM oxidized glutathione were 3.85×10^{-2} and 3.47×10^{-2} min^{-1}, respectively. When these rate constants are compared to the pseudo-first-order rate constant of 4.95×10^{-2} min^{-1} obtained in the control experiment, a K_d of ca. 102 (±13) mM is obtained. Therefore, it appears that the poor substrate activity of glutathione is in large part due to its inability to bind at the enzyme active site.

Charge-Transfer Complex. To determine if two-electron-reduced trypanothione reductase forms a charge-transfer complex after reduction of the internal redox-active disulfide, the enzyme was reduced under anaerobic conditions with dithionite, and spectral changes were monitored. Initial titrations showed that a kinetic barrier existed for dithionite reduction; thus, 3.3 μM methylviologen was added to mediate electron transfer. When a 2 electron equiv of dithionite was added to trypanothione reductase, an increase in absorbance at 530 nm was observed (Figure 4A). Further reduction with dithionite subsequently resulted in the decrease in absorbance at this wavelength. These results are similar to those obtained with several analogous disulfide-containing flavoprotein reductases such as mercuric reductase (Fox & Walsh, 1982), lipoamide dehydrogenase (Matthews & Williams, 1976), and glutathione reductase (Massey & Williams, 1965). The stoichiometry of reduction was examined by plotting the

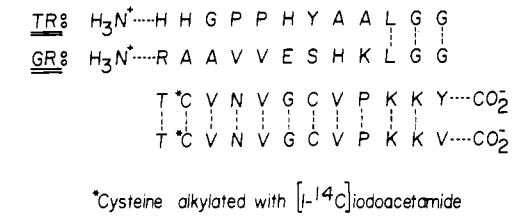


FIGURE 5: Comparison of the active-site peptides from trypanothione reductase (TR) and human erythrocyte glutathione reductase (GR). The homologous residues are indicated with dashed lines.

change in absorbance at both 464 and 530 nm as a function of dithionite equivalents added. The resulting plot, shown in Figure 4B, indicates that 4 electron equiv are necessary to reduce completely trypanothione reductase. Whereas the two-electron-reduced enzyme was found to have isosbestic points at 356, 396, 444, and 508 nm, the fully reduced enzyme showed isosbestic points at 328 nm and at ~390 nm, the latter obscured by the absorbance of reduced methylviologen. Once the trypanothione reductase was reduced with 2 electron equiv, the absorbance of reduced methylviologen both at 395 and at 600 nm was observed. This suggests that the $E^{\circ\prime}$ for the reduction of the two-electron-reduced enzyme to the four-electron-reduced enzyme is similar to that of methylviologen with $E^{\circ\prime} = -440$ mV.

Taken together, these results are consistent with the presence of a redox-active disulfide in the active site of trypanothione reductase. Upon reduction, a charge-transfer complex between one of the newly generated thiols and the enzyme-bound FAD is formed. It should be noted that initial dithionite titrations of trypanothione reductase yielded a stoichiometry of 3 dithionite equiv for complete reduction, with 1 equiv of dithionite necessary for the formation of the charge-transfer complex. The titration data shown in Figure 4B were obtained with enzyme that had previously been titrated and then regenerated by air oxidation and dialysis. The additional dithionite equivalent used in the first titration may have reduced a second disulfide present in the native protein.

Active-Site Peptide. When trypanothione reductase was monoalkylated with iodo[1-^{14}C]acetamide followed by treatment with trypsin and HPLC separation of the peptides, only a single peptide showed incorporation of a radioactive label. These data are consistent with the labeling stoichiometry of 0.85 mol of iodo[1-^{14}C]acetamide/mol of FAD found by trichloroacetic acid precipitation of the labeled enzyme (see Materials and Methods). The sequence of the purified labeled peptide is shown in Figure 5. All residues could be clearly identified except for the penultimate amino acid. The data for this cycle, however, were suggestive of a lysine residue. Most striking is that the active-site tetradecapeptides of trypanothione reductase and human erythrocyte glutathione reductase (Krohne-Ehrich et al., 1971; Untucht-Grau et al., 1982) show identical sequence homology (Figure 5). Furthermore, in similar fashion to analogous disulfide-containing flavoenzyme reductases (Williams et al., 1978; Fox & Walsh, 1982), the cysteine closer to the N-terminus was exclusively alkylated by iodo[1-^{14}C]acetamide. The low reactivity of the cysteine closer to the C-terminus toward alkylation by iodoacetamide suggests that it takes part in the charge-transfer complex with enzyme-bound FAD.

Discussion

Trypanothione, N^1,N^8-bis(glutathionyl)spermidine, is a newly discovered derivative of glutathione that has been found in various trypanosomatids including the human pathogens *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leshmania mexicana*. The reduction of oxidized glutathione in dialyzed cell-free extracts of these organisms requires the addition of trypanothione, thus raising the prospect that these various trypanosomatids do not contain a classical glutathione reductase. Since a high intracellular ratio of reduced to oxidized glutathione is essential for many cell types to contend with oxidant stress (Meister & Anderson, 1983; Meister, 1981), we desired to purify the reductase and determine its catalytic properties. The insect trypanosomatid *Crithidia fasciculata* was chosen for an initial biochemical model of the human pathogens *Trypanosoma brucei* and *Trypanosoma cruzi* because of its nonpathogenic nature and ease of mass culture.

Table III: Comparison of Structural and Catalytic Parameters of Trypanothione Reductase and Human Erythrocyte Glutathione Reductase

property	trypanothione reductase	glutathione reductase[a]
flavin	FAD	FAD
pyridine nucleotide	NADPH	NADPH
M_r of monomer	53800	50000
oligomeric structure	dimer	dimer
E_{ox}, λ_{max} (nm)	464	460
ϵ at λ_{max} (mM^{-1} cm^{-1})	11.3	11.3
redox-active disulfide	yes	yes
charge transfer in EH_2	yes	yes
λ_{max} (nm)	530	530[b]
ϵ (mM^{-1} cm^{-1})	3.63	3.6[b]
turnover no. (min^{-1})		
glutathione	3.1[c]	12000
trypanothione	31000	9.6[d]
active-site peptide	e	e

[a] Data taken from Pai et al. (1978) unless otherwise indicated. [b] Values for the yeast enzyme (Bulger & Brandt, 1971). [c] Value determined at 50 mM glutathione. [d] Value determined at 0.28 mM trypanothione. [e] Fourteen residues around active-site cysteines are identical.

The purification to homogeneity of trypanothione reductase from *Crithidia fasciculata* supports the initial observations of Fairlamb and Cerami (1985) which demonstrated that a classical glutathione reductase did not exist in this trypanosomatid. Furthermore, the pure enzyme, that is highly specific for trypanothione, is at least 10000-fold less active at glutathione reduction. Also, oxidized glutathione was found to have a dissociation constant from NADPH-reduced enzyme of greater than 0.1 M, a value that is ~2000-fold higher than the K_m of 53 μM determined for trypanothione. These results heighten the prospect that trypanothione reductase, as a parasite-specific enzyme, may serve as a new target for antiparasitic drug development.

The work reported here demonstrates that trypanothione reductase is a new member of the family of disulfide-containing flavoenzymes that includes glutathione reductase, mercuric reductase, and lipoamide dehydrogenase. Numerous structural and catalytic homologies between the latter three disulfide-containing flavoenzymes have been found (Williams, 1975; Fox & Walsh, 1982; Williams et al., 1982). In similar fashion, trypanothione reductase appears to have striking homology to glutathione reductase. Table III compares the various structural and catalytic parameters of trypanothione reductase to those of human erythrocyte glutathione reductase for which there is a high-resolution crystal structure. These comparisons, taken together with the sequence homology of the active-site peptides of the two enzymes (Figure 5), suggest that trypanothione reductase is a catalyst built along the same lines as glutathione reductase.

Further characterization of trypanothione reductase would be facilitated by gene cloning and sequencing to get at the full primary structure. Our research efforts are now focused in

this direction. Differences between the substrate binding domains of glutathione and trypanothione reductases may reveal information that will aid in the design of highly specific active-site-directed inhibitors. Ultimately, an X-ray structure may be required to aid in the rational design of antitrypanosomatid drugs targeted against this enzyme. However, the mutually exclusive specificity of parasite and host enzymes for their respective disulfide-containing substrates should augur success in that effort.

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Registry No. L-C, 52-90-4; NADPH, 53-57-6; GR, 9001-48-3; TR, 102210-35-5; trypanothione, 96304-42-6.

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