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Substrate Specificity of the Flavoprotein Trypanothione Disulfide Reductase from *Crithidia fasciculata*†

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ABSTRACT: The substrate specificity of the trypanosomatid enzyme trypanothione reductase has been studied by measuring the ability of the enzyme to reduce a series of chemically synthesized cyclic and acyclic derivatives of N^1,N^8-bis(glutathionyl)spermidine disulfide (trypanothione). Kinetic analysis of the enzymatic reduction of these synthetic substrates indicates that the mutually exclusive substrate specificity observed by the NADPH-dependent trypanothione disulfide reductase and the related flavoprotein glutathione disulfide reductase is due to the presence of a spermidine binding site in the substrate binding domain of trypanothione reductase. Trypanothione reductase will reduce the disulfide form of N^1-monoglutathionylspermidine and also the mixed disulfide of N^1-monoglutathionylspermidine and glutathione. The Michaelis constants for these reactions are 149 μM and 379 μM, respectively. Since the disulfide form of N^1-monoglutathionylspermidine and the mixed disulfide of N^1-monoglutathionylspermidine and glutathione could be formed in trypanosomatids, the binding constants and turnover numbers for the enzymatic reduction of these acyclic disulfides are consistent with these being potential alternative substrates for trypanothione reductase in vivo.

The tripeptide L-γ-glutamyl-L-cysteinylglycine (glutathione; GSH) is maintained in high concentration within the cells of eukaryotic and prokaryotic organisms and frequently constitutes the major thiol metabolite within these cells. Important general functions of this peptide are thought to include maintenance of the intracellular thiol redox balance (RSH/RSSR), the scavenging of free-radical species, and functioning as a cofactor in hydrogen peroxide metabolism (Meister,

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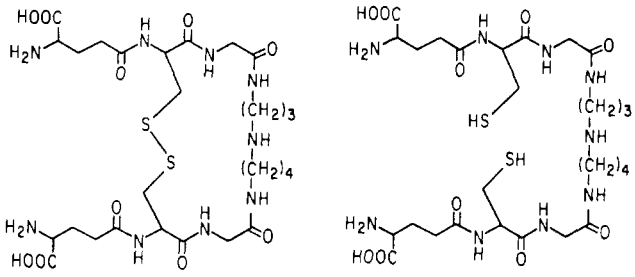


FIGURE 1: Structures of trypanothione disulfide [$T(S)_2$] and dihydrotrypanothione [$T(SH)_2$].

1983). In most cells, a high intracellular ratio of reduced to oxidized glutathione (GSH/GSSG) is maintained by the NADPH-dependent flavoprotein glutathione disulfide reductase (Williams et al., 1978).

Organisms of the trypanosomatid family, including *Trypanosoma brucei*, *Trypanosoma cruzi*, and various *Leishmania* species, are highly unusual in that although these organisms contain glutathione, they lack glutathione reductase and glutathione peroxidase (Fairlamb & Cerami, 1985). In these organisms glutathione and possibly other thiol metabolites are maintained in the reduced (thiol) form through the mediation of the novel glutathione–spermidine conjugate N^1,N^8-bis(glutathionyl)spermidine (trypanothione, see Figure 1) and a NADPH-dependent disulfide reductase (trypanothione reductase). Thus in trypanosomatids, GSSG and other disulfides are reduced in the following manner:

$$T(S)_2 + NADPH + H^+ \rightarrow T(SH)_2 + NADP^+ \quad (1)$$

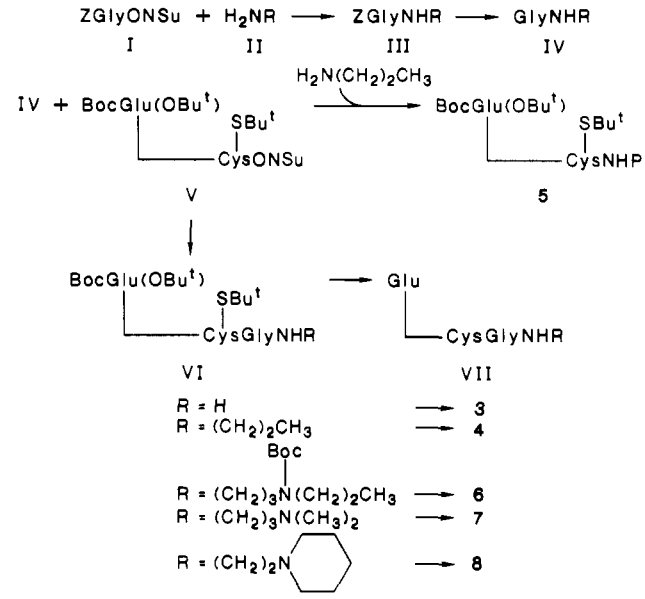
$$T(SH)_2 + RSSR \rightarrow T(S)_2 + 2RSH \quad (2)$$

where reaction 1 is catalyzed by trypanothione disulfide reductase and reaction 2 can occur nonenzymatically by thiol–disulfide exchange reactions with the dithiol [dihydrotrypanothione [$T(SH)_2$]] (Fairlamb et al., 1985).

The enzyme trypanothione reductase has recently been purified to homogeneity from the insect trypanosomatid *Crithidia fasciculata* (Shames et al., 1986). The pure *C. fasciculata* trypanothione reductase was shown to have similar physical and chemical properties to glutathione disulfide reductase. Each of these enzymes contains in its active site both a flavin and a redox-active disulfide which are essential for their respective catalytic function. Also, the two enzymes have similar amino acid compositions and subunit molecular weights. Furthermore, amino acid sequence analysis of their respective catalytic centers shows complete homology in 14 of 23 amino acid residues. Although trypanothione reductase and glutathione reductase share a common catalytic mechanism, the two enzymes display mutually exclusive substrate specificities; trypanothione reductase does not reduce glutathione disulfide at a significant rate, and conversely trypanothione disulfide is not a substrate for either yeast or human glutathione reductase. An understanding of the molecular basis of these enzyme substrate specificities is of key importance to the development of strategies for the specific inhibition of the parasite enzyme and possibly to the design of new therapeutic agents against parasite infection.

We report here that the substrate specificity displayed by trypanothione reductase is in part due to the presence in the substrate binding domain of a binding region for the spermidine portion of trypanothione. The presence of such a spermidine binding site in trypanothione reductase enables N^1-monoglutathionylspermidine disulfide and also the mixed disulfide of N^1-monoglutathionylspermidine and glutathione to be highly active substrates for this enzyme.

Scheme I



MATERIALS AND METHODS

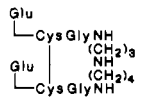
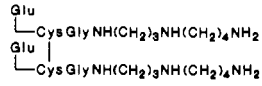
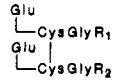
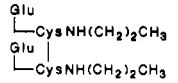
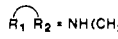
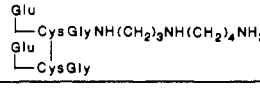
Materials. All reagents and chemicals were of the highest grade commercially available.

Enzymes. Human erythrocyte glutathione reductase and partially purified *T. cruzi* trypanothione reductase were the kind gifts of Dr. Heiner Schirmer (Biochemistry Institute, University of Heidelberg). *C. fasciculata* trypanothione reductase was purified as described previously (Shames et al., 1986).

Synthetic Disulfides. Trypanothione and N^1-monoglutathionylspermidine were synthesized as described previously (Henderson et al., 1986); the other disulfide derivatives used in this study (Table I, structures **3–9**) were synthesized by modification of this procedure. The general synthetic route to the various disulfides is shown in Scheme I. Condensation of the succinimido carbobenzoxyglycinate ester (I) with the required amino derivative II gave the glycyl amide III. After removal of the carbobenzoxy protecting group (Z) from III (by hydrogenolysis), the resulting amines IV were reacted with the functionally protected succinimido γ-glutamylcysteinate ester (V) to give the tripeptides VI. The *tert*-butoxycarbonyl (Boc) and *tert*-butylthio (Bu^tS) protecting groups were removed from the tripeptides VI by sequential treatment with trifluoroacetic acid and dithiothreitol, and the resulting thiols VII were oxidized to their respective disulfide forms by treatment with methanolic iodine. Before use the synthetic disulfides (Table I, structures 3–9) were purified to homogeneity by fast protein liquid chromatography (FPLC) on a Pharmacia Mono S ion-exchange column. The disulfides were analytically pure by high-performance liquid chromatography (HPLC) and amino acid analysis (Fairlamb et al., 1986), and chemical ionization mass spectrometric analyses were consistent with the proposed structures.

Synthesis of N^1-Monoglutathionylspermidine Glutathione Disulfide (**10**). N^1-Monoglutathionylspermidine disulfide (5 μmol) was dissolved in 0.3 mL of 0.1 M potassium phosphate buffer (pH 8) and was reduced by the addition of 20 μmol of DTT. The solution was acidified to pH 3 and extracted with ethyl acetate (4 × 2 volumes). Glutathione (100 μmol) was added, and the solution was adjusted to pH 7 and cooled to 5 °C. This solution was oxidized by the addition of iodine

Table I

compd no.	disulfide	K_m ($\times 10^{-6}$ M)	rel V_{max}	K_{cat}/K_m ($\times 10^7$ M^{-1} s^{-1})
1		51	1	1
2		149	2.28	0.79
3	 $R_1, R_2 = NH_2$	ND[a]	ND	ND
4	$R_1, R_2 = NH(CH_2)_2CH_3$	9130	0.324	0.002
5		ND	ND	ND
6	$R_1, R_2 = NH(CH_2)_3NH(CH_2)_2CH_3$	92	2.90	1.63
7	$R_1, R_2 = NH(CH_2)_3N(CH_3)_2$	185	2.57	0.72
8	$R_1, R_2 = NH(CH_2)_2N$ 	242	1.83	0.39
9	 $R_1 R_2 = NH(CH_2)_5NH$	296	2.43	0.42
10		380	0.81	0.167

[a] ND, not determined.

in methanol to slight excess. The foregoing mixture of disulfides was fractionated by FPLC as described above. A small amount of the symmetrical disulfide of glutathionylspermidine present in the mixture and the bulk of glutathione disulfide were readily separated from the mixed disulfide. HPLC analysis showed that the purified mixed disulfide contained no N^1-monoglutathionylspermidine or N^1-monoglutathionylspermidine disulfide but did contain approximately 4% glutathione disulfide.

Enzyme Assays and Kinetic Analyses. Trypanothione reductase activity was assayed spectrophotometrically by monitoring substrate-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. Absorbance changes were monitored on a Beckman ACTA spectrophotometer with a thermostated cuvette chamber. Kinetic runs were carried out at 27 °C and at pH 7.80, the pH optimum for trypanothione reduction by *C. fasciculata* trypanothione reductase. The standard assay mixture contained 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.25 mM NADPH, and 0.1–0.3 unit/mL trypanothione reductase. All solutions were preincubated at 27 °C before analysis commenced. Initial velocity measurements were made at six or more substrate concentrations which normally ranged from 0.5 to 5 or to 10 times the K_m of the particular substate. Michaelis contants (K_m) and maximum initial velocities (V_{max}) were calculated from these measurements by Hanes–Woolf plots (Segal, 1976). Linear regression analyses gave r values >0.999 in each case.

Results

The disulfide form of N^1-monoglutathionylspermidine (Table I, structure **2**) was found to be a substrate of the homogeneously purified trypanothione reductase from *C. fasciculata*. Normal Michaelis–Menten saturation kinetics were displayed by N^1-monoglutathyionylspermidine disulfide, and K_m was determined to be 149 μM. In parallel experiments the K_m for trypanothione disulfide was determined to be 51 μM, in good agreement with the value reported previously (53 μM) (Shames et al., 1986).

The Michaelis constant for N^1-monoglutathionylspermidine disulfide, an acyclic disulfide, was sufficiently close to the binding constant determined for trypanothione disulfide to indicate that the spermidine portion of the two substrates (or some part of that moiety) must have a key role in the binding of these compounds to the active site of trypanothione reductase. Thus putative disulfide substrates of trypanothione reductase do not necessarily have to be locked in cyclic conformations.

To further define the trypanothione substrate binding domain, the disulfides shown in Table I were chemically synthesized as outlined in Scheme I. These compounds were prepared by modification of synthetic procedures developed previously for the synthesis of trypanothione and the isomeric monoglutathionylspermidines (Henderson et al., 1986). In the simplest case (Table I, structure **3**) the two glycine carboxylate groups of glutathione disulfide were converted to the corresponding carboxamide of ammonia. When tested with trypanothione reductase, compound **3** did not inhibit trypanothione disulfide reduction at 1 or 4 mM concentration, and at 4 mM the rate of reduction of this compound was approximately 1% that observed with 50 μM trypanothione disulfide.

Enzymatic reduction of the dipropyl amide (Table I, structure **4**) by trypanothione reductase could be readily measured, and the apparent K_m for this substrate was calcu-

lated to be 9.13 mM. The related compound (Table I, structure **5**) that lacks glycyl residues was not reduced by trypanothione reductase at 4 or 8 mM; however, the compound did display a weak inhibitory effect on the rate of reduction of trypanothione disulfide. The apparent K_m values for trypanothione disulfide in the presence of 4 and 8 mM concentrations of structure **5** were 106 μM and 151 μM, respectively, resulting in a calculated K_I for compound **5** of 3.9 mM. The addition of an amine function to the dipropyl amide disulfide (compound **5**) led to a series of compounds (Table I, structures **6–8**) which when tested with trypanothione reductase showed dramatically improved binding constants and turnover numbers. To further assess the importance of the N^4 amino function in trypanothione disulfide or glutathionylspermidine disulfide to substrate binding in the active site, the cyclic disulfide (Table I, structure **9**) was synthesized in which the two glutathionyl, units are linked by N^1,N^8-diaminooctane. Although the cyclic diaminooctane derivative was reduced by trypanothione reductase, K_m for this substrate was determined to be almost 6-fold higher (296 μM) than for trypanothione disulfide (K_m = 51 μM).

These synthetic disulfide derivatives were also tested as substrates for pure glutathione reductase from yeast and from human erythrocytes. Of the synthetic disulfides shown in Table I, only the disulfide form of glutathione amide (Table I, structure **3**) had activity with glutathione reductase. This compound was found to have approximately 3% of the activity of glutathione disulfide with the human enzyme (both substrates tested at 250 μM) and was a weak inhibitor of GSSG reduction at higher concentrations. With the corresponding reductase from yeast the compound had <1% of the glutathione disulfide activity.

Since glutathione and N^1-monoglutathionylspermidine are cometabolites in trypanosomatids, the mixed disulfide of these two thiols (Table I, structure **10**) was prepared and tested with trypanothione reductase. We were unable to remove all contaminating glutathione disulfide from this synthetic material (4% by HPLC analysis) and consequently could not determine the activity of this material with glutathione reductase. Since N^1-monoglutathionylspermidine disulfide could not be detected in the sample and since glutathione disulfide is neither a substrate nor an inhibitor of trypanothione reductase, the presence of this impurity did not affect analysis of the mixed disulfide activity with the parasite enzyme. The mixed disulfide was found to be a substrate for trypanothione reductase with a K_m of 380 μM (see Table I).

Discussion

The mammalian enzyme glutathione reductase is extremely specific for its natural peptide substrate, the only known alternative disulfide of comparable activity being the mixed disulfide of GSH and coenzyme A (Mannervik et al., 1978). In contrast, as shown in Table I, trypanothione reductase displays high catalytic efficiency with various analogues of trypanothione disulfide in which the spermidine moiety has been replaced by aliphatic side chains possessing a basic amino function. The kinetic constants K_m, K_{cat}/K_m, and V_{max} for the enzymatic reaction of trypanothione reductase with these disulfides highlights the importance of the spermidine portion of the natural substrate disulfide(s) to binding in the catalytic center. The lack of an equivalent binding site in glutathione reductase would account for the complete absence of activity observed when these disulfides were tested with this enzyme. The almost negligible substrate activity of the disulfide form of glutathione amide (Table I, structure **3**) with human erythrocytes and, more strikingly, with yeast glutathione reductase would indicate that the glycyl carboxylate groups of glutathione disulfide are important for binding of this substrate in the active site of these enzymes. This is supported by the previous report that glycyl esters of glutathione disulfide are poor substrates for glutathione reductase (Anderson et al., 1985). It is of interest that the V_{max} values for the reaction of the disulfides **6–9** exceed that of trypanothione disulfide. It is possible that the higher catalytic rates observed with these substrates represent a less tightly bound enzyme–product complex so that product is released more quickly.

From these analogue studies several important conclusions about the substrate specificity of trypanothione reductase may be drawn. In particular, the enzyme is not specific for cyclic (conformationally constrained) disulfide substrates, and high catalytic activities are obtained when GSSG is attached to at least one basic amino function that is 2–3 methylene groups removed from the glycyl carboxylate. Furthermore, the amine-bearing side chain need not be structurally analogous to spermidine as shown by the relatively low K_m values for the reaction of the tertiary amino *N*-(dimethylpropyl)- and *N*-ethylpiperidine derivatives (Table I, structures **7** and **8**, respectively).

Of the various disulfides shown in Table I, the high substrate activity of N^1-monoglutathionylsperimidine disulfide and of the mixed disulfide of glutathione and N^1-monoglutathionylspermidine (Table I, structure **10**) with trypanothione reductase is of particular importance. Both N^1-monoglutathionylsperimidine and glutathione are cometabolites and biosynthetic precursors of trypanothione in trypanosomatids (Fairlamb et al., 1986). These disulfides are, therefore, potential substrates of the enzyme in vivo. In other experiments with partially purified trypanothione reductase from the mammalian parasites *T. brucei* and *T. cruzi*, N^1-monoglutathionylspermidine disulfide was also found to be an excellent substrate for these enzymes. The K_m and K_{cat}/K_m values for the three natural substrates indicate that trypanothione is formally the best substrate for the *C. fasciculata* enzyme when tested in vitro. However, the importance of the three potential substrates in vivo will depend on the relative intracellular concentrations of the disulfides and also on their intracellular location relative to the enzyme. The distribution of the various disulfide permutations within the cell will also depend on rates of nonenzymatic thiol–disulfide exchange and possible disulfide isomerase activity.

The unique nature of the "trypanothione system" in trypanosomatids (Fairlamb & Henderson, 1987) and the enzyme trypanothione reductase in particular represents an important new target for the developement of chemotherapeutic drugs against these parasites. By taking advantage of the mutually exclusive substrate specificities of trypanothione reductase and glutathione reductase, it should be possible to target reactive functional groups into the active site of the parasite reductase which are capable of reacting with the catalytically active thiol residues or flavin prosthetic group. Thus, inhibitors of the parasite reductase that would not affect host cell glutathione reductase might be prepared. In the present study we have concentrated on analogues of the substrate trypanothione, but in principle the structural features of the enzyme that confer substrate activity to these peptide disulfides could be used to prepare nonpeptide inhibitors of the parasite enzyme. We are currently investigating this approach.

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Adenosine Deaminase: Viscosity Studies and the Mechanism of Binding of Substrate and of Ground- and Transition-State Analogue Inhibitors†

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ABSTRACT: We have studied the effects of viscosogenic agents, sucrose and ficoll, on (1) the hydrolysis of adenosine and of 6-methoxypurine riboside catalyzed by adenosine deaminase and (2) the rates of association and dissociation of ground-state and transition-state analogue inhibitors. For adenosine, V_{max}/K_m is found to be inversely proportional to the relative viscosity with sucrose, an agent affecting the microscopic viscosity, while no effect is found with ficoll, an agent affecting the macroscopic viscosity. Viscosogenic agents have no effect on the kinetic constants for 6-methoxypurine riboside. Thus, the bimolecular rate constant, $V_{max}/K_m = 11.2 \pm 0.8\ \mu M^{-1}\ s^{-1}$, for the reaction with adenosine is found to be at the encounter-controlled limit while that for the reaction with the poor substrate 6-methoxypurine riboside, $0.040 \pm 0.004\ \mu M^{-1}\ s^{-1}$, is limited by some other process. Viscosity-dependent processes do not make a significant (<10%) contribution to V_{max}. The dissociation constants for inhibitors are unaffected by viscosity. The ground-state analogue inhibitor purine riboside appears to bind at a rate comparable to that of adenosine. However, the slower rates of association ($0.16–2.5\ \mu M^{-1}\ s^{-1}$) and dissociation ($5 \times 10^{-6}$ to $12\ s^{-1}$) of transition-state analogue inhibitors are affected by the viscosity of the medium to approximately the same extent as the encounter-controlled rates of association and dissociation of adenosine. A two-step mechanism, a weak prior-equilibrium binding step followed by a viscosity-dependent enzyme conformation change, seems to adequately account for both the low absolute magnitude and the viscosity dependence of the apparent second-order rate constants for transition-state analogue binding.

Adenosine deaminase (EC 3.5.4.4) catalyzes the hydrolysis of (deoxy)adenosine to (deoxy)inosine. An addition–elimination mechanism (Figure 1) has been proposed which proceeds via the addition of water to the 6-position of the purine ring, forming a tetrahedral intermediate (Wolfenden, 1968). Some of the evidence in support of this mechanism is based on the transition-state analogue inhibitor concept (Pauling, 1946; Wolfenden, 1972) in which molecules with structural features of the proposed transition state show unusually high affinity for the enzyme (Figure 2). Thus, nucleoside analogues (Wolfenden et al., 1977; Frieden et al., 1980; Frick et al., 1986) such as (deoxy)coformycin and 1,6-dihydro-6-(hydroxymethyl)purine riboside (DHMPR) with a tetrahedral carbon at the 6-position of the heterocyclic ring (as in the proposed tetrahedral intermediate or its adjacent transition states) have been shown to be unusually effective inhibitors (K_i for deoxycoformycin $< 10^{-12}$ M).

We have studied (Frieden et al., 1980) the kinetic behavior of the interaction of transition-state and ground-state analogue inhibitors with adenosine deaminase. The transition-state analogue systems exhibit unusual kinetics in that they typically bind 1–3 orders of magnitude more slowly than substrates or their analogues (Frieden et al., 1980; this work). Similar slow binding has been observed in several other enzyme–transi-

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