Mutational Analysis of Parasite Trypanothione Reductase: Acquisition of Glutathione Reductase Activity in a Triple Mutant†

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ABSTRACT: African trypanosomes contain a cyclic derivative of oxidized glutathione, N^1,N^8-bis(glutathionyl)spermidine, termed trypanothione. This is the substrate for the parasite enzyme trypanothione reductase, a key enzyme in disulfide/dithiol redox balance and a target enzyme for trypanocidal therapy. Trypanothione reductase from these and related trypanosomatid parasites is structurally homologous to host glutathione reductase but the two enzymes show mutually exclusive substrate specificities. To assess the basis of host vs parasite enzyme recognition for their disulfide substrates, the interaction of bound glutathione with active-site residues in human red cell glutathione reductase as defined by prior X-ray analysis was used as the starting point for mutagenesis of three residues in trypanothione reductase from *Trypanosoma congolense*, a cattle parasite. Mutation of three residues radically alters enzyme specificity and permits acquisition of glutathione reductase activity at levels 10^4 higher than in wild-type trypanothione reductase.

Trypanosomatid parasites cause serious diseases in both humans and animals, including in the old world African human sleeping sickness (*Trypanosoma gambiense* and *Trypanosoma rhodesiense*) and the cognate cattle disease nagana (*Trypanosoma congolense* and *Trypanosoma brucei*), while in the new world *Trypanosoma cruzi* is the causative agent for Chagas' disease (Marr & Docampo, 1986). Leishmaniasis is also caused by trypanosomatid *Leishmania* species. It has been suggested that trypanosomatids are susceptible to oxidative stress by reduced ability to deal with superoxide, hydrogen peroxide, and hydroxyl radicals generated in normal metabolism or by redox cycling of trypanocidal drugs such as nifurtimox (Meshnick et al., 1978; Docampo & Moreno, 1984; Biaglow et al., 1976, 1977). Lack of detectable parasite hemeprotein peroxidases in trypanosomatids focused attention on the anticipated alternate peroxide scavenging path involving coupled glutathione peroxidase/glutathione reductase. However, Fairlamb, Cerami, and colleagues made the unexpected discovery that no parasite glutathione reductase activity was detectable (Fairlamb & Cerami, 1985) and that glutathione levels were very low (Shim & Fairlamb, 1988). Instead the cyclic compound N^1,N^8-bis(glutathionyl)spermidine, termed trypanothione (Fairlamb et al., 1985) (Figure 1), was found to be the main glutathione derivative in trypanosomatid parasites, and this suggested that trypanothione redox metabolism might be a target for antitrypanosomal strategies. Trypanothione in its oxidized form is a 24-membered macrocycle that lacks the negatively charged glycine carboxylate termini of glutathione and has a cationic secondary amine in the spermidine bridge.

To characterize this potential parasite target enzyme we initially purified to homogeneity the trypanothione reductase from the insect trypanosome parasite *Crithidia fasciculata* and characterized it as a flavoprotein dimer with homology to glutathione reductase (Shames et al., 1986). We further demonstrated mutally exclusive specificity of the *C. fasciculata* reductase for oxidized trypanothione over oxidized glutathione (k_{cat} 31 000 min^{-1} vs 3.1 min^{-1}) compared to human red cell glutathione reductase, which reduces glutathione but not trypanothione (k_{cat} 12 000 min^{-1} vs 9.6 min^{-1}). To begin to elucidate the molecular basis of host vs parasite reductase specificity, the gene for trypanothione reductase (from *T. congolense*) was then cloned, its primary sequence (492 amino acids) determined, and high homology to the 478 amino acid human red blood cell glutathione reductase noted throughout the two proteins (Shames et al., 1988). Optimal alignments, including comparison to the *Escherichia coli* glutathione reductase, suggested at least two features of note. First, it appeared that the *T. congolense* enzyme had a 20 amino acid extension at the C-terminus, and second, of the several key amino acid residues implicated in glutathione reductase substrate recognition and catalysis (Karplus et al., 1989; Karplus & Schulz, 1989), two arginine residues (Arg_{37} and Arg_{347}) were not conserved in the parasite enzyme. Most recently we reported expression and overproduction of active *T. congolense* trypanothione reductase in a glutathione reductase deletion strain of *E. coli* and its purification to homogeneity in quantity (Sullivan et al., 1989), setting the stage for analysis of structure/function questions by mutational analysis.

In the absence of a crystal structure for trypanothione reductase, we have drawn heavily on the extensive high-resolution structural studies of human glutathione reductase, at 1.54 Å for the native enzyme and at 2 Å for enzyme ligand complexes, most particularly for the E·NADP·glutathione[1] complex recently reported by Karplus and Schulz (1989) and by Karplus et al. (1989). These X-ray studies delineate the conformation of bound glutathione and differential interaction of the two

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[1] Abbreviations: GS, reduced glutathione; GSSG, oxidized glutathione; TSST, oxidized trypanothione; TR, trypanothione reductase; GR, glutathione reductase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid); NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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Table I: Amino Acids of Glutathione Reductase Involved in GSSG Binding and Their Cognates in Trypanothione Reductase[a]

GSSG residue	human GR residue	residue changes: in Eco GR	residue changes: in *T. congolense* TR	GSSG residue	human GR residue	residue changes: in Eco GR	residue changes: in *T. congolense* TR
Glu_1	Thr_{339} (side)	Thr	Thr_{335}	Glu_2	Lys_{67} (side)	Lys	Lys_{61}
	Ile_{343} (side)	Val	Ile_{339}		$Met_{406'}$ (all)	Met	Leu_{399}
	$\underline{Arg_{347}}$ (side)	Arg	$\underline{Ala_{343}}$		$Pro_{468'}$ (all)	Pro	Pro_{462}
	$His_{467'}$ (side)	His	His_{461}		$Thr_{469'}$ (main)	Thr	Thr_{463}
	$Glu_{473'}$ (side)	Glu	Glu_{467}		$Ser_{470'}$ (side)	Ala	Ser_{464}
	$Thr_{476'}$	Thr	Ser_{472}		$Glu_{472'}$ (side)	Glu	Glu_{466}
Cys_1	Ser_{30} (side)	Ser	Ser_{14}		$Glu_{473'}$ (side)	Glu	Glu_{467}
	Val_{59} (side)	Val	Val_{53}	Cys_2	Leu_{110} (side)	Ile	Ile_{106}
	Val_{64} (side)	Val	Val_{59}		Tyr_{114} (side)	Tyr	Tyr_{110}
	Tyr_{114} (side)	Tyr	Tyr_{110}		$His_{467'}$ (side)	His	His_{461}
	$His_{467'}$ (side)	His	His_{461}	Gly_2	Ile_{113} (side)	Ser	Ser_{109}
Gly_1	$\underline{Ala_{34}}$ (main)	Ala	$\underline{Glu_{18}}$		Tyr_{114} (side)	Tyr	Tyr_{110}
	$\underline{Arg_{37}}$ (side)	Asn	$\underline{Trp_{21}}$		Asn_{117} (side)	Val	Met_{113}
	Tyr_{114} (side)	Tyr	Tyr_{110}				

[a] Residues in TR mutated in this paper are underlined. (main) indicates an interaction between the reductase backbone and the substrate. (side) indicates a side-chain interaction between the reductase and substrate. Eco GR, *E. coli* glutathione reductase. This table is adapted from Karplus et al. (1989) and Janes and Schulz (1990).

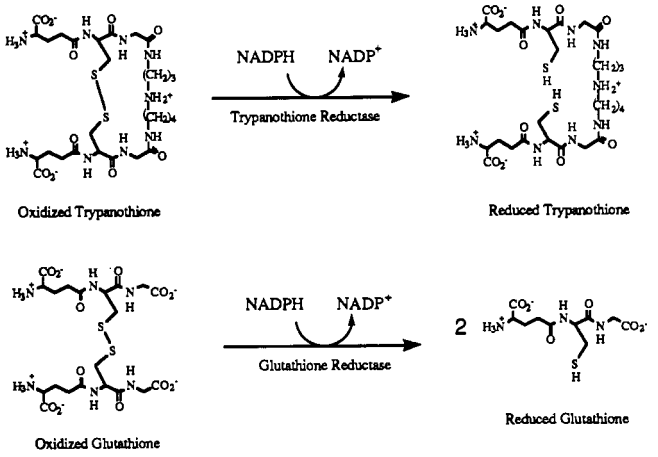


FIGURE 1: Structures of oxidized and reduced trypanothione and oxidized and reduced glutathione and reactions catalyzed by their specific reductases. The macrocyclic trypanothione is lacking the two negative charges of the Gly_1 and Gly_2 moieties and has in its spermidine bridge a cationic secondary ammonium group, a site of possible interaction with the reductase.

GS moieties with the enzyme. The GS-I moiety is in a V-shaped conformation and interacts with the active site via the γ-Glu_1 amino acid residue while the GS-II moiety is in extended conformation and there are strong interactions of the γ-Glu_2 residue (to the other subunit) but only a very weak interaction of Gly_2 to the enzyme. In particular, Karplus, Pai, and Schulz point out that Arg_{347} forms a tight ion pair with the GS-I γ-Glu and that Arg_{37} makes a corresponding ion pair with the Gly_1 carboxylate ion. Ala_{34} also is listed as interacting with the buried Gly_1 residue of bound glutathione. While they note that 14 out of 19 residues interacting with glutathione are conserved between human glutathione reductase and *T. congolense* trypanothione reductase, the Arg_{347}, Arg_{37}, and Ala_{34} contacts are not (see Table I). Our gene sequence alignment has corresponding Ala_{343} (vs Arg_{347}), Trp_{21} (vs Arg_{37}), and Glu_{18} (vs Arg_{34}) in the *T. congolense* trypanothione reductase (Shames et al., 1988). A molecular graphic display of the orientation of bound glutathione and the interacting side chains of Arg_{347}, Arg_{37}, and Ala_{34} in the glutathione reductase active site is displayed in Figure 2.

To assess the basis for exclusive recognition of glutathione by host and trypanothione by trypanosomatid parasite reductase, we report here the results of two kinds of mutations and the properties of mutant enzyme forms of *T. congolense* trypanothione reductase after expression in *E. coli* and purification to homogeneity. First, we have investigated the role of the C-terminal 20 amino acid extension in the parasite enzyme by successive deletions of 5, 10, 15, and 20 residues, and second, we report the results of single, double, and triple combinations of E18A, W21R, and A343R mutations on the catalytic activity of the *T. congolense* reductase toward trypanothione and acquisition of glutathione reductase activity.

Materials and Methods

Materials. The oligonucleotide-directed in vitro mutagenesis system was obtained from Amersham. The Sequenase DNA sequencing kit was obtained from U.S. Biochemicals. Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs and BRL/Gibco. Radionucleotides were obtained from Amersham and New England Nuclear. 2′,5′-ADP–sepharose was obtained from Sigma Chemical Co. MonoQ HR5/5 and phenyl-Superose HR5/5 chromatography columns were obtained from Pharmacia/LKB. Trypanothione was purchased from Bachem Bioscience Inc. (Philadelphia, PA). 4*S*-[4-^{2}H]NADPH was prepared from D-glucose-*1-d* (Aldrich) by using hexokinase and glucose 6-phosphate dehydrogenase according to Bergmeyer et al. (1974). All other reagents were from Sigma Chemical Co. *E. coli* glutathione reductase was a generous gift of Prof. R. Perham (Cambridge University, Cambridge, England).

Vectors and Bacterial Strains. pT7TR-3+, a vector containing the *T. congolense* TR gene under transcriptional control of the T7 RNA polymerase promoter, was described in Sullivan et al. (1989). pGP1-2, a plasmid containing the T7 RNA polymerase gene under transcriptional control of the temperature-sensitive cI857 λ repressor (Tabor & Richardson, 1985), was a generous gift of Dr. Stan Tabor (Harvard Medical School, Boston, MA). *E. coli* strain SG5 (Greer & Perham, 1986) contains a chromosomal deletion of the glutathione reductase gene and was used for expression of all trypanothione reductases. Strain SG5 was a generous gift of Prof. R. Perham (Cambridge University, Cambridge, England). *E. coli* strain XL1 Blue was obtained from Stratagene and used in all routine propagation of plasmid DNAs. *E. coli* strain TG1 was obtained from Amersham and used in the oligonucleotide site-directed mutagenesis.

Construction of Mutants. Mutant genes for all the single mutations and the double mutant E18A/W21R, were generated by the phosphorothioate procedure (Taylor et al., 1985) and according to the manufacturers (Amersham) directions

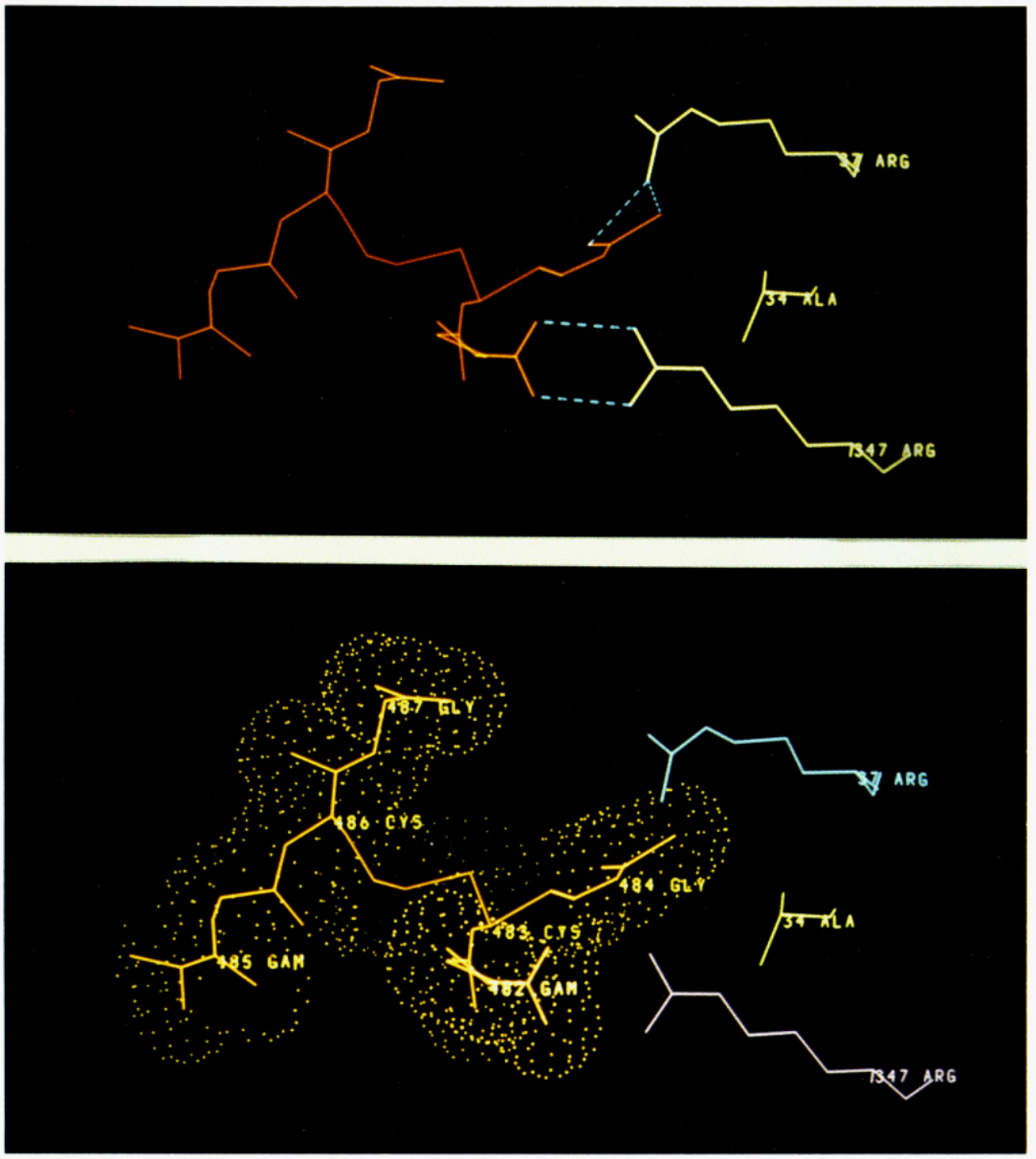


FIGURE 2: Molecular graphic representations of bound glutathione at 3.0-Å resolution in the glutathione reductase map at 1.54 Å, produced from coordinates provided by Professors P. A. Karplus and G. E. Schulz by using FRODO on an Evans & Sutherland PS390 graphics terminal. (Top) The glutathione I moiety (482GAM, 483CYS, and 484GLY represent γ-Glu_1, Cys_1, and Gly_1) is in a V-shaped conformation while the glutathione II moiety (485GAM, CYS, and 487GLY represent γ-Glu_2, Cys_2, and Gly_2) is in extended conformation. The residues highlighted, Ala_{34}, Arg_{37}, and Arg_{347}, interact with the substrate glutathione. The guanidinium of Arg_{347} hydrogen-bonds to the carboxylate of γ-Glu_1 in glutathione, the guanidinium of Arg_{37} hydrogen-bonds to the carboxylate of Gly_1 in glutathione I, and Ala_{34} appears to have main-chain interactions with glutathione I. (Bottom) Same portions with van der Waals radii map shown. In trypanothione reductase the highlighted side chains would correspond to Glu_{18}, Trp_{21}, and Ala_{343} from optimal sequence alignment. These interactions have directed the mutagenesis to the triple mutant E18A/W21R/A343R, which as discussed in the text turned glutathione over at a rate of 980 min^{-1}.

with the single stranded template pT7TR-3+ and the following oligonucleotides:

E18A	TCAGGAGGACTGGCAGCTGGTTGGAAC
W21R	CTGGAAGCTGGTCGCAACGCCGCTACA
A343R	ATTAATGAGGGACGCAGTGTCGTAGAT
E18A/W21R	GGAGGACTGGCAGCTGGTCGCAACGCCGCT
P488*	ATGGAAACACTATGAGACTCGAGCCTC
K483*	ATAAAAGGGGAGTGAATGGAAACACTA
Y478*	CCCTCTCACTACTGAATAAAAGGGGAG
T473*	TGCTCCATGCGCTGACCCTCTCACTAC

The double-mutant (E18A/A343R and W21R/A343R) and the triple-mutant (E18A/W21R/A343R) genes were generated by enzymatic digest of the appropriate single or double mutant genes with the restriction enzymes *Eco*O109, which cuts at a unique site in the vector, and *Nsi*I, which cuts at a unique site in the gene. The fragments were gel purified and the appropriate fragments were religated with T4 DNA ligase at 16 °C for 12 h. The mutant genes generated by oligonucleotide-directed mutagenesis were all sequenced in their entirety by using 10 short synthetic DNA primers. The mutant genes constructed by restriction fragment swapping were sequenced in the region of the mutations and at the junction where the restriction fragments were joined.

Expression and Purification of Mutant Trypanothione Reductases. The genes were expressed in *E. coli* strain SG5 by using the two plasmid system of Tabor and Richardson (1985) and the proteins purified to homogeneity as previously described (Sullivan et al., 1989) with the omission of the DEAE-Sephacel column. A typical preparation yielded ~100 mg of essentially pure enzyme from 40 g of cells after a single chromatographic step (2′,5′-ADP–Sepharose). Two of the tail

mutants (T473* and Y478*) were expressed at low levels, yielding ~40 μg of enzyme from 40 g of cells. These two reductases were further purified after the 2′,5′-ADP–Sepharose column by chromatography on MonoQ and phenyl-Superose columns. Both columns were run in 10 mM bistrispropane buffer, pH 7.0. The MonoQ column was developed with a 100-mL gradient of potassium chloride (0–1 M KCl, 0.25 mL/min). The phenyl-Superose column was developed with a 100-mL gradient of ammonium sulfate (1.7–0 M, 0.5 mL/min).

Enzyme Assays and Kinetic Analysis. Trypanothione and glutathione reductase activities were assayed by the decrease in NADPH absorbance at 340 nm, in 100 mM sodium phosphate/50 mM sodium citrate buffer, pH 6.6, as this buffer was observed to optimize activity. [The tail mutants P488*, K483*, Y478*, and T473* were assayed in a buffer of 100 mM Hepes, pH 7.8, and 1 mM EDTA (Sullivan et al., 1986)]. All assays were performed at 25 °C with HP 8452A or Gilford 260 spectrophotometers. Trypanothione reductase concentrations were quantified by using the absorption coefficient of the FAD cofactor (ϵ_{464} = 10.6 mM^{-1} cm^{-1}) (Shames et al., 1986; Sullivan et al., 1989). The concentrations of stock solutions of oxidized trypanothione and glutathione were determined by complete reduction with NADPH and the appropriate reductase. With two exceptions, the kinetic data were analyzed by nonlinear methods, either with the Cleland program HYPER (Cleland, 1979) or the program supplied with the HP kinetics software. Substrate concentrations ranged from 0.5 to 5 or 10 K_m and standard errors (in Tables II and III) were less than 10%. K_m values for disulfide substrate were determined with saturating concentrations of NADPH (150 μM). K_m for NADPH was determined with saturating concentrations of trypanothione (5 times K_m for the particular mutant). The values of K_m and k_{cat} for wild-type and the triple-mutant trypanothione reductase for both trypanothione, glutathione, and NADPH determined this way were identical with those obtained through more extensive steady-state analyses.

In two cases, wild-type TR and mutant E18A/W21R with glutathione, the K_m for the oxidized glutathione substrate was too large to allow for a complete kinetic analysis. In these cases k_{cat}/K_m was determined by varying both enzyme and substrate concentrations, with substrate concentration at least 100-fold less than the estimated K_m.

Anaerobic Assays. Anaerobic assays were performed in 13 × 100 mm test tubes sealed with rubber septa. Buffer and substrate solutions were degassed by purging with argon. Enzyme and reagents were added with gastight syringes.

Kinetic Isotope Effects. Solvent kinetic isotope effects were determined by using variable concentrations of either GSSG or TSST and at saturating concentrations of NADPH. ^{D_2O}V and $^{D_2O}V/K$ represent the effect observed on maximum velocity and V/K, respectively. Similarly, ^{D}V and $^{D}V/K$ represent the kinetic isotope effects observed when using 4*S*-[4-^{2}H]NADPH as the saturating substrate in water with variable concentrations of TSST or GSSG (Northrop, 1975). Control reactions were performed with NADPH prepared identically with the deuterated substrate. The kinetic data were analyzed by nonlinear methods as supplied with the HP kinetics software.

RESULTS

C-Terminal Deletion Effects on Trypanothione Reductase. In analysis of the homology of *T. congolense* trypanothione reductase to human red blood cell and *E. coli* glutathione reductase, one initial distinction (Shames et al., 1988) was the presence in the parasite enzyme of the last 20 residues (473–492), which had no counterpart in the shorter human (478 amino acids) and *E. coli* (450 amino acids) glutathione reductases (Figure 3). These C-terminal 20 residues in trypanothione reductase have three negatively charged residues, Glu_{482}, Glu_{485}, and Asp_{489}, which could perhaps provide interaction with the cationic ammonium group in the spermidine bridge of the macrocyclic oxidized trypanothione. Using the coordinates of glutathione bound at the active site of glutathione reductase as a starting point and knowing that the C-terminus of the other subunit provides $His_{467'}$ as the crucial base in an intersubunit active site in that enzyme (Karplus et al., 1989; Karplus & Schulz, 1989), we reasoned that the C-terminus of the second subunit of trypanothione reductase (with its conserved $His_{461'}$) could also loop around and perhaps provide a flap to the trypanothione reductase active site and a specificity-contributing interaction to bound trypanothione.

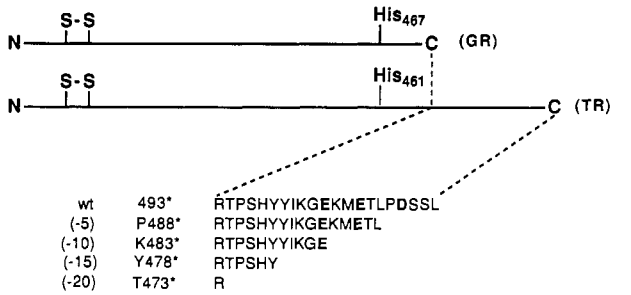


FIGURE 3: Amino acid sequence of the tail extension of trypanothione reductase, highlighting the negatively charged residues that could interact with the secondary amine in trypanothione and their removal in the successive deletion mutants. These sequences are diagrammed with respect to glutathione reductase. S–S represents the redox-active disulfide.

Table II: Effect of Removing the C-Terminal 20 Amino Acids on the Catalytic Efficiency of Trypanothione Reductase[a]

enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m [min^{-1} M^{-1} ($\times 10^6$)]
wild type	25	5300	212
P488*	22	4000	184
K483*	11	3400	309
Y478*	33	nd	nd
T473*	12	5300	312

[a] The reactions were all performed in 100 mM Hepes buffer, pH 7.8, as described under Methods. nd, not determined.

To assess the role of Glu_{482}, Glu_{485}, and Asp_{489}, stepwise deletions of five residues from the C-terminal end of the trypanothione reductase gene were constructed to yield trypanothione reductases P488*, K483*, Y478*, and T473*, deleting 5, 10, 15, and 20 residues, respectively, and removing one, two, and three of the negatively charged side chains (Figure 3). T473* is a deletion mutant that has essentially the same length as human glutathione reductase. After purification of each mutant protein to homogeneity, the catalytic efficiency of each as a trypanothione reductase was analyzed (Table II). There is essentially no loss in catalytic efficiency as one proceeds from full-length trypanothione reductase to the T473* mutant with truncation of the 20 C-terminal residues. Clearly the extension serves no crucial purpose in catalysis. Further, evaluation of the mutant enzymes for relaxation of specificity toward glutathione revealed no detectable glutathione reductase activity above background oxidase activity. The three anionic side chains in the C-terminus are not providing a specificity determinant for trypanothione vs glutathione. One notable consequence of the deletions in the heterologous *E. coli* expression system was a dramatic

Table III: Kinetic Constants for Wild-Type and Mutant Trypanothione Reductase and Human Glutathione Reductase with Both Glutathione and Trypanothione[a]

	TSST			GSSG	
enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})	k_{cat}/K_m (min^{-1} M^{-1})	K_m (μM)
WT TR	18	9100	505 000 000	0.84	
E18A	266	3800	14 000 000	≤1	
W21R	600	2100	3 100 000	≤1	
A343R	9	1700	188 000 000	≤1	
E18A/W21R	5200	3000	570 000	4400	>100 000
E18A/A343R	375	495	1 300 000	≤1	
W21R/A343R	2700	4200	1 500 000	≤1	
E18A/W21R/A343R	4500	1800	400 000	14 000	70 000[b]
WT GR	nd	3[c]	nd	180 000 000	100

[a] The reactions with TR were all performed in 100 mM sodium phosphate/50 mM sodium citrate buffer, pH 6.6, as described under Methods. Human GR data are from Pai et al. (1978). [b] ±15 mM. The concentration range of GSSG used in K_m determination was 20–200 mM. The k_{cat}/K_m ratio determined in this manner was identical with that derived at low substrate concentration, with varying enzyme concentrations; see Materials and Methods. [c] Determined at 280 μM TSST (Shames et al., 1986).

decrease in overproduction of T473* and Y478*, the −20 and −15 truncations, which were detected in 2500-fold lower levels than the full-length and less extensively truncated mutants (P488* and K483*). Since so little pure protein was available for Y478* and T473*, we have not yet determined if the low yields reflect heightened sensitivity to thermal denaturation or protease sensitivity.

Site-Directed Mutants: Single Mutations. To evaluate the importance of specific residues in trypanothione reductase substrate recognition and catalysis, on the basis of the interactions of the highly homologous glutathione reductase with glutathione, the three residues Glu_{18}, Trp_{21}, and Ala_{343} were mutated singly to the congeneric residues found to interact with either the Gly_1 moiety (Ala_{34}, Arg_{37}) or the γ-Glu_1 moiety (Arg_{347}) of bound glutathione with glutathione reductase. This yielded the single-mutant trypanothione reductase enzymes E18A, W21R, and A343R. Each was purified (in ca. 50–100-mg quantities) from an *E. coli* overexpression construct, and steady-state kinetic constants K_m, for trypanothione and NADPH, and k_{cat} were determined. As noted in Table III, the A343R mutation by itself had little adverse effect, only 3-fold lowered k_{cat}/K_m with a 5-fold reduction in k_{cat}. The E18A mutant has a ca. 36-fold diminished k_{cat}/K_m ratio, mostly due to a 15-fold increase in K_m for trypanothione. The W21R single mutant yields the most impaired enzyme at ca. 0.6% the k_{cat}/K_m for wild type with 35-fold larger K_m for trypanothione and only a 4-fold diminishment in k_{cat}. The introduction of the bulky cationic guanidinium side chain in the W21R mutant enzyme appears to be problematic with respect to substrate binding. On the other hand none of these single-mutant enzymes possessed detectable reductase activity with glutathione, above the background oxidase rates. Indeed none of these mutant enzymes were even inhibited by 20 mM glutathione. The lower limit of detection was constrained in aerobic assays by the intrinsic NADPH oxidase activity of trypanothione reductase, which ranged from 1 min^{-1} for wild-type enzyme to up to 3 min^{-1} for the E18A mutant. On anaerobic assay, an upper limit on k_{cat}/K_m of 0.8 M^{-1} min^{-1} could be determined for wild-type enzyme acting as a glutathione reductase, a (6×10^8)-fold difference for TSST vs GSSG.

Site-Directed Mutants: Double and Triple Mutations and Acquistion of Glutathione Reductase Activity. With singly mutated trypanothione reductase genes constructed, two double mutants and one triple mutant could the be constructed by swapping of appropriate restriction fragments. The other double mutant was prepared by in vitro mutagenesis as described previously. All these enzymes were purified to homogeneity in >50-mg quantities. The E18A/A343R double-mutant enzyme is substantially poorer as a catalyst than either constituent single mutant (Table III), with a 20-fold higher K_m (vs 15-fold for E18A and no effect for A343R) and only 5% of the k_{cat} of wild type (whereas neither single mutant showed any substantial k_{cat} effect) for a k_{cat}/K_m ratio for trypanothione of 1/400 vs wild type. The double mutant W21R/A343R has a K_m for oxidized trypanothione 150-fold higher than wild-type, ca. 4-fold over the W21R single mutant's poor K_m, consistent again with the W21R alteration having a major disadvantage in trypanothione recognition. The k_{cat} for W21R/A343R is 4200 min^{-1}, not dramatically distinct from wild-type (9100 min^{-1}) or from the single mutant W21R (2100 min^{-1}) or A343R (1700 min^{-1}) k_{cat} values.

The third of the double mutants, E18A/W21R, combines both changes that are thought to be crucial determinants for recognition of the glycyl carboxylate function of the GS-I moiety of glutathione bound to the active site of glutathione reductase (Figure 2). In this double mutant, K_m for trypanothione is 5200 μM, about a 10-fold increase over both the W21R and E18A single mutants, but the k_{cat} (3000 min^{-1}) of ca. 30% of wild type is not a simple translation from the essentially wild-type level k_{cat} values for the constituent single mutants. This is the poorest enzyme of the set of three double mutants, with the k_{cat}/K_m value some 900-fold worse than wild type with trypanothione.

This E18A/W21R double mutant may be a substantially poorer catalyst of trypanothione reductase, but it is the only one of the three single or three double mutants to display detectable glutathione reductase activity (Table III). This is the first inkling that one can switch specificity but this enzyme species is still a relatively poor catalyst for glutathione recognition. The K_m value could not be measured; it is at least 100 mM. However, at concentrations of glutathione less than K_m, one can calculate a k_{cat}/K_m value from the experimental data and a k_{cat}/K_m of 4400 M^{-1}/min^{-1} was obtained (Table III).

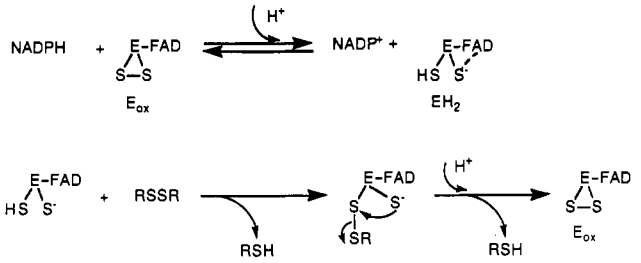
The triple-mutant enzyme E18A/W21R/A343R was also constructed by restriction fragment swapping and overproduced, and 110 mg was purified to homogeneity. The simultaneous presence of all three residue changes moves the K_m for trypanothione from 18 μM for wild type to 4500 μM in this triple mutant, a 250-fold effect. The k_{cat} value of 1800 min^{-1} is ca 20% that of wild type, still a substantial catalyst. The k_{cat}/K_m value of 400 000 M^{-1} min^{-1} is now 1/1250 the value of wild-type trypanothione reductase, lower than any of the double mutants.

What has addition of the A343R mutation to the E18A/

W21R combination done for glutathione reductase activity? This enzyme is 3-fold better as a glutathione reductase by the k_{cat}/K_m criterion, at 14000 M^{-1} min^{-1} (Table III) vs 4400 M^{-1} min^{-1} for E18A/W21R. The K_m for glutathione is now measurable, if still high, at 70 mM and k_{cat} is 980 min^{-1}.

The absorbance spectra of all mutant enzymes were identical with those of wild-type enzyme, within experimental error, and K_m's for NADPH were between 5 and 20 μM.

Initial Evaluation of Rate-Determining Steps in Wild-Type and Triple-Mutant Trypanothione Reductases. In the redox catalysis effected by the glutathione reductase and trypanothione reductase class of flavoprotein disulfide dehydrogenation enzymes, NADPH acts to reduce an E_{ox} species (containing FAD and an active-site disulfide) to a dihydroenzyme species (EH_2) that involves initial reduction of FAD and then accumulation of the active-site cysteine dithiol, FAD species as the predominant EH_2 form (Schirmer et al., 1989; Williams, 1976). This EH_2 form has the capacity to carry out accelerated dithiol/disulfide interchange on GSSG or TSST (shown as RSSR) to yield 2RSH and regenerate E_{ox}.



Given the 1200-fold decrease in k_{cat}/K_m for the triple-mutant vs wild-type trypanothione reductase, both substrate deuterium isotope effects, via 4*S*-[4-^{2}H]NADPH, and solvent deuterium isotope effects, via D_2O, were measured to determine if changes in rate-determining steps in wild-type vs E18A/W21R/A343R enzyme could be monitored. 4*S*-[4-^{2}H]NADPH monitors the degree to which the hydride transfer step to N_5 of FAD is kinetically significant. There was no ^DV or $^DV/K$ effect significantly different from unity for wild-type enzyme, but there was a $^DV/K$ effect of 1.8 ± 0.3 for the triple mutant with essentially no ^DV effect. When the triple mutant is assayed as a glutathione reductase, with k_{cat} approximately one-tenth that of wild-type trypanothione reductase, there is now a $^DV/K$ of 2.3 ± 0.2, essentially all on K_m (data not shown). Hydride transfer is beginning to attain greater kinetic importance in the mutant with its relaxed substrate specificity and its lessened catalytic efficiency. In D_2O the wild-type trypanothione reductase displayed a substantial (3.0 ± 0.1)-fold ^{D_2O}V, while now the triple mutant had a ^{D_2O}V of only 1.1 ± 0.3. Clearly the proton transfer steps in catalysis to give EH_2 or in the release of either RSH product molecule limit catalysis in the wild-type but not the mutant enzyme, where the EH_2 formation step appears to be selectively slowed. For comparison, the triple mutant acting as a glutathione reductase shows a ^{D_2O}V effect that is intermediate at 1.7 ± 0.3. Human red blood cell glutathione reductase has been reported to show a ^{D_2O}V effect of 2.8 ± 0.1 (Wong et al., 1988). Further assignment of changes in rate-determining steps can be addressed in stopped-flow UV/visible spectroscopic studies where EH_2 formation and reoxidation in the wild-type and mutant enzymes can be analyzed separately and by careful analysis of half-reaction solvent isotope effects.

Consistent with the kinetic mechanism for human erythrocyte glutathione reductase (Pai & Schulz, 1983), both wild-type trypanothione reductase and the trypanothione reductase triple mutant show ping-pong kinetic patterns in initial velocity kinetics (data not shown), indicating no gross changes in relative order of release of NADP and binding of RSSR cosubstrate.

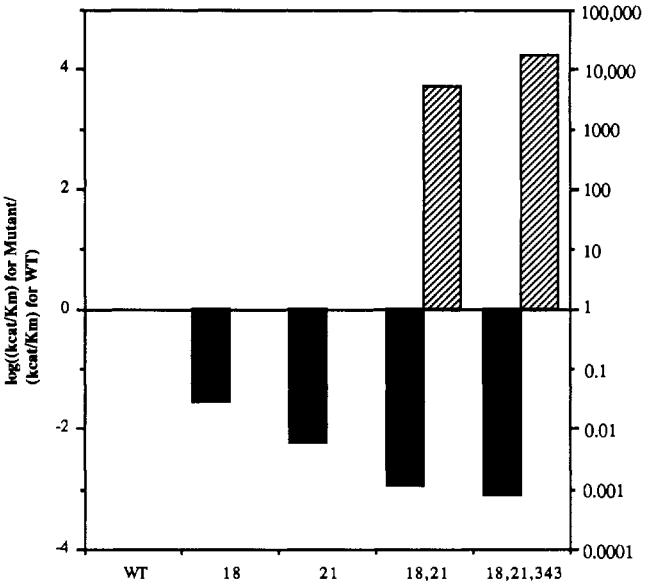


FIGURE 4: Histogram illustration of the effects of mutation on k_{cat}/K_m for enzyme activity with trypanothione and glutathione for selected mutants. Solid bars, with TSST; hatched bars, with GSSG. WT = wild type; 18 = E18A; 21 = W21R; 18,21 = E18A/W21R; 18,21,343 = E18A/W21R/A343R. The right-hand scale lists relative order of magnitude changes in k_{cat}/K_m.

Discussion

Conclusions on Reengineering of a Parasite Enzyme to Acquire Host Enzyme Selectivity. Wild-type *T. congolense* trypanothione reductase shows a 6 × 10^8/1 ratio in k_{cat}/K_m for TSST/GSSG reduction. The three mutations introduced by consideration of the glutathione reductase–glutathione X-ray have dramatically affected the specificity constants such that E18A/W21R/A343R now has a TR/GR ratio of only 28/1, a change in ratio of (2 × 10^7)-fold. This is dissectable into a (1.2 × 10^3)-fold decrease in k_{cat}/K_m as a trypanothione reductase and a (1.7 × 10^4)-fold increase as a glutathione reductase.

Only the triple and the E18A/W21R double mutant have measurable glutathione reductase activities. The triple mutant is in a sense an enzyme with relaxed or hybrid specificity: K_m(GSSG)/K_m(TSST) = 16/1 and k_{cat}(TSST)/k_{cat}(GSSG) = 2/1. Put another way, the triple-mutant trypanothione reductase has acquired substantial glutathione reductase activity both by k_{cat} increase from essentially undetectable levels up to 980 min^{-1} and by the (1.7 × 10^4)-fold increase in k_{cat}/K_m starting from wild-type trypanothione reductase. Viewed against the wild-type human glutathione reductase (k_{cat}/K_m of 1.8 × 10^8 M^{-1} min^{-1}; Pai et al., 1978), the triple mutant is still 4 orders of magnitute worse; however, by the same measure, it is 4 orders of magnitude better than wild-type trypanothione reductase (Figure 4).

In terms of recent reports of enzyme specificity reengineering, these results are substantial. The reengineering of lactate dehydrogenase to malate dehydrogenase resulted in a 10^4-fold drop in k_{cat}/K_m for pyruvate and a (8.4 × 10^3)-fold increase in oxaloacetate recognition by a Q102R mutation (Clarke et al., 1989). In aspartate aminotransferase, the R292D mutant enzyme has a 10^6-fold decreased activity as an aspartate transaminase and is only 15.5-fold better as an arginine transaminase (Cronin et al., 1987). Most recently and relevantly, *E. coli* glutathione reductase has been subjected

to directed mutagenesis to alter NADPH to NADH coenzyme recognition. A septuple mutant had a 250-fold drop in k_{cat}/K_m for NADPH and a 72-fold increase in k_{cat}/K_m for NADH (Scrutton et al., 1990).

To interpret the molecular basis of this 17000-fold k_{cat}/K_m acquistion of glutathione reductase activity will require X-ray analyses of wild-type and mutant trypanothione reductase with and without complexed TSST and GSSG (for E18A/W21R/A343R) to further decipher the recognition issues. Nonetheless, the views of alignment of GSSG at the active site of glutathione reductase have proven to be predictively effective blueprints for reengineering trypanothione reductase to accept GSSG. It may be that TSST assumes a bound conformation that mimics the V-shaped conformation of bound GSSG (in which the two glycyl-COO^- groups are close enough to be spanned by a spermidine bridge).

That these three mutations do not completely convert substrate specificity for E18A/W21R/A343R implies that there are additional interactions responsible for substrate recognition/binding that have yet to be changed in the triple mutant. These contacts may involve residues on the reductase thought to take part in substrate binding as noted in Table I. Of these, the two remaining residues that differ most dramatically between human GR and the triple mutant are Met_{113}, which is an Asn in human GR, and Ser_{109}, which is an Ile in human GR. Alternatively, the contacts may involve differences in the backbone structures of the two proteins at their active sites.

A complementary reengineering approach to that described here is to undertake to turn a glutathione reductase into a trypanothione reductase. Inspection of the sequence variation between human red blood cell and *E. coli* glutathione reductase (Table I) led Karplus and Schulz (1989) to note that the Arg_{347} of human glutathione reductase that makes a salt bridge to the γ-Glu of the GS-I moiety of GSSG is converted to an Asn in the corresponding site in *E. coli* glutathione reductase. While this apparently does not impair the *E. coli* glutathione reductase as a catalyst [k_{cat} = 16000 min^{-1} (Scrutton et al., 1990)] for glutathione reduction, we wondered if that natural mutation at one of the three residues we changed in trypanothione reductase might relax specificity for TSST recognition and reduction by *E. coli* glutathione reductase. Indeed, while almost no recognition of TSST by human glutathione reductase is observed (Shames et al., 1986), when pure *E. coli* glutathione reductase is assayed for TSST reduction, a K_m of 2.7 mM for TSST (vs 0.1 mM for GSSG) reduction and a k_{cat} of 8900 min^{-1} can be determined. Thus *E. coli* glutathione reductase displays almost 60% the k_{cat} of GSSG against the parasite metabolite TSST, while the human glutathione reductase acts on TSST at only 9.6 min^{-1} (Shames et al., 1986). These data augur that just as the parasite enzyme can be reengineered to show the specificity of mammalian host reductase, so the reciprocal reengineering should be successful.

We plan to use these insights and those already gained in the design of substrate analogues (Henderson et al., 1988; Fairlamb et al., 1989) for selective inhibition of trypanothione redox metabolism, now known to be a site for classical trypanocidal arsenical-based drugs such as melarsen B (Fairlamb et al., 1989).

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