Catalytic and Potentiometric Characterization of E201D and E201Q Mutants of *Trypanosoma congolense* Trypanothione Reductase†

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ABSTRACT: Trypanothione reductase is a member of the structurally and functionally well-characterized family of flavoprotein reductases, which catalyze the reduced pyridine nucleotide dependent reduction of their disulfide, peroxide, or metal ion substrates. Trypanothione reductase is found in a wide variety of *Trypanosoma* species, where the enzyme serves physiologically to protect the organism from oxidative stress and assists in maintaining low intracellular levels of hydrogen peroxide. The redox potential of the flavin and the hydride ion transfer reaction of the *pro-S* hydrogen of NADPH to N5 of FAD have been proposed to be influenced by the presence of a conserved Lys-Glu (K60-E201) ion pair at the bottom of the nicotinamide binding pocket. We have evaluated this hypothesis by making modest substitutions for both the Lys and Glu residues using site-directed mutagenesis. Replacement of the K60 residue with an arginine led to a poorly expressed, and completely inactive, enzyme. Replacement of the Glu201 residue with either a glutamine (E201Q) or an aspartate (E201D) residue led to expressed enzymes which could be readily purified in >20 mg amounts using protocols developed for the WT enzyme, and which had significant residual trypanothione-reducing activity. These enzymes have now been characterized to determine their redox potentials, catalytic activities, and nucleotide specificities. Relative to the WT enzyme, both E201D and E201Q exhibit ca. 5% of WT trypanothione-reducing activity using NADPH as reductant, but significantly enhanced quinone reductase activity. The oxidase activity of both mutants is enhanced by over 50-fold compared to that of the WT. The redox potential of the WT enzyme has been determined to be −273 mV, while both the E201D and E201Q exhibit more positive redox potentials (−259 and −251 mV, respectively). These data confirm the modulating effect of the K60-E201 ion pair on the redox potential of the flavin, and the reactivity of the enzyme toward alternate reducible substrates.

Trypanothione reductase is a member of the family of FAD-containing pyridine nucleotide−disulfide oxidoreductases (Williams, 1992) and catalyzes the NAD(P)H dependent reduction of trypanothione, N^1,N^8-bis(glutathionyl)spermidine, $T(S)_2$,[1] a cyclic glutathione analog unique to trypanosomatid parasites (Scheme 1). This enzyme serves a function similar to that of glutathione reductase in the antioxidant system of trypanosomatidae, the causative pathogens responsible for African sleeping sickness *(Trypanosoma gambiense* and *Trypanosoma rhodiense)* and Chagas disease *(Trypanosoma cruzi)* in humans and nagana *(Trypanosoma congolense* and *Trypanosoma brucei)* in cattle (Fairlamb & Cerami, 1992). The enzyme from *T. congolense* is a dimer of identical 55 kDa subunits, each containing a noncovalently bound FAD and a redox active disulfide consisting of cysteine residues 52 and 57 (Shames et al., 1986), responsible for reversible electron storage and disulfide reduction. The three-dimensional structures of trypanothione reductase purified from *Crithidia fasciculata* (Kuriyan et al., 1991) and *T. cruzi* (Lantwin et al., 1994) are extremely similar to the three-dimensional structure of human erythrocyte glutathione reductase (Pai & Schulz, 1983; Pai et al., 1988), with similarly distinct binding sites for pyridine nucleotide and disulfide substrate, separated by the isoalloxazine ring of FAD. The reduction of the enzyme with 1 equiv of NADPH produces a charge transfer complex between FAD and the Cys57 thiolate anion, with an absorption maximum at 530 nm (Krauth-Siegel et al., 1987; Sullivan et al., 1989). The steady-state kinetic mechanism of enzyme is "ping-pong", and the oxidation of two-electron-reduced enzyme by $T(S)_2$ is the rate-limiting step (Leichus et al., 1992).

The comparison of the amino acid sequences of trypanothione reductases from *C. fasciculata* (Kuriyan et al., 1991), *T. cruzi* (Lantwin et al., 1994), and *T. congolense* (Shames et al., 1988) suggested the existence of a conserved ion pair in the pyridine nucleotide-binding site of trypanothione reductases, formed by lysine 60 and glutamate 201 in *T. congolense*. This ion pair is additionally conserved in the family of FAD-containing pyridine nucleotide−disulfide oxidoreductases, e.g. Lys53-Glu188 in *Escherichia coli* lipoamide dehydrogenase (Stephens et al., 1983) and Lys66-Glu201 in human glutathione reductase (Krauth-Siegel et al., 1982; Karplus & Schulz, 1989). The ϵ-amino group of the conserved lysine residue is located within 3.5 Å of the flavin O4α and N5 atoms and C4 and N7 of bound NADPH in *T. cruzi* trypanothione reductase (Lantwin et al., 1994). It has

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[1] Abbreviations: WT, wild-type; $T(S)_2$, oxidized trypanothione; E, oxidized enzyme; EH_2, two-electron-reduced enzyme; E_o, two-electron reduction potential of enzyme; E_h, redox potential of the media; FAD, flavin adenine dinucleotide; 3APADP$^+$, β-3-acetylpyridine adenine dinucleotide phosphate; t-NADP$^+$, β-thionicotinamide adenine dinucleotide phosphate.

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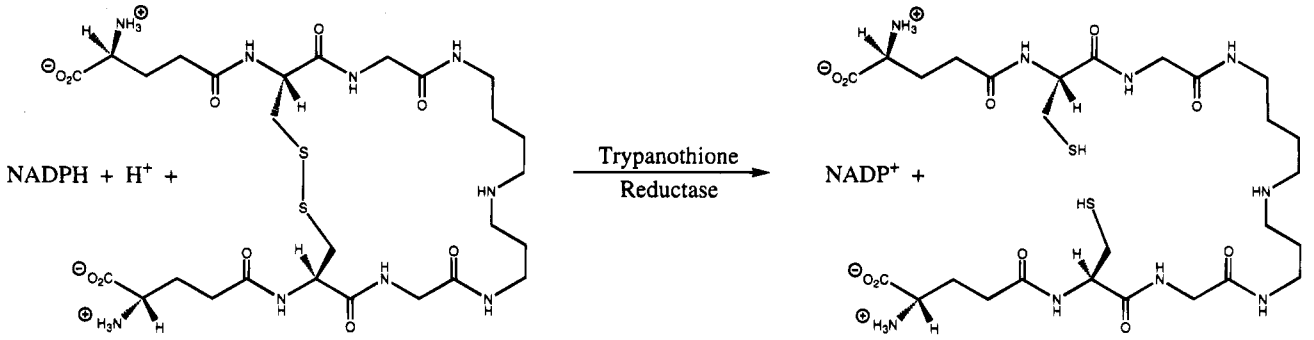
Scheme 1



been proposed that this ion pair may modulate the redox potential of the noncovalently bound FAD (Pai & Schulz, 1983) and could stabilize the transition state for hydride transfer from NADPH to FAD (Sweet & Blanchard, 1991).

In order to evaluate the role of the Lys60-Glu201 ion pair in redox catalysis by trypanothione reductase, we conservatively replaced either the Lys60 residue with arginine or the Glu201 residue with either an aspartate (E201D) or glutamine (E201Q) residue, expressed and purified these enzymes, and determined catalytic activities, redox potentials, and nucleotide specificities of mutant enzymes.

EXPERIMENTAL PROCEDURES

Reagents. The "Altered Sites *in vitro* Mutagenesis System" was from Promega, and DNA sequencing was performed using materials supplied by U.S. Biochemicals. Restriction endonucleases and T4 polynucleotide kinase were from New England Biolabs. pT7TR-3+ (Sullivan et al., 1989), a derivative of PBS+ containing the *T. congolense* trypanothione reductase gene under control of a T7 promoter, was the generous gift of Prof. C. Walsh (Harvard Medical School). *E. coli* strain HMS174(DE3), containing a chromosomal copy of the gene for T7 RNA polymerase, was obtained from Novagen. 2′,5′-ADP Sepharose and all chromatographic supports were from Pharmacia. All pyridine nucleotides, cytochrome *c*, xanthine oxidase, hypoxanthine, quinones, and buffer components were purchased from Sigma or Aldrich and were used as received. Phenosafranine was from Fluka AG, and oxidized trypanothione was from Bachem Bioscience (Switzerland). Reduced forms of t-NADP$^+$and 3APADP$^+$ were prepared as described previously (Leichus et al., 1992).

Mutagenesis, Cell Growth, and Enzyme Purification. The mutant genes for E201D, E201Q, and K60R were constructed by site-directed mutagenesis (Hutchinson et al., 1978) according to the manufacturer's (Promega) instructions using single-stranded template pT7TR-3+. The synthetic oligonucleotides used for mutagenesis were

5′-GGT TTT ATT TCC GTT GAG TTT GCT GG-3′
(WT)

5′-GGT TTT ATT TCC GTT GAT TTT GCT GG-3′
(E201D)

5′-GGT TTT ATT TCC GTT CAG TTT GCT GG-3′
(E201Q)

and

5′-GGT TGC GTG CCG AAG AAG CTC ATG GT-3′
(WT)

5′-GGT TGC GTG CCG AGG AAG CTC ATG GT-3′
(K60R)

where the mismatched bases are underlined. The mutated genes were sequenced by the dideoxy chain termination method (Sanger et al., 1977) to confirm that no unexpected mutations had been introduced during the procedure. The mutated and WT genes were expressed in *E. coli* strain HMS174 (DE3). After induction with 1 mM IPTG for 4 h, the cells were harvested by centrifugation, washed, and stored at −70 °C.

WT and mutant trypanothione reductases were purified essentially as previously described (Sullivan et al., 1989). All operations were performed at 4 °C, and 0.02 M TEA-HCl, pH 7.8, was used as buffer throughout the purification procedure. Briefly, cells were suspended in 0.02 M TEA-HCl, pH 7.8, containing 1 mM EDTA and 20 μg/mL of lysozyme. Cells were disrupted by sonication and centrifuged and nucleic acids precipitated from the cell-free supernatant using streptomycin sulfate (1% w/v final). The dialyzed streptomycin supernatant was loaded onto a 5 × 35 cm Fast Flow Q Sepharose anion exchange column and eluted with a linear 0−0.8 M NaCl gradient. Active fractions were pooled, concentrated by ultrafiltration, and applied to a 1.6 × 70 cm Superdex 200 gel filtration column. Active fractions were pooled, applied to a 20 mL 2′,5′-ADP-Sepharose affinity column, and eluted with a linear 0−0.4 M NaCl gradient. Active fractions were pooled, dialyzed, and applied to a 1 × 10 cm Mono Q high-resolution anion exchange column to remove minor contaminating species.

Steady-State Kinetic Studies. Initial velocities were measured by the monitoring of the decrease in the absorbance of reduced pyridine nucleotide using $\Delta\epsilon_{340} = 6.2$ mM^{-1} cm^{-1} for NAD(P)H, $\Delta\epsilon_{395} = 11.3$ mM^{-1} cm^{-1} for t-NADPH, and $\Delta\epsilon_{363} = 9.1$ mM^{-1} cm^{-1} for 3APADPH. A Gilford 260 spectrophotometer equipped with thermospacers and connected to a circulating water bath was used. Typically, 10−100 μM reduced pyridine nucleotide and 20−100 μM T(S)$_2$ were used, and assays were performed in 0.05 M Hepes, pH 7.5, containing 1 mM EDTA, at 25 °C. Quinone reductase activity, determined using 1,4-benzoquinone or 9,10-phenanthrenequinone as substrates, was monitored as described previously (Cenas et al., 1994). The benzosemiquinone-mediated reduction of cytochrome *c* (50 μM) was monitored as an increase in the absorbance due to reduced cytochrome *c* at 550 nm ($\Delta\epsilon = 20$ mM^{-1} cm^{-1}) in 0.05 M Hepes, pH 7.0 (Nakamura & Yamazaki, 1972). Steady-state

kinetic data were graphically analyzed by Lineweaver–Burk analysis and fitted to the appropriate rate equations using the FORTRAN programs of Cleland (1979).

Spectral Measurements. The concentration of enzyme active sites was determined using an extinction coefficient of 11.3 mM^{-1} cm^{-1} for the enzyme-bound FAD in WT (463 nm), E201D (458 nm), and E201Q (442 nm) trypanothione reductases. Absorption spectra were recorded with a Milton Roy 3000 diode array spectrophotometer. Sample solutions for anaerobic experiments were prepared by sequential cycling between vacuum and oxygen-free nitrogen gas (10 times), the final cycle being equilibration with nitrogen gas. High-purity nitrogen gas was pretreated by passage through an oxygen trap (Labclear).

Determination of Redox Potentials. The standard two-electron reduction potentials of WT and mutant trypanothione reductases were determined using xanthine oxidase and 0.2 mM hypoxanthine as the source of redox equivalents (Massey, 1991). Typically, the anaerobic cell contained 10 μM phenosafranine as redox indicator, 2 μM benzyl viologen, and 10–20 μM trypanothione reductase. Under the conditions of experiment, sufficient xanthine oxidase was added so that the reduction of redox indicator and trypanothione reductase was complete in more than 3–4 h. The extent of reduction of phenosafranine was monitored at 508 nm (the isobestic point for the E to EH_2 transition for WT and E201Q) or 512 nm (the isobestic point for the E to EH_2 transition for E201D). The extent of reduction of WT trypanothione reductase was determined as an increase in absorbance at 600 nm and as a decrease in absorbance at 460 nm. The extent of reduction of mutant trypanothione reductases was determined as a decrease at 440–460 nm only. At these wavelengths, the data were corrected by subtraction of absorbance changes due to phenosafranine reduction.

RESULTS

Expression and Purification of WT and Mutant Trypanothione Reductase. E. coli strain HMS174 (DE3) was chosen for expression of WT and mutant trypanothione reductase genes, since significantly higher yields of cell mass and activity could be obtained. Although *E. coli* strain HMS174 (DE3) carries a chromosomal copy of the glutathione reductase gene (*gor*$^+$), these two disulfide reductases were well-separated by the initial anion exchange chromatography. WT and mutant trypanothione reductases, expressed in HMS174, showed no contamination by *E. coli* glutathione reductase. A typical purification yielded approximately 40 mg of WT enzyme and 20–30 mg of mutant enzyme from 60 g of cells, respectively.

Spectroscopic and Potentiometric Characteristics of Mutant Trypanothione Reductase. The absorption maxima (Figure 1) of the FAD chromophore in the oxidized forms of E201Q trypanothione reductase (λ_{max} = 442 nm) and E201D trypanothione reductase (λ_{max} = 458 nm) are blue-shifted compared to that of the WT enzyme (λ_{max} = 463 nm). The anaerobic reduction of enzyme by 1 equiv of NADH, to form the EH_2 form of the enzyme, results in a partial bleaching of the 440–460 nm band and the appearance of absorbance at 530–700 nm (Figure 1). These spectral changes, characteristic of the two-electron-reduced form of disulfide reductases, can be attributed to a charge

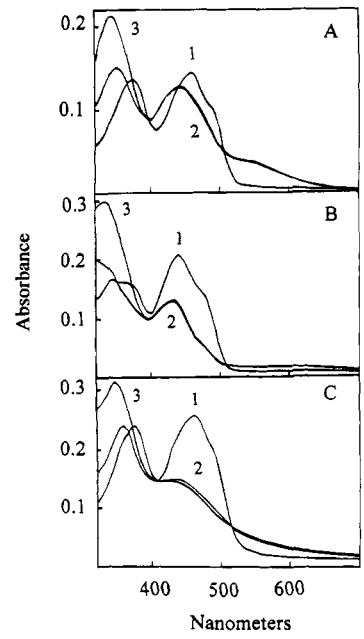


FIGURE 1: Visible absorption spectra of WT (A), E201Q (B), and E201D (C) trypanothione reductases. Oxidized enzyme (1), reduction with 1 equiv of NADH (2), addition of second equiv of NADH (3). Enzymes were in 0.05 M Hepes, pH 7.5, containing 0.3 mM EDTA and made anaerobic as described in the text.

transfer complex between FAD and one of the thiols of the redox active cysteine residues (Williams, 1992). The addition of a second equivalent of NADH does not result in further reduction of the enzyme to form an EH_4 form of either WT or the mutant trypanothione reductases. The spectra of the two-electron-reduced mutant trypanothione reductases exhibit a less pronounced charge transfer absorption band at 550 nm and a more pronounced decrease in absorbance at 440–460 nm, compared to that of WT enzyme (Figure 1).

In the presence of phenosafranine, benzyl viologen, and xanthine oxidase/xanthine as a source of reducing equivalents, the reduction of phenosafranine is accompanied by the parallel reduction of added trypanothione reductase. For the WT enzyme, this is most clearly indicated by the absorbance increase at 550–600 nm (Figure 2). Since, for the mutant enzymes, the absorbance increases at 550–600 nm are less pronounced (Figure 1), the extent of their reduction was estimated by the monitoring of absorbance changes at 440–460 nm, after the substraction of absorbance changes due to phenosafranine reduction. For experiments with mutant enzymes (Figure 2B,C), these changes contributed ca. 35% of the total absorbance change. The linearization of the absorbance data was performed by the plotting of log $[E]/[EH_2]$ versus log $[Dye_{ox}]/[Dye_{red}]$ and gave a slope close to unity, indicating that complete equilibration had occurred between the two-electron-accepting enzyme and two-electron-accepting redox mediator (Massey, 1991; data not shown). At pH 7.5, E_o for phenosafranine is −0.267 V (Massey, 1991; Prince et al., 1981), and using this value for the calculation of E_h, the redox potential of the media (Figure 3), we have determined the redox potential of WT trypanothione reductase to be −273 ± 3 mV (600 nm data). These data can be compared to the data obtained at 460 nm (Figure 3, filled triangles), where corrections for the absorbance changes accompanying phenosafranine reduction (Figure 2A) were made. The redox potentials of both the E201D and E201Q

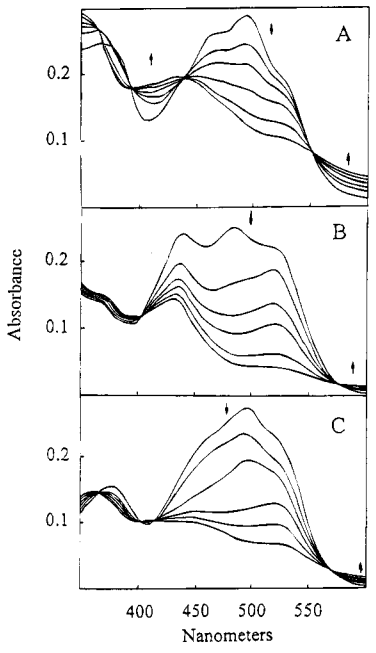


FIGURE 2: Time course of the anaerobic potentiometric titration of WT (A), E201Q (B), and E201D (C) trypanothione reductase, using xanthine oxidase/xanthine as the source of redox equivalents and phenosafranine as redox indicator (Massey, 1991). Enzyme, 20 μM, in 0.05 M Hepes, pH 7.5, contained 0.3 mM EDTA and 10 μM phenosafranine. The curves were recorded at 30 min intervals.

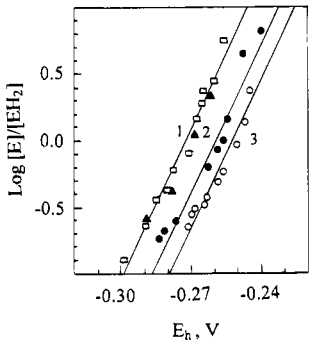


FIGURE 3: Linearization of potentiometric data of WT (1, squares), E201D (2, filled circles), and E201Q (3, open circles) trypanothione reductase in Nernst coordinates, where E_h is the redox potential of the media. The WT data were obtained at 600 nm (open squares) and at 460 nm (filled triangles), while the mutant data were obtained at 440−460 nm.

mutants were more positive at −259 ± 6 and −251 ± 5 mV, respectively.

Steady-State Kinetics of Mutant Trypanothione Reductases. The E201D and E201Q mutant trypanothione reductases exhibit markedly altered steady-state kinetic properties compared to WT enzyme. At saturating concentrations of NADPH (50 μM), the maximal velocity, V_m, for the reduction of trypanothione by both mutants is ca. 5% of the maximal rate of WT enzyme (Table 1). There is no significant difference in the steady-state K_m values for NADPH or $T(S)_2$. On the other hand, the oxidase activity of both mutant enzymes is increased by more than 50-fold (Table 1). Among other potential oxidants of trypanothione reductase, quinones are of some interest. Using 1,4-benzoquinone and 9,10-phenanthrenequinone as oxidants, we have found that the maximal rate of their reduction is increased 7−30-fold in mutant enzymes, and V/K_m is increased by about 1 order of magnitude (Table 1). The efficiency of enzymatic one-electron reduction of quinones was estimated by the monitoring of the 1,4-benzosemiquinone-mediated reduction of cytochrome *c* (50 μM) at pH < 7.2 (Nakamura & Yamazaki, 1972). Under these conditions, the rate of reduction of cytochrome *c* by 1,4-benzohydroquinone is negligible, and the percentage of single-electron flux is calculated from the ratio of reduction of cytochrome *c* to twice the rate of oxidation of NADPH in the presence of quinone.

Replacement of Glu201 by glutamine increases the percentage of one-electron reduction of benzoquinone to 100% (Table 1). This reaction is not primarily superoxide-mediated since the formation of superoxide from the NADPH dependent reduction of molecular oxygen by mutant enzymes, estimated from the rate of superoxide dismutase inhibitable reduction of added cytochrome *c*, makes up less than 10−15% of total electron flux.

Nucleotide Specificity. In addition to the normal, physiological reductant NADPH, we measured the kinetic parameters exhibited by WT and mutant trypanothione reductases with the higher potential reductants 3APADPH and t-NADPH. In contrast to the WT enzyme, where both substrates are poorer substrates as assessed by maximum velocity determinations, the mutant enzymes use these two reduced nucleotide substrates better than the WT (Table 2). The E201Q mutant uses 3APADPH and t-NADPH at 98 and 87%, respectively, of the rate of NADPH, while the E201D mutant uses 3APADPH as a better substrate than NADPH, but uses t-NADPH only poorly.

To extend these observations, we determined the inhibition constants of the oxidized forms of these nucleotides against NADPH. With the exception of t-$NADP^+$ using the E201D mutant, all oxidized nucleotides exhibit linear, competitive inhbition versus NADPH. $NADP^+$ inhibits all three enzymes with statistically equivalent K_i values (~11 μM). $3APADP^+$ is bound 2.5−4 times more tightly by the two mutant enzymes compared to the wild type enzyme.

DISCUSSION

The chemical mechansin of FAD-containing pyridine nucleotide dependent disulfide reductases involves two half-reactions, which occur on opposite faces of the isoalloxazine ring of the bound flavin cofactor. The reductive half-reaction involves formation of the binary enzyme-reduced nucleotide complex, followed by hydride transfer of the S4 hydrogen of reduced pyridine nucleotide to the N5 position of the isoalloxazine ring. This generates transiently a charge transfer complex between $FADH^-$ and $NAD(P)^+$ whose absorbance characteristics have been previously described (Williams, 1992). Intramolecular electron transfer to the disulfide occurs via a C4a covalent intermediate, to generate the two-electron-reduced enzyme, EH_2, which has a characteristic charge transfer absorbance feature at ~550 nm resulting from the interaction of the proximal thiolate anion of the redox active disulfide and the oxidized flavin (Wong et al., 1988; Williams, 1992; Reitveld et al., 1994). The reoxidation of two-electron-reduced enzyme by the disulfide substrate, to form the first of the thiol products, occurs via the initial nucleophilic attack of the distal thiolate anion of the redox active disulfide and a mixed enzyme−substrate disulfide. This is subsequently attacked by the proximal thiolate to release the second product and regenerate the oxidized enzyme (Wong et al., 1988; Leichus et al., 1992).

Table 1: Electron Acceptor Specificity of WT and Mutant *T. congolense* Trypanothione Reductases[a]

	WT		E201Q		E201D	
electron acceptor	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
trypanothione	122 ± 4	$(7.2 \pm 0.3) \times 10^6$	5.1 ± 0.1	$(5.7 \pm 1.0) \times 10^5$	5.3 ± 0.1	$(6.7 \pm 0.6) \times 10^5$
9,10-phenanthrenequinone	2.9 ± 0.4	$(3.5 \pm 0.2) \times 10^4$	9.4 ± 1.3	$(2.9 \pm 0.4) \times 10^5$	29 ± 12	$(2.2 \pm 0.2) \times 10^5$
1,4-benzoquinone	4.8 ± 1.3	$(2.9 \pm 0.3) \times 10^4$	24.5 ± 1.7	$(9.4 \pm 1.2) \times 10^5$	13.6 ± 1.0	$(2.6 \pm 0.3) \times 10^5$
% $1e^-$ reduction (benzoquinone)		44		100		50
oxygen	<0.02	–	1.5	–	1.06	–

[a] All reactions were performed in 50 mM Hepes, pH 7.5, containing 50 μM NADPH and 0.3 mM EDTA at 25 °C.

Table 2: Nucleotide Specificity of WT and Mutant Trypanothione Reductases[a]

	WT		E201Q		E201D	
substrate	rel V_m (%)	K_m (μM)	rel V_m (%)	K_m (μM)	rel V_m (%)	K_m (μM)
NADPH	100[b]	1.2 ± 0.2	100[b]	1.5 ± 0.9	100[b]	2.0 ± 0.5
3-APADPH	13	3.9 ± 1.1	98	3.2 ± 0.9	124	3.4 ± 0.6
t-NADPH	39	11.3 ± 1.1	87	2.3 ± 0.3	2.5	6.6 ± 0.6

[a] All reactions were performed in 50 mM Hepes, pH 7.5, containing 100 μM $T(S)_2$ and 0.3 mM EDTA at 25 °C. [b] See Table 1 for values of V in s^{-1}.

Site-directed mutagenesis studies of a number of disulfide reductases have been reported. *E. coli* glutathione reductase has been subjected to extensive mutagenic examination of nucleotide specificity (Scrutton et al., 1990), kinetic mechanism (Berry et al., 1989), and general acid catalysis (Deonarian et al., 1989). The *E. coli* (Henderson et al., 1988) and human (Bradley et al., 1991) glutathione reductases have been mutagenized to enhance their catalytic abilities to reduce trypanothione, and the *T. congolense* trypanothione reductase has been engineered to perform glutathione reduction (Sullivan et al., 1991). Of most interest to the present study are two studies of *E. coli* lipoamide dehydrogenase examining the effects of mutations of the conserved Lys-Glu ion pair positioned in the nicotinamide binding pocket of glutathione reductase (Pai & Schulz, 1983), lipoamide dehydrogenase (Mattevi et al., 1992), and trypanothione reductase (Kuriyan et al., 1991). The replacement of Glu188 of *E. coli* lipoamide dehydrogenase with aspartate caused a reduction of the maximum velocity to 0.3% of that of WT but had only modest effects on the K_m values determined for NADH (Allison et al., 1988), while replacement of Lys53 with arginine causes a "profound" change in the activity and properties of the expressed mutant enzyme (Maeda-Yorita et al., 1994). Given previous suggestions that this ion pair modulates the redox potential of the flavin (Pai & Schulz, 1983) and may contribute to the stabilization of the hydride ion transfer transition state (Sweet & Blanchard, 1991), we have prepared and characterized the E201Q and E201D mutant forms of *T. congolense* trypanothione reductase. While we could prepare the K60R mutated gene, we were unable to obtain reasonable levels of expression and could not demonstrate catalytic activity in either crude extracts or chromatographically purified fractions.

Significant differences are apparent in the optical spectra of the oxidized forms of both the E201D and especially the E201Q mutant enzymes, as seen in Figure 1. Although the shoulder at 480 nm, indicative of the hydrophobic environment of the FAD (Harbury et al., 1959), is observed in both mutants, the peaks at 380 and 450 nm observed for the WT are significantly blue-shifted in the E201Q mutant (Figure 1B). These shifts are consistent with predictions based on the hydrophobicity of the flavin environment (Shinkai et al., 1981). More striking differences are observed in the visible spectra of the two-electron-reduced forms of the E210Q and E201D mutants. Both mutants exhibit reduced intensities of the charge transfer band at 550 nm and substantial bleaching of the flavin absorbance at 430 nm compared to that of the WT enzyme. This suggests that, in the two-electron-reduced forms of these mutant enzymes, the distribution of electron density is shifted from the dithiols to the FAD (Williams, 1992), possibly reflecting the more positive redox potential of the $FAD/FADH^-$ pair.

The redox potentials for a number of pyridine nucleotide dependent disulfide reductases have been determined and range from −265 mV for glutathione reductase (Arscott et al., 1991) to −269 mV for mercuric reductase (Fox & Walsh, 1982) to −280 mV for pig heart lipoamide dehydrogenase (Matthews & Williams, 1976). The value that we have determined for WT trypanothione reductase of −273 mV falls within this range. The measured redox potentials of the E/EH_2 pair for both mutants are more positive, being raised by 14 and 22 mV for the E201D and E201Q mutants, respectively (Figure 3), confirming the role of the conserved Lys-Glu pair in modulating the flavin redox potential. These measured macroscopic potentials must be carefully interpreted since they can reflect either the flavin ($FAD/FADH^-$) or enzymic disulfide/dithiol potential, unless the two potentials are widely separated, as is the case for pig heart lipoamide dehydrogenase (Matthews & Williams, 1976). In order to assess the change in the potentials observed for the mutant forms of trypanothione reductase, we compared the ability of mutants to catalyze reduction of quinones and molecular oxygen, since these reactions are thought to occur with the flavin and do not rely on the redox active disulfide (Cenas et al., 1994).

While trypanothione-reducing activity is reduced to ca. 5% in both the mutants compared to WT, the quinone reductase activity of the mutant trypanothione reductases is enhanced by 7−30-fold compared to that of the WT enzyme. In addition, the percentage of benzoquinone reduction occurring via one-electron transfer increases from 44% for the WT enzyme to 100% for the E201Q mutant. These reactions have previously been shown to occur when the redox active disulfide is reduced and alkylated or when the nucleotide binding pocket is occupied by $NADP^+$, suggesting that quinone reduction occurs predominantly by quinone binding at a third site and via a one-electron transfer reaction (Cenas et al., 1994). These data suggest that the observed more positive macroscopic redox potentials of the mutants are specific effects on the redox potential of the flavin and therefore reactions occcurring at the flavin. These perturbations of the normal distribution of reducing equivalents between the flavin and disulfide in the two-electron-reduced

enzyme should enhance reactions known to be performed by reduced flavins and can account for the 20-fold decrease in trypanothione-reducing activity, which occurs via interaction of the trypanothione disulfide with the enzymic dithiols. While WT disulfide reductases catalyze molecular oxygen reduction slowly, both two-electron-reduced forms of the mutants are 50-fold more reactive to oxidation by molecular oxygen (Table 1). Similar results have been reported for mercuric reductase, where site-directed mutagenesis of the redox active cysteines to serine enhanced the oxidase activity of the mutants relative to the WT enzyme (DiStefano et al., 1989).

It has been suggested that, in addition to its role in stabilizing the positive charge on Lys66 and thus influencing the flavin redox potential, Glu 201 may contribute to the hydride transfer reaction, both electrostatically (Sweet & Blanchard, 1991) and by making specific interactions with bound nucleotide (Karplus & Schulz, 1989). Thus, in *T. cruzi* trypanothione reductase, one of the carboxyl oxygens (OE2) of Glu202 (equivalent to Glu201 in *T. congolense* trypanothione reductase) is within hydrogen-bonding distance (3.0 Å) of the nitrogen (N7) of the carboxamide side chain of bound NADPH, while the other carboxyl oxygen of Glu202 (OE1) is 3.5 Å from C4 (Lantwin et al., 1994). The latter interaction may stabilize the transiently developing positive charge at C4 as a result of C4−H_s cleavage occurring during the hydride transfer step in the reductive half-reaction. The interaction of the glutamate carboxyl with the carboxamide nitrogen suggests that the E201Q and E210D mutants might exhibit different selectivity for reduced nucelotide substrate analogs. This is in fact observed, although in both cases, the mutants use reduced 3-acetylpyridine adenine dinucleotide phosphate as well, or better, than NADPH, compared to the wild-type enzyme (98 and 124% for E201Q and E201D, respectively, compared to 13% for WT; Table 2). Similar results have been reported for *E. coli* lipoamide dehydrogenase, where 3APADH was a better substrate for the E201D mutant enzyme (Allison et al., 1988). Although 3APAD(P)H has a more positive redox potential than NAD(P)H (−258 versus −320 mV), these data suggest that there may be changes in the interaction of the enzyme with this nucleotide substrate, which cannot participate in hydrogen-bonding interactions due to the replacement of the NH_2 group with the isosteric methyl group. To test this possibility, we determined the inhibition constants of $NADP^+$ and $3APADP^+$ versus that of NADPH for WT and mutant forms of trypanothione reductase. While the determined K_i values for $NADP^+$ were statistically indistinguishable for WT, E201Q, and E201D enzymes, the K_i values for $3APADP^+$ were 2−3 times lower for the mutant enzymes compared to WT (Table 3). This suggests some favorable interaction between this nucleotide analog and the mutant enzymes, although we are unsure what the nature of the interaction is from this limited data. The K53R mutant of *E. coli* lipoamide dehydrogenase has also recently been shown to exhibit altered binding of nucleotide substrates (Maeda-Yorita et al., 1994), suggesting that these phenomena may be relevant to other members of the disulfide reductase family.

Together, the potentiometric and kinetic characterization of the E201Q and E201D mutants of *T. congolense* trypanothione reductase reported here supports the hypothesis that the conserved Lys-Glu ion pair in the family of flavoenzyme disulfide reductases modulates the redox potential, and thus chemical reactivity, of the noncovalently bound FAD (Pai & Schultz, 1983; Williams, 1992). The reduced disulfide-reducing activity and enhanced quinone reductase and oxidase activity of the E201D, and especially E201Q, mutant support a role for the glutamate carboxyl maintaining Lys60 in its protonated, and correctly positioned, form. The redox potential of the FAD and the equilibrium distribution of reducing equivalents in the two-electron-reduced enzyme between flavin and redox active disulfide are thus affected. The basis for the observed nucleotide selectivity in the mutant enzymes is unknown at this time but is under investigation.

Table 3: Inhibition of WT and Mutant Trypanothione Reductases by Oxidized Pyridine Nucleotides[a]

	K_i (μM)[b]		
	WT	E201Q	E201D
$NADP^+$	11 ± 2.0	13.6 ± 2.5	11.1 ± 5.5
$3\text{-}APADP^+$	8.4 ± 1.7	1.7 ± 0.3	3.3 ± 0.8
$t\text{-}NADP^+$	2.9 ± 0.4	11 ± 3.5	5.04 ± 2.2 (slopes) 51.3 ± 8.2 (intercepts)

[a] All reactions were performed in 50 mM Hepes, pH 7.5, containing 50 μM NADPH and 0.3 mM EDTA at 25 °C. [b] All oxidized nucleotides exhibited linear competitive inhibition versus NADPH, except $t\text{-}NADP^+$ which exhibited linear, noncompetitive inhibition.

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