Mechanism of Reduction of Quinones by *Trypanosoma congolense* Trypanothione Reductase†

Narimantas K. Cenas,‡,§ David Arscott,⊥ Charles H. Williams, Jr.,⊥ and John S. Blanchard*,§

Institute of Biochemistry, Lithuanian Academy of Sciences, 2600 Vilnius, Mokslininku 12, Lithuania, Department of Veteran Affairs Medical Center and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48105, and Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Received October 18, 1993; Revised Manuscript Received December 27, 1993®

ABSTRACT: A number of quinones were analyzed as substrates for trypanothione reductase from *Trypanosoma congolense*, an enzyme responsible for the protection of trypanosomes against oxidative stress. Using NADPH as substrate, the maximal rate of the steady-state reaction at pH 7.5 was between 24 and 1.6 s^{-1} for 11 quinone substrates. The biomolecular steady-state rate constants for quinone reduction, V/K_m, ranged from 240 to 1.9×10^5 M^{-1} s^{-1}, and their logarithms exhibited a hyperbolic dependence on the one-electron-reduction potentials of the quinone substrate. The addition of $NADP^+$ stimulated these rates, with V/K_m values increasing with an increasing $NADP^+$/NADPH ratio. The results of alkylation of the cysteine residue in the two-electron-reduced enzyme by iodoacetamide indicate that these residues are not primarily involved in the reduction of these quinones. Single-electron reduction of benzoquinone constitutes 40% of the total electron transfer from NADPH to quinone in the absence of $NADP^+$, and increases to 80% at $NADP^+$/NADPH ratios greater than 10. These steady-state results were confirmed in pre-steady-state rapid reaction experiments. The rate of reduced enzyme oxidation by 1,4-benzoquinone is approximately 100 times faster in the presence of $NADP^+$ than in its absence. The reactivities of various pyridine nucleotide liganded forms of EH_2 for quinone reduction are presumably affected by the electron density at FAD. We suggest that one-electron reduction of quinones occurs at a site distinct from the two active sites involved in hydride ion transfer and disulfide reduction.

Trypanosomes are the causative parasites of several tropical diseases, including African sleeping sickness (*Trypanosoma gambiense* and *Trypanosoma rhodiense*) and Chagas disease (*Trypanosoma cruzi*) in humans, and nagana (*Trypanosoma congolense* and *Trypanosoma brucei*) in cattle. Trypanothione reductase catalyzes the NADPH-dependent reduction of trypanothione, N^1,N^8-bis(glutathionyl)spermidine, $T(S)_2$,[1] a glutathione analog unique to trypanosomatid parasites. This enzyme performs similar functions to those of glutathione reductase in these parasites, including maintenance of intracellular thiol redox poise and protection against oxidative stress resulting from an increased steady-state concentration of radicals and other reactive reduced forms of oxygen [for a review, see Fairlamb and Cerami, (1992)]. The enzyme from *T. congolense* is a dimer of identical 55-kDa subunits, each containing a noncovalently bound FAD cofactor and a redox-active disulfide consisting of cysteine residues 52 and 57 (Shames et al., 1986). A 2.4-Å-resolution three-dimensional structure of trypanothione reductase purified from *Crithidia fasciculata* (Kuriyan et al., 1991) reveals a substantial structural similarity to human erythrocyte glutathione reductase (Pai et al., 1988), with structurally distinct binding sites for pyrdine 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 Cys 57 thiolate anion, with an absorption maximum at 530 nm (Krauth-Siegel et al., 1987; Sullivan et al., 1989), which is subsequently reoxidized by $T(S)_2$. The steady-state kinetic mechanism is "ping-pong" (Leichus et al., 1992), and amino acid residues responsible for the selective binding of $T(S)_2$, and its discrimination against glutathione, have been recently identified (Sullivan et al., 1991).

It has been suggested that trypanosomes are more sensitive to oxidative stress than their hosts (Walsh et al., 1991), and the selective inhibition of trypanothione reductase is one basis for the search of trypanocidal compounds. It has been reported that quinones and nitrofurans act as inhibitors of trypanothione reductase (Henderson et al., 1988; Jockers-Scherübl et al., 1989) and of the structurally and functionally related flavoproteins glutathione reductase (Grinblat et al., 1989; Cènas et al., 1991; Bironaitè et al., 1991) and thioredoxin reductase (Mau & Powis, 1992). Quinone and nitrofuran substrates are reduced by these enzymes, and subsequently redox cycle via reaction of the reduced quinones and nitrofurans with molecular oxygen to form reactive oxygen species (Henderson et al., 1988; Jockers-Scherübl et al., 1989; Cènas et al., 1989; Mau & Powis, 1992). They act as "subversive substrates" of antioxidant enzymes (Henderson et al., 1988), converting the normally protective functions of these enzymes into toxic ones. It is believed that heteroaromatic, positively-charged inhibitors of trypanothione reductase bind at or close to the $T(S)_2$-binding site (Jockers-Scherübl et al., 1989; Benson et al., 1992), although binding at other sites (Karplus et al., 1989; Kuriyan et al., 1991) cannot be excluded. The mechanism of reduction of these compounds, especially the formation of their one-electron-reduced forms, is poorly

† This work was supported by NIH Grant GM33449 and the Hirschl Foundation (to J.S.B.) and NIH Grant GM 21444 (to C.H.W.).

* To whom correspondence should be addressed. Phone: (718)430-3096. FAX: (718)892-0703.

‡ Lithuanian Academy of Sciences.

§ Albert Einstein College of Medicine.

⊥ University of Michigan.

® Abstract published in *Advance ACS Abstracts,* February 15, 1994.

[1] Abbreviations: $T(S)_2$, oxidized trypanothione; k_{obsd}, first-order rate constant in the stopped-flow experiments; k_{ox}, second-order oxidation rate constant in the stopped-flow experiments; E, oxidized enzyme; EH_2, two-electron-reduced enzyme; E_1, single-electron-reduction potential of the quinones; FAD, flavin adenine dinucleotide.

0006-2960/94/0433-2509$04.50/0

Table 1: Dependence of the Kinetic Parameters of Reduction of Quinones by Trypanothione Reductase on Their Single-Electron-Reduction Potentials (E^1)[a]

quinone	E^1, V[b]	V, s^{-1}	V/K_m, M^{-1} s^{-1}
1,4-benzoquinone, **1**	0.09	4.8 ± 1.3	(2.9 ± 0.3) × 10^4
2-methyl-1,4-benzoquinone, **2**	0.01	1.6 ± 0.1	(1.1 ± 0.1) × 10^4
2,3-dichloro-1,4-naphthoquinone, **3**	−0.03	24 ± 9	(1.9 ± 0.2) × 10^5
2,5-dimethyl-1,4-benzoquinone, **4**	−0.08	2.4 ± 0.2	(5.9 ± 0.3) × 10^3
5-hydroxy-1,4-naphthoquinone, **5**	−0.09	3.4 ± 0.6	(3.2 ± 0.4) × 10^4
5,8-dihydroxy-1,4-naphthoquinone, **6**	−0.11	4.4 ± 1.0	(4.1 ± 0.5) × 10^4
9,10-phenanthrenequinone, **7**	−0.12	2.9 ± 0.4	(3.5 ± 0.2) × 10^4
1,4-naphthoquinone, **8**	−0.15	8.8 ± 3.2	(6.3 ± 0.4) × 10^3
2-methyl-5-hydroxy-1,4-naphthoquinone, **9**	−0.16	2.4 ± 0.4	(5.8 ± 0.3) × 10^3
2-methyl-1,4-naphthoquinone, **10**	−0.20	1.6 ± 0.2	(1.6 ± 0.1) × 10^3
tetramethyl-1,4-benzoquinone, **11**	−0.26		(2.4 ± 0.3) × 10^2

[a] All data obtained in 50 mM Hepes, pH 7.5, at 25 °C. [b] From O'Brien (1991) and Cènas et al. (1989).

understood. As an initial attempt to understand the mechanism of reduction of quinones by trypanothione reductase, we have analyzed these reactions using steady-state and pre-steady-state kinetic methods.

EXPERIMENTAL PROCEDURES

Materials. The overexpressed trypanothione reductase from *T. congolense* was isolated as previously described (Sullivan et al., 1989). The concentration of enzyme was determined spectrophotometrically using ϵ_{464} = 11.3 mM^{-1} cm^{-1} for the absorbance of enzyme-bound FAD (Shames et al., 1986). All pyridine nucleotides, cytochrome *c*, 2′,5′-ADP, iodoacetamide, and buffer components were purchased from Sigma. Quinones were purchased from Sigma or Aldrich and used as received (numbers refer to Table 1). Oxidized trypanothione was purchased from Bachem Bioscience (Switzerland).

Steady-State Kinetic Studies. Initial velocities were measured by monitoring the decrease in the absorbance at 340 nm of NADPH (ϵ_{340} = 6.2 mM^{-1} cm^{-1}) in a Gilford 260 spectrophotometer equipped with thermospacers and connected to a circulating water bath. Typically, 10–100 μM NADPH was used, and assays were performed in 0.05 M Hepes, pH 7.5, containing 1 mM EDTA, at 25 ± 0.1 °C (Leichus et al., 1992). For the determination of steady-state values of V and V/K_m of quinones, 5–7 concentrations of the quinone substrate were used. The maximal concentrations of quinones were limited by their water solubility, e.g., 30 μM 2,3-dichloro-1,4-naphthoquinone, 50 μ-M 9,10-phenanthrene quinone, 400 μM 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, or tetramethyl-1,4-benzoquinone, or by high rates of nonenzymatic oxidation of NADPH (i.e., 100 μM 1,4-benzoquinone, 200 μM 2-methyl-1,4-benzoquinone). Under the conditions used, the rates of the latter reactions did not exceed 5–10% of the enzyme-catalyzed reaction rate, and were subtracted from the enzyme-catalyzed rate. Quinones were dissolved in either acetonitrile or ethanol at concentrations which permitted the final concentration of organic solvent to be less than 1% of the total reaction volume. Control experiments demonstrated that this content of organic solvent did not affect enzyme activity. 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 6.5–7.0 (Nakamura & Yamazaki, 1972).

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

Rapid Reaction Studies. Rapid reaction kinetics of quinone reduction by two-electron-reduced trypanothione reductase were measured with a stopped-flow spectrophotometer designed for anaerobic work. This instrument has a 2-cm optical path and a bifurcated quartz light pipe to direct the emerging light beam to a Tracor Northern TN-6500 rapid scan spectrometer diode array detector (350–700 nm) and to a double monochromator (variable wavelength)/photomultiplier. The photomultiplier tube is interfaced to a Gateway2000 486/33C computer through a Data Translation DT2801-A high-speed data acqusition board, with software for data acquisition written in ASYST (D. Arscott and C. H. Williams, Jr., unpublished results). Trypanothione reductase at a concentration of 30–50 μM active sites in 0.05 M Hepes, pH 7.5, was made anaerobic by 10 cycles of evacuation and flushing with oxygen-free nitrogen. The enzyme was reduced by tipping 5–8 mL of the enzyme solution into a side arm of the anaerobic tonometer which had a ca. 50-fold excess of sodium borohydride in ca. 10 μL of 0.2 M NaOH. Excess borohydride was allowed to hydrolyze for 30–40 min ($k_{hydrolysis}$ = 0.03–0.04 s^{-1}; Davis & Swain, 1960) prior to reaction with oxidant. Complete enzyme reduction was assessed spectrophotometrically. Reactions were initiated by rapidly mixing equal volumes of borohydride-reduced enzyme and anaerobic 1,4-benzoquinone solutions, either alone or containing 2 equiv of $NADP^+$ per enzyme-bound FAD at 25 °C. Reactions were performed in duplicate at each benzoquinone concentration.

Stopped-flow spectrophotometric traces at 450 and 530 nm were analyzed as a sum of the exponential process (Marquardt, 1963) using a PASCAL program developed in Professor D. Ballou's laboratory at the University of Michigan. Data were first fitted to a single exponential and then to multiple exponentials. The appropriateness of the fit was determined by visual inspection of the experimental and simulated traces, and from plots of the residuals. The first-order rate constants (k_{obsd}) were plotted against the concentration of benzoquinone to obtain the second-order rate constant of enzyme oxidation (k_{ox}).

Alkylation of Reduced Trypanothione Reductase with Iodoacetamide. The alkylation of reduced trypanothione reductase with iodoacetamide was performed as described previously (Shames et al., 1986). The anaerobic mixture contained 1.2 μM trypanothione reductase, 2.0 mM iodoacetamide, and 0.25 mM NADPH, and was allowed to proceed for 1 h at 25 °C. Residual trypanothione and quinone reducing activities were measured as described above.

RESULTS

Enzymatic Activities of Trypanothione Reductase in $T(S)_2$ Reduction. At saturating concentrations of NADPH (50 μM), the variation of $T(S)_2$ concentrations between 10 and 100 μM gives a linear Lineweaver–Burk plot, allowing the calculation

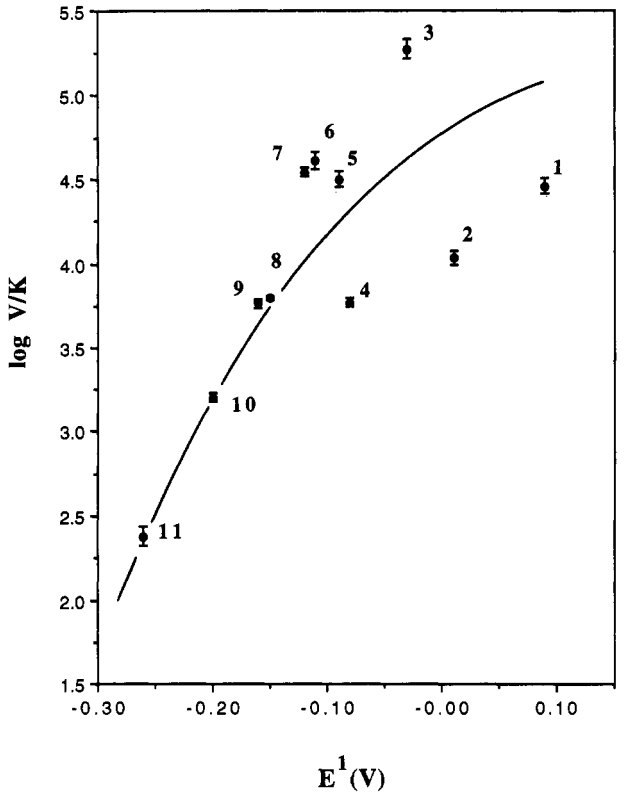


FIGURE 1: Dependence of steady-state V/K_m of reduction of quinones by trypanothione reductase on their single-electron-reduction potentials (E^1): 1,4-benzoquinone (**1**), 2-methyl-1,4-benzoquinone (**2**), 2,3-dichloro-1,4-naphthoquinone (**3**), 2,5-dimethyl-1,4-benzoquinone (**4**), 5-hydroxy-1,4-naphthoquinone (**5**), 5,8-dihydroxy-1,4-naphthoquinone (**6**), 9,10-phenanthrenequinone (**7**), 1,4-naphthoquinone (**8**), 2-methyl-5-hydroxy-1,4-naphthoquinone (**9**), 2-methyl-1,4-naphthoquinone (**10**), tetramethyl-1,4-benzoquinone (**11**).

of the maximal velocity, V_m = 122 ± 4 s^{-1}, and the K_m value for $T(S)_2$ of 17 ± 2 μM, which are similar to those reported previously (Leichus et al., 1992; Fairlamb & Cerami, 1992). At a fixed concentration of 100 μM $T(S)_2$, $NADP^+$ and 2′,5′-ADP act as linear competitive inhibitors vs NADPH (data not shown). Calculated K_i values for $NADP^+$ and 2′,5′-ADP were 11.1 ± 2 and 8.9 ± 1.9 μM, respectively, compared to the K_m value of NADPH of 2.4 ± 0.5 μM under these conditions. At a fixed concentration of NADPH of 25 μM (~10K_m), $NADP^+$ and 2′,5′-ADP act as uncompetitive inhibitors versus $T(S)_2$ (data not shown). Calculated K_i values of $NADP^+$ and 2′,5′-ADP versus $T(S)_2$ were 67 ± 3 and 75 ± 2 μM, respectively, at this concentration of NADPH.

Steady-State Reduction of Quinones by Trypanothione Reductase. The steady-state kinetic parameters for reduction of quinones by trypanothione reductase are shown in Table 1. The calculated values of V and V/K_m were independent of the concentration of NADPH used (10–100 μM). The data of Figure 1 indicate that there is a hyperbolic dependence of the log V/K_m values of quinones on their single-electron-reduction potentials (E^1). This observed dependence of the rate on the redox potential is characteristic of an outer-sphere single-electron-transfer reaction (Marcus & Suttin, 1985; Meyer et al., 1983), and indicates that semiquinones may be formed as products during the reduction of quinones by trypanothione reductase.

The efficiency of a one-electron reduction of quinones may be estimated quantitatively using the observation that, at pH values <7.2, the rate of reduction of cytochrome c by 1,4-benzohydroquinone is negligible, but that benzosemiquinone rapidly reduces cytochrome c at these pH values (k = 1.5 × 10^6 M^{-1} s^{-1}; Nakamura & Yamazaki, 1972). This reaction is not mediated via superoxide, and the rate of comproportionation of benzoquinone and benzohydroquinone is small, especially under these initial velocity steady-state conditions. At the high concentrations of cytochrome c used in these experiments (~50 μM), the percentage of single-electron flux is calculated from the ratio of the rate of reduction of cytochrome c to twice the rate of oxidation of NADPH in the presence of quinone. In the absence of added $NADP^+$, the percentage of trypanothione reductase catalyzed single-electron reduction of benzoquinone is 40% at pH 6.5–7.0, with the remaining 60% accounted for by two-electron reduction.

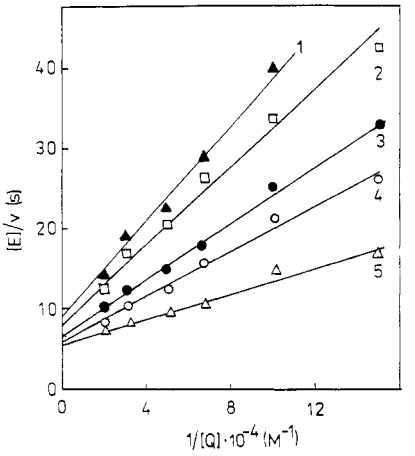


FIGURE 2: Kinetics of reduction of phenanthrenequinone by trypanothione reductase using NADH as substrate. Concentration of NADH: 15.6 mM (1), 21.5 μM (2), 32.3 μM (3), 64.6 μM (4), 109 μM (5).

Using NADH as substrate, the maximal activity of $T(S)_2$ reduction is decreased about 30-fold compared to using NADPH as reductant (Leichus et al., 1992). For several of the quinones investigated, the maximal rates of their enzymatic reduction using NADH as reductant were determined, and decreased as well, being 0.156 ± 0.01 s^{-1} for 5-hydroxy-1,4-naphthoquinone (**5**; cf. Table 1) and 0.12 ± 0.02 s^{-1} for 9,-10-phenanthrenequinone (**7**; Figure 2). However, V/K values of these quinones were not significantly different from those determined using NADPH as reductant (cf. Table 1), being (4.0 ± 2.6) × 10^4 M^{-1} s^{-1} for 5-hydroxy-1,4-naphthoquinone (**5**), and (2.4 ± 0.78) × 10^4 M^{-1} s^{-1} for 9,10-phenanthrenequinone (**7**).

The anaerobic alkylation of Cys 52 of reduced trypanothione reductase by iodoacetamide at pH 7.5 led to a loss of 98% of the $T(S)_2$ reducing activity. However, enzyme treated this way maintained considerable quinone reductase activity. Thus, the maximum velocity for phenanthrenequinone (**7**) reduction by alkylated trypanothione reductase was 1.5 ± 0.2 s^{-1} (52% of normal; see Table 1) while the maximal velocity for 5-hydroxy-1,4-naphthoquinone (**5**) reduction was 2.9 ± 0.9 s^{-1} (85% of normal; see Table 1). The V/K values of both quinones were also reduced to 55% and 24% of their values for nonalkylated enzyme.

$NADP^+$ as Activator of the Quinone Reductase Reaction of Trypanothione Reductase. In contrast to the reduction of $T(S)_2$, where $NADP^+$ inhibited the reaction, the reduction of quinones was activated by $NADP^+$ (Figure 3). The addition of $NADP^+$ increased both the V/K_m values of quinones as well as the maximum velocity of quinone reduction. It was observed that the V/K_m values of quinones depended on the $[NADP^+]/[NADPH]$ ratio, acquiring constant values at

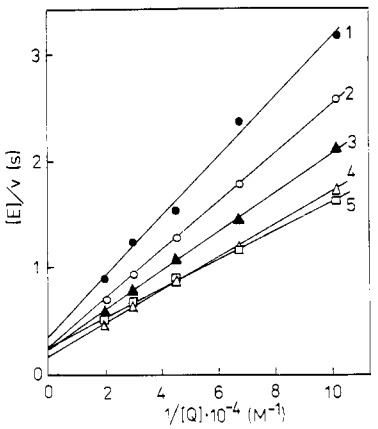


FIGURE 3: Activation by $NADP^+$ of reduction of 9,10-phenanthrenequinone by trypanothione reductase. Concentration of NADPH: 90 mM. Concentration of $NADP^+$: 0 (1), 10 μM (2), 30 μM (3), 300 μM (4), 1000 μM (5).

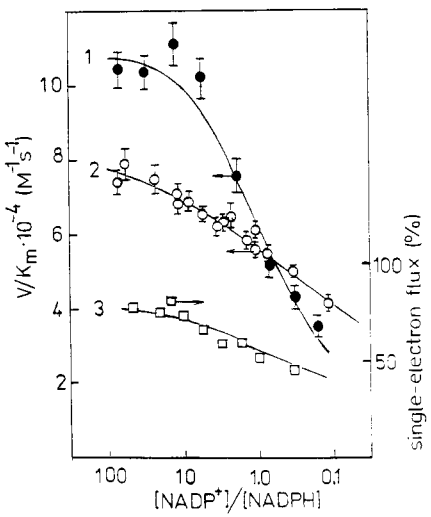


FIGURE 4: Dependence of the V/K_m value of 1,4-benzoquinone (1, left scale) and 9,10-phenanthrenequinone (2, left scale) reduction and the efficiency of the single-electron reduction of benzoquinone (3, right scale) by trypanothione reductase on the $[NADP^+]/[NADPH]$ ratio.

$[NADP^+]/[NADPH] > 10$ (Figure 4). The degree of activation was dependent on the quinone substrate, being 3.4–3.6 for 1,4-benzoquinone (**1**), 2-methyl-1,4-benzoquinone (**2**), and 2,3-dichloro-1,4-naphthoquinone (**3**), 2.6 for phenanthrenequinone (**7**) and 2-methyl-5-hydroxy-1,4-naphthoquinone (**9**), and 1.6 for dimethyl-1,4-benzoquinone (**4**), 1,4-naphthoquinone (**8**), and 2-methyl-1,4-naphthoquinone (**10**). The V/K_m values for reduction of 5-hydroxy-1,4-naphthoquinone (**5**) and 5,8-dihydroxy-1,4-naphthoquinone (**6**) were not affected by added $NADP^+$. The increase in the $[NADP^+]/[NADPH]$ ratio also increases the percentage of a single-electron reduction of benzoquinone, paralleling its effect on V/K_m of quinones (Figure 4). At $[NADP^+]/[NADPH]$ ratios greater than 10, reduction of benzoquinone by a one-electron transfer occurs with 80% of the electrons introduced into trypanothione reductase by NADPH. At 1 mM $NADP^+$, the V/K_m value for NADPH, determined for both $T(S)_2$ reductase and phenanthrenequinone reductase reactions, are experimentally indistinguishable and equal to $(5.55 \pm 0.25) \times 10^5$ and $(5.14 \pm 0.66) \times 10^5$ M^{-1} s^{-1}, respectively. Neither 2′,5′-ADP nor 3-amino adenine dinucleotide, a redox-inactive analog of $NADP^+$, activates quinone reduction, and, in fact, inhibits this reaction.

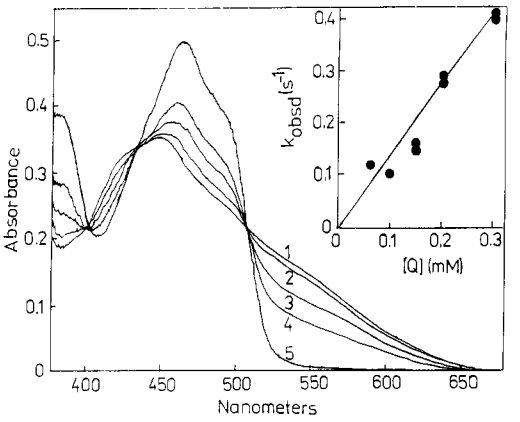


FIGURE 5: Stopped-flow kinetics of oxidation of reduced trypanothione reductase (EH_2) by 1,4-benzoquinone (2-cm optical path). Final concentration of trypanothione reductase: 23 μM. Final concentration of benzoquinone: 300 μM. Times after mixing: 9.5 ms (1), 0.22 s (2), 0.83 s (3), 1.66 s (4), 10.31 s (5). Inset: dependence of the first-order oxidation rate constant (k_{obsd}) on the concentration of benzoquinone (monitored at 530 nm).

Rapid Reaction Studies of Reoxidation of Reduced Trypanothione Reductase by 1,4-Benzoquinone. The anaerobic reduction of trypanothione reductase with sodium borohydride leads to a decrease of FAD absorbance at 460 mm and an increase in absorbance at 530 nm (Figure 5, spectra 1), indicating the formation of a two-electron-reduced enzyme (EH_2) with a charge-transfer complex between FAD and the thiol of Cys 57 (Shames et al., 1986). The spectrum of EH_2 is analogous to those obtained with other disulfide-containing flavoproteins, such as glutathione reductase and lipoamide dehydrogenase (Williams, 1992). We have found in pre-steady-state rapid reaction experiments that 1,4-benzoquinone oxidizes EH_2 extremely slowly (Figure 5). The reaction rate monitored at 530 nm is best fit by a single exponential, and the dependence of this first-order rate constant (k_{obsd}) on the concentration of benzoquinone (Figure 5, inset) gives a second-order rate constant (k_{ox}) of $(1.48 \pm 0.15) \times 10^3$ M^{-1} s^{-1}.

The complex of EH_2, produced by sodium borohydride reduction, and $NADP^+$ was formed by the addition of a 2-fold excess of $NADP^+$ over the concentration of enzyme active sites. The spectral characteristics of EH_2–$NADP^+$ (a further bleaching of absorbance at 450 and 530 nm, and increased absorbance at longer wavelengths, (Figure 6) were similar to those of the complex of $NADP^+$ and reduced glutathione reductase (Bulger & Brandt, 1971). Since in control experiments the EH_2–$NADP^+$ complex was formed within the mixing time of the apparatus (≪4 ms), $NADP^+$ was added to a syringe containing benzoquinone and rapidly mixed with EH_2. 1,4-Benzoquinone oxidized EH_2–$NADP^+$ rapidly (Figure 6), without the noticeable appearance of reaction intermediates absorbing at other wavelengths. Monitoring the reaction at either 450 or 530 nm gave almost identical results (Figure 6, inset), and allowed the calculation of the second-order rate constant for quinone reduction, $k_{ox} = (1.31 \pm 0.06) \times 10^5$ M^{-1} s^{-1}. Thus, the second-order rate constant for reduction of 1,4-benzoquinone is 90 times faster in the presence of $NADP^+$ bound at the active site of trypanothione reductase.

DISCUSSION

The mutually exclusive substrate specificities of trypanothione reductase and the related host enzyme glutathione reductase have led to this parasite enzyme's targeting for inhibitor design (Walsh et al., 1991; Benson et al., 1992; Fairlamb, 1989). The gene encoding trypanothione reductase

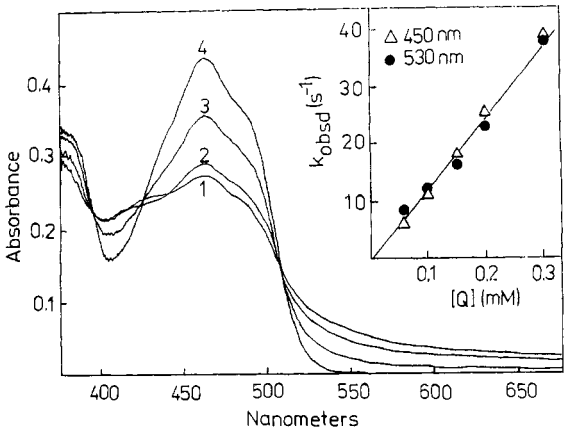


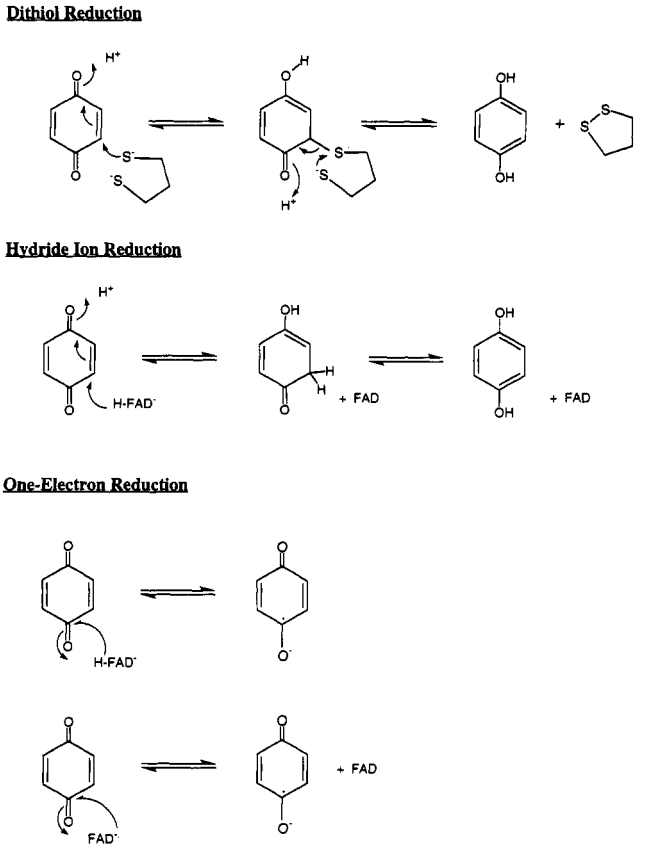
FIGURE 6: Stopped-flow kinetics of oxidation of the complex of reduced trypanothione reductase and NADP+ (EH_2–NADP+) by 1,4-benzoquinone (2-cm optical path). Final concentration of trypanothione reductase: 20.5 μM. Final concentration of benzoquinone: 300 μM. Times after mixing: 9.5 ms (1), 14.9 ms (2), 31.5 ms (3), 280 ms (4). Inset: dependence of the first-order oxidation rate constant (k_{obsd}) on the concentration of benzoquinone.

has been cloned and sequenced from the insect trypanosomatid *C. fasiculata* (Field et al., 1992) and the cattle parasite *T. congolense* (Shames, 1988). The derived amino acid sequences are 68% identical, but only 34% identical to the amino acid sequence of human glutathione reductase (Krauth-Siegel et al., 1982). Alignment of the sequences of trypanothione and glutathione reductases reveals that essentially all of the catalytically important residues have been conserved, including the redox-active disulfide, composed of Cys 52 and 57 in *T. congolense* trypanothione reductase, the His461′–Glu466′ ion pair responsible for protonation of the thiolate anion of trypanothione produced during reduction (Leichus et al., 1992), and Tyr 197 which hydrogen bonds to the flavin N10 position, effectively capping the nicotinamide binding site. The recently determined 2.4 Å-resolution-three-dimensional structure of *C. fasiculata* trypanothione reductase (Kuriyan et al., 1991) reveals an extraordinary degree of structural relatedness to human erythrocyte glutathione reductase (Karplus & Schulz, 1987), with root mean squared deviations in equivalent C_α positions of <1 Å.

The treatment of trypanosomiasis using organic arsenicals (Ehrlich & Bertheim, 1912), such as melarsoprol, has been shown to be due to the interaction of these compounds with the reduced, dithiol form of trypanothione (Fairlamb et al., 1989). Due to the sensitivity of the bloodstream forms of trypanosomatids to oxidative stress, and the central role of trypanothione reductase in maintaining an intracellular reducing environment, new approaches to treatment have focused on trypanothione reductase. In particular, the trypanothione reductase catalyzed reduction of naphthoquinone and nitrofuran derivatives leads to products which are readily oxidized intracellularly by molecular oxygen (Henderson et al., 1988). The sum of this futile "redox cycling" leads to the oxidation of cellular NADPH and the generation of reactive oxygen species. The mechanism by which trypanothione reductase reduces these compounds, in particular quinones, remains unresolved, and has led to the present investigations.

Quinone reduction by trypanothione reductase could occur via several different mechanisms. The reduction of quinones by dithiols has been demonstrated (Anusevicius & Cenas, 1993), and is presumed to occur via initial nucleophilic attack on the α,β-unsaturation to yield an intermediate which can subsequently be attacked to yield the dihydroquinone (quinol)

Scheme 1



and disulfide products (Scheme 1). The enzyme-catalyzed reduction of cationic napthoquinones and nitrofurans (Henderson et al., 1988) may occur by this mechanism in the $T(S)_2$-binding site, since cationic tricyclic antidepressants are competitive inhibitors versus $T(S)_2$ (Benson et al., 1992). A second mechanism involves quinone binding at the nicotinamide binding pocket defined by the parallel aromatic isoalloxazine and phenol rings of FAD and Tyr 197 (Karplus et al., 1989). Hydride transfer from the N5 position of $FADH^-$ (Manstein et al., 1986) to the α,β-unsaturation would generate the ketone shown, which would aromatize via enolization to form the quinol. A third mechanism could involve sequential one-electron transfers from the flavin to the quinone substrates, since both have thermodynamically accessible semiquinone forms.

Our first approach to distinguish between these mechanisms was the reductive alkylation of Cys 52 of trypanothione reductase. The carboxamidomethylation of the "distal" catalytic cysteine residue results in the nearly complete loss of $T(S)_2$ reducing activity, but alkylated trypanothione reductase retains the ability to reduce quinone substrates. Quinone reduction can thus be catalyzed to a significant extent by the enzyme without the covalent involvement of the reduced catalytic disulfide invoked in $T(S)_2$ reduction. The equal values of V/K_{NADPH}, determined at high concentrations of NADP+, for both the $T(S)_2$ and quinone reductase reactions, requires that quinones are reduced by a two-electron-reduced trypanothione reductase, in analogy to studies performed with glutathione reductase (Arscott et al., 1981; Cènas et al., 1989).

The stimulation of the steady-state rate of reduction of quinones by trypanothione reductase in the presence of added NADP+ (Figures 3 and 4) is analogous to previously observed effects of added NADP+ on the rate of reduction of trinitrobenzenesulfonate (Carlberg & Mannervik, 1986) and

quinones (Cènas et al., 1989) by glutathione reductase. The latter observation was explained by the higher electron density on FAD in the EH_2–$NADP^+$ complex as compared to the EH_2–NADPH complex, and by a competition between $NADP^+$ and NADPH for EH_2 binding (Cènas et al., 1989). $NADP^+$ binds tightly (K_i = 11 μM) to the EH_2 form of trypanothione reductase as seen in Figure 6. NADPH also binds to EH_2 tightly as evidenced by the differences in the spectrum of the EH_2 charge-transfer complexes at 530 nm in the presence and absence of NADPH (Krauth-Siegel et al., 1987) and data on the pre-steady-state reduction of *T. congolense* trypanothione reductase by NADPH (J. S. Blanchard, unpublished observations). The half-maximal activation of quinones occurs at $[NADP^+]/[NADPH] \approx 1$, and this value reflects the ratio of K_d values for pyridine nucleotides from the EH_2–$NADP^+$ and EH_2–NADPH complexes. The K_d value of NADPH in the EH_2–NADPH complex (ca. 1 μM) was determined from the analysis of the inhibition of phenanthrenequinone by 2′,5′-ADP, allowing us to calculate the K_d of the enzyme–NADPH complex at a zero concentration of 2′,5′-ADP.

To extend these steady-state observations, we preformed anaerobic pre-steady-state rapid reaction experiments. The second-order rate constant of oxidation of $NaBH_4$-reduced trypanothione reductase (EH_2) by benzoquinone was 1.5×10^3 M^{-1} s^{-1} (Figure 5), while the second-order rate constant for oxidation of $NaBH_4$-reduced trypanothione reductase, in the presence of 2 equiv of $NADP^+$ (EH_2–$NADP^+$), was 1.3×10^5 M^{-1} s^{-1} (Figure 6). These data confirm that occupancy of the nicotinamide-binding pocket by $NADP^+$ increases the rate of quinone reduction. The presence of $NADP^+$ also increases the proportion of quinone reduction effected by a one-electron process (Figure 4). Moreover, alkylation of the nascent thiol that interacts with $T(S)_2$ does not greatly diminish the quinone reductase activity, indicating that most of the quinone reduction takes place elsewhere. These two findings suggest that quinone reduction occurs at a third site. This third site on *T. congolense* trypanothione reductase may be similar to the site on the crystallographic 2-fold axis in human erythrocyte glutathione reductase where safranin and menadione bind (Karplus et al., 1989).

The reactivities of various free and pyridine nucleotide-bound forms of reduced trypanothione reductase toward benzoquinone decrease in the order EH_2–$NADP^+$ > EH_2–NADPH > EH_2, which is probably related, by analogy to glutathione reductase (Bulger & Brandt, 1971), to a decrease of the electron density on the isoalloxazine ring of the FAD cofactor of the corresponding complex, as evidenced by changes in the extinction coefficient at 460 nm. The V/K_m value of benzoquinone, determined in steady-state experiments at high $[NADP^+]/[NADPH]$ ratios (Figure 4), is very close to the value of k_{ox} for benzoquinone reduction by EH_2–$NADP^+$ determined in stopped-flow experiments (Figure 6, inset), and thus represents a true bimolecular rate constant. Similarly, the V/K_m values of quinones shown in Table 1 represent k_{ox} for quinone reduction by EH_2–NADPH, which seems to be the most important catalytic form of enzyme under steady-state conditions and for the reduction of $T(S)_2$. It seems, however, that quinones, in contrast to $T(S)_2$, oxidize the nucleotide-liganded forms of reduced trypanothione reductase most efficiently when significant electron density is localized on the isoalloxazine ring of partially reduced FAD and not on the enzymic dithiols which participate covalently in $T(S)_2$ reduction. The rate-limiting step of the quinone reductase reaction catalyzed by trypanothione reductase remains unclear, although isotopic investigations suggest that the oxidative half-reaction is rate-limiting in $T(S)_2$ reduction (Leichus et al., 1992). Thus, the steady-state maximal velocity of 1,4-benzoquinone reduction at $[NADP^+]/[NADPH]$ = 10–30 is 10–12 s^{-1} (data not shown), a value significantly less than k_{obsd} measured in pre-steady-state rapid reaction experiments.

The data presented indicate that, under similar conditions, trypanothione reductase is 10-fold more efficient in the one-electron reduction of quinones than is glutathione reductase. Although glutathione reductase reduces quinones with similar V and V/K_m values, the percentage of single electron reduction in the absence of $NADP^+$ is only 3.6%, but increases to 44% at $[NADP^+]/[NADPH]$ = 100 (Cènas et al., 1989). The increased efficiency of single-electron reduction by trypanothione reductase in the presence of added $NADP^+$ could be the result of changes of the single-electron reduction potentials of $FADH_2$ ($FADH_2 \rightarrow FADH^\bullet$) in the EH_2–$NADP^+$ complex, an observation reported for the flavoproteins NADH: cytochrome b_5 reductase (Iyanagi et al., 1984) and flavocytochrome b_2 (Tegonni et al., 1984). It seems that the predominant factor determining the reactivity of the series of uncharged quinones and hydroxyquinones studied here is their single-electron-reduction potential (Figure 1). In comparison to the data of Henderson et al. (1988) for *C. fasciculata* trypanothione reductase, neutral and anionic quinones possessing E^1 of –0.2 to –0.26 V (Table 1) are 1–2 orders of magnitude less active than the hydrazonoamidine-substituted naphthoquinones possessing similar E^1 values. However, their reactivities are similar to those of the negatively charged dicarboxylate derivatives of naphthoquinone tested with the *Crithidia* reductase (Henderson et al., 1988). It is possible, that the reactivity of hydrazonoamidine-substituted naphthoquinones is indeed caused by their binding to, or close to, negatively charged amino acid residues implicated in the binding of $T(S)_2$ (Henderson et al., 1988).

The ability of trypanothione reductase to catalyze the efficient one-electron reduction of quinones has important physiological consequences for the organism. The formation of semiquinone products will enhance the rate of formation of superoxide and other reactive oxygen species, including hydrogen peroxide. This additional stress on the trypanosomal peroxide reducing system, composed of trypanothione peroxidase and trypanothione reductase (Henderson et al., 1987), will deplete the cell of reducing equivalents, and will cause the intracellular levels of $NADP^+$ to rise. Higher $[NADP^+]/[NADPH]$ ratios will *inhibit* the normal and protective $T(S)_2$ reducing activity of trypanothione reductase, but, as shown in this study, will *enhance* the rate and proportion of one-electron reduction of quinones. The escalating flux of reactive oxygen species would be expected to eventually overwhelm the organism's protective mechanisms and damage critical macromolecular components within the cell. Future experiments will be aimed at clarifying and extending the results presented here, and evaluating the *in vivo* consequences of the reduction of quinones by trypanothione reductase.

REFERENCES

Anusevicius, Z. J., & Cènas, N. K. (1993) *Arch. Biochem. Biophys. 302*, 420.

Arscott, L. D., Thorpe, C., & Williams, C. H., Jr. (1981) *Biochemistry 20*, 1513.

Benson, T. J., McKie, J. H., Garforth, J., Borges, A., Fairlamb, A. H., & Douglas, K. T. (1992) *Biochem. J. 286*.

Bironaitè, D. A., Cènas, N. K., Kulys, J. J., Medentsev, A. G., & Akimenko, V. K. (1991) *Z. Naturforsch. 46c*, 966.

Bulger, J. E., & Brandt, K. G. (1971) *J. Biol. Chem. 246*, 5578.
Carlberg, I., & Mannervik, B. (1986) *J. Biol. Chem. 261*, 1629.
Cènas, N. K., Rakauskienè, G. A., & Kulys, J. J. (1989) *Biochim. Biophys. Acta 973*, 399.
Cènas, N. K., Bironaitè, D. A., Kulys, J. J., & Sukhova, N. M. (1991) *Biochim. Biophys. Acta 1073*, 195.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 104.
Davis, R. E., & Swain, C. G. (1960) *J. Am. Chem. Soc. 82*, 5949.
Ehrlich, P., & Bertheim, A. (1912) *Ber. Dtsch. Chem. Ges. 45*, 756.
Fairlamb, A. H. (1989) *Parasitology 99S*, 93.
Fairlamb, A. H., & Cerami, A. (1992) *Annu. Rev. Microbiol. 46*, 695.
Field, H., Cerami, A., & Henderson, G. B. (1992) *Mol. Biochem. Parasitol 50*, 47.
Grinblat, L., Sreider, C. M., & Stoppani, A. O. M. (1989) *Biochem. Pharmacol. 38*, 767.
Henderson, G. B., Fairlamb, A. H., & Cerami, A. (1987) *Mol. Biochem. Parasitol. 24*, 39.
Henderson, G. B., Ulrich, P., Fairlamb, A. H., Rosenberg, I., Pereira, M., Sela, M., & Cerami, (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5374.
Jockers-Scherübl, M. C., Schirmer, R. H., & Krauth-Siegel, R. L. (1989) *Eur. J. Biochem. 180*, 267.
Iyanagi, T., Watanabe, T., & Anan, K. F. (1984) *Biochemistry 23*, 1418.
Karplus, P. A., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 701.
Karplus, P. A., Pai, E. F., & Schulz, G. E. (1989) *Eur. J. Biochem. 178*, 693.
Krauth-Siegel, L., Blatterspiel, R., Saleh, M., Schiltz, E., Schirmer, R. H., & Untucht-Grau, R. (1982) *Eur. J. Biochem. 121*, 259.
Krauth-Siegel, L., Enders, B., Henderson, G. B., Fairlamb, A. H., & Schirmer, R. H. (1987) *Eur. J. Biochem. 164*, 123.
Kuriyan, J., Kong, X. P., Krishna, T. S. R., Sweet, R. M., Murgolo, N. J., Field, H., Cerami, A., & Henderson, G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8764.
Leichus, B. N., Bradley, M., Nadeau, K., Walsh, C. T., & Blanchard, J. S. (1992) *Biochemistry 31*, 6414.
Manstein, D. J., Pai, E. F., Schopfer, L. M., & Massey, V. (1986) *Biochemistry 25*, 6807.
Marcus, R. A., & Suttin, N. (1985) *Biochim. Biophys. Acta 811*, 265.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431.
Mau, B. L., & Powis, G. (1992) *Biochem. Pharmacol. 43*, 1613.
Meyer, T. E., Przysiecki, C. T., Watkins, J. A., Bhatatacharyya, A., Simondsen, R. P., Cusanovich, M. A., & Tollin, G. (1983) *Proc. Natl. Acad. U.S.A. 80*, 6740.
Nakamura, M., & Yamazaki, Y. (1972) *Biochim. Biophys. Acta 267*, 249.
O'Brien, P. J. (1991) *Chem.-Biol. Ineract. 80*, 1.
Pai, E. F., Karplus, P. A., & Schulz, G. E. (1988) *Biochemistry 27*, 4465.
Shames, S. L., Fairlamb, A. H., Cerami, A., & Walsh, C. T. (1986) *Biochemistry 25*, 3519.
Shames, S. L., Kimmel, B. E., Peoples, O. P., Agabian, N., & Walsh, C. T. (1988) *Biochemistry 27*, 5014.
Sullivan, F. X., Shames, S. L., & Walsh, C. T. (1989) *Biochemistry 28*, 4986.
Sullivan, F. X., Sobolov, S. B., Bradley, B., & Walsh, C. T. (1991) *Biochemistry 30*, 2761.
Tegonni, M., Janot, J. M., & Labeyrie, F. (1984) *Biochimie 66*, 127.
Walsh, C., Bradley, M., & Nadeau, K. (1991) *Trends Biochem. Sci. 16*, 305.
Williams, C. H., Jr. (1992) in *Chemistry and Biochemistry of Flavoenzymes 3* (Müller, F., Ed.) pp 123–211, CRC Press, Boca Raton, Ann Arbor, New York.