Functional and Physicochemical Characterization of the Thioredoxin System in *Trypanosoma brucei*

Heide Schmidt and R. Luise Krauth-Siegel†

From the Biochemie-Zentrum Heidelberg, Universität Heidelberg, 69120 Heidelberg, Germany

*Trypanosoma brucei*, the causative agent of African sleeping sickness, possesses a single thioredoxin that has an unusually high pI value of 8.5 and lacks a conserved aspartyl residue claimed to be involved in catalysis in other thioredoxins. Despite these peculiarities, *T. brucei* thioredoxin behaves like classical thioredoxins. It is reduced by thioredoxin reductases from different species, serves as donor of reducing equivalents for the ribonucleotide reductase of the parasite, and catalyzes the reduction of protein disulfides. The redox potential of $-267 \text{ mV}$ was obtained from protein-protein redox equilibration with *Escherichia coli* thioredoxin. The pK value of *T. brucei* thioredoxin was determined by two different methods. Carboxamidomethylation of the reduced protein yielded a pK value of 7.4 and generated mono-alkylated protein. The thiolate absorption at 240 nm resulted in a pK of 7.6 and, based on the extinction coefficient of 11.6 $\text{mM}^{-1} \text{cm}^{-1}$, there are two (or three) cysteines titrating with very similar pK values. A thioredoxin reductase has not yet been detected in any organism of the order Kinetoplastida. *T. brucei* thioredoxin is spontaneously reduced by trypanothione (bis(glutathionyl)spermidine). Obviously, a specific thioredoxin reductase is not required as thioredoxin reduction can be conducted by the parasite-specific trypanothione/trypanothione reductase system.

Trypanosomatids such as *Trypanosoma brucei*, the causative agent of African sleeping sickness, have a unique thiol metabolism in which the ubiquitous glutathione/glutathione reductase couple is replaced by trypanothione (N$^\gamma$,N$^\delta$-bis(glutathionyl)spermidine), and the flavoenzyme trypanothione reductase (1, 2). The parasite dithiol is much more reactive than glutathione. It is a spontaneous reductant of dehydroascorbate as well as the disulfide forms of glutathione and ovothiol (2). It reduces trypanothione, in a 16-kDa dithiol protein with a CPPC active site motif, which distantly belongs to the thioredoxin protein family (3). The trypanothione/trypanothione couple is the donor of reducing equivalents for the detoxification of hydroperoxides catalyzed by a cascade of proteins that are composed of trypanothione reductase, trypanothione, tryparredoxin, and a peroxiredoxin-type tryparredoxin peroxidase. Tryparredoxin is also involved in the reduction of ribonucleotide reductase (4) and glutathione peroxidase-like parasite peroxidases (5, 6). The trypanothione metabolism is essential for the parasite. Down-regulation of trypanothione reductase in bloodstream *T. brucei* by more than 90% results in growth arrest and hypersensitivity toward hydrogen peroxide as well as the loss of infectivity in a mouse model (7).

Besides trypanothione, *T. brucei* possesses a single classical thioredoxin with a $M_\text{r}$ of 12,000 and the conserved WCGPC catalytic site (8). The parasite protein is unusual in having a calculated pI value of 8.5 instead of the acidic pI found for most thioredoxins and a conserved functional aspartate (Asp-26 in *Escherichia coli* thioredoxin) (9, 10) is replaced by a tryptophan residue. In addition, *T. brucei* thioredoxin has a third thiol (Cys-68) not occurring in the bacterial proteins. The corresponding Cys-69 in human thioredoxin has recently been shown to be accessible to nitrosylation (11). *T. brucei* thioredoxin catalyzes the reduction of insulin disulfide and the parasite ribonucleotide reductase by dithioerythritol and is a substrate of human thioredoxin reductase (8). In all known organisms, oxidized thioredoxin, Trx-S$_2$ (1), is reduced by NADPH and the flavoenzyme thioredoxin reductase (12, 13), whereby two different types of enzymes have evolved (14). Bacterial and yeast thioredoxin reductases such as the *E. coli* enzyme are homodimers with a subunit molecular mass of about 35 kDa, whereas the human, *Drosophila melanogaster*, and *Plasmodium falciparum* thioredoxin reductases are larger proteins of about 2 $\times$ 55 kDa (14). In trypanosomes and other kinetoplastid organisms a specific thioredoxin reductase has not yet been detected that raises the question as to the physiological electron donor for thioredoxin in these parasites.

Here we report on the catalytic and physicochemical properties of *T. brucei* thioredoxin. We provide strong evidence that the trypanothione system can keep the parasite thioredoxin in the dithiol state.

**EXPERIMENTAL PROCEDURES**

Trypanothione disulfide and glutathionylspermidine disulfide were purchased from Bachem, bovine pancreas insulin, GDP, dTTP, and iodoacetamide were from Sigma, and $[^1\text{H}]\text{GDP}$ was from Amersham Biosciences. Dihydrolipoamide was prepared as described earlier (15). *E. coli* thioredoxin was obtained from Calbiochem. *E. coli* thioredoxin reductase was a kind gift of Dr. Charles H. Williams Jr., Ann Arbor, MI. Human thioredoxin reductase was obtained from Drs. Katja Becker, Giessen, and R. Heiner Schirmer, Heidelberg. Dr. R. Heiner Schirmer also provided *D. melanogaster* thioredoxin reductase. *Crithidia fasciculata* tryparredoxin peroxidase was a kind gift of Dr. Leopold Flohé, Braunschweig. The plasmids encoding the R1 and R2 subunits of *T. brucei* ribonucleotide reductase were provided by Drs. Anders Hofer

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† To whom correspondence should be addressed: Biochemie-Zentrum Heidelberg, Universität Heidelberg, Im Neuenheimer Feld 504, 69120 Heidelberg, Germany. Tel.: 49-6221-544187; Fax: 49-6221-545586; E-mail: krauth-siegel@urz.uni-heidelberg.de.

‡ To whom correspondence should be addressed: Biochemie-Zentrum Heidelberg, Universität Heidelberg, Im Neuenheimer Feld 504, 69120 Heidelberg, Germany. Tel.: 49-6221-544187; Fax: 49-6221-545586; E-mail: krauth-siegel@urz.uni-heidelberg.de.

The abbreviations used are: Trx-S$_2$, oxidized thioredoxin; DTT, dithiothreitol; TSH$_{2}$, trypanothione; Trx, thioredoxin; Trx(HS)$_{2}$, reduced thioredoxin; HP/LC, high performance liquid chromatography; MES, 2-morpholinoethane sulfonic acid; MOPS, 3-morpholinopropane sulfonic acid.
Purification of Recombinant T. brucei Thioredoxin—E. coli SG13009 cells with the pQE32/trx plasmid were grown as described (8) but terrific broth medium was used. Cells from 1 liter of bacterial culture were harvested at a density of 3.5 × 10^9 cells/ml and directly lysed in sample buffer containing tris(2-carboxyethyl)phosphate (Bond-Breaker, Pierce). Protein samples were separated on a 15% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane by wet blotting (Bio-Rad) and probed with 1:1000 diluted polyclonal rabbit antibodies against recombinant T. brucei thioredoxin (Eurogentec). The secondary antibody (anti-rabbit IgG conjugated with horseradish peroxidase, Santa Cruz) was diluted 1:20000. The blots were developed using a chemiluminescence system (SuperSignal West Pico, Pierce).

Construction of a C6SS Mutant of T. brucei Thioredoxin—Two overlapping PCR products both containing the C6SS mutation were synthesized. The first fragment was amplified using the TrxHis forward (5'-TCTGGATGGGATTATGACG-3') and the Trx S/C reverse (3'-TGCTTGGCTGCAGGTCGACC-5') primers and the second one with the Trx S/C forward (5'-CCTGAGCTCAGCGACGTCCTAC-3') and the TrxHis reverse (5'-GTCGACAACATTGCTAGGTCGACC-3') primers. The PCR product was purified and cloned into the Smal and PstI sites of the pQE32 vector (Qiagen) and completely sequenced in both directions. An unmutated C64S mutant was synthesized. The first fragment was amplified using the TrxHis forward (5'-TCTGGATGGGATTATGACG-3') and the Trx S/C reverse (5'-TGCTTGGCTGCAGGTCGACC-3') primer and the second one with the TrxHis forward (5'-TCTGGATGGGATTATGACG-3') and the Trx S/C reverse (5'-TGCTTGGCTGCAGGTCGACC-3') primer. Codons representing the mutation are given in bold letters. The bold italic codon marks an altered Lys codon to introduce a PvuII restriction site. PCR was carried out with Pfu DNA polymerase (95 °C, 2 min; 31 times (94 °C, 30 s; 55 °C, 30 s; 72 °C, 3 min); 72 °C, 3 min) using the pQE 32/trx plasmid as template (8). The two purified fragments were used as templates in the third PCR, amplifying the complete 3'hex gene with the TrxHis forward and pQE32 reverse primer. The PCR product was purified and cloned into the Smal and PstI sites of the pQE32 vector (Qiagen) and completely sequenced in both directions. This mutant gene was expressed in E. coli SG13009 cells and the expressed Trx C68S mutant was purified as described above for wild type thioredoxin.

Chemical Reduction of Thioredoxin—400–800 μM T. brucei and E. coli thioredoxin, respectively, in 300 μl of 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazol, pH 8.0, was incubated with a 100-fold molar excess of DTT for 1 h at room temperature. The reduced protein was desalted on a 15-ml Sephadex G-25 (fine) column equilibrated in the respective buffer.

Measurement of the Thiolute Anion by UV Absorption—The pH-dependent cysteine ionization was followed by the absorption of the thiolute anion at 240 nm (19). Spectra of 10–18 μM oxidized and reduced Thioredoxin A were recorded between 200 and 400 nm at 25 °C in 1.3 ml of 1 mM citrate, 1 mM borate, and 1 mM phosphate, 0.2 mM KCl, pH 5.1, purged with argon. The pH value was adjusted by adding defined volumes of 0.2 or 0.1 M KOH from pH 5.1 to 10.5. The spectra were measured against air in a stopped quartz cuvette in a Beckman DU-7400 spectrophotometer. The buffer spectrum recorded in the same cuvette was subtracted from the spectrum of the respective protein solution. The absorbance was converted into molar extinction coefficients and corrected for the dilution because of the pH adjustment. The protein concentration was determined from the absorbance at 280 nm. An A_280 = 1 corresponds to 1 mg/ml pure T. brucei thioredoxin.

pH Dependent Carboxamidomethylation—50 μM Reduced thioredoxin A or C. fasciculata thioredoxin were incubated for 10 min each with 50 μM iodoacetate, MES, MOPS, Tris, and 0.2 mM KCl between pH 5 and 9.7 at 25 °C in a total volume of 150 μl. The reaction was stopped at different time points by mixing a 10-μl sample and 10 μl of 500 mM DTT. Reduced and carboxamidomethylated thioredoxin were separated by HPLC on a C_18/VYDAC column (2.1 mm × 25 cm, 200T502) with a C_8 pre-column (2.1 mm × 5 cm). The absorbance was recorded at 280 nm. A 5-μl sample was injected. After 15 min of isocratic washing, the proteins were eluted by a 30-min gradient from 38.5 to 49% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.2 ml/min and 25 °C. The proteins were detected at 214 nm.

Determination of the Redox Potential—The redox potential of T. brucei thioredoxin was derived from the absorption of Thioredoxin A, pH 7.5 was used. Thioredoxin was reduced by 0.1 M K_2CO_3 protein–protein redox equilibration (20, 21). Different concentrations of reduced and oxidized T. brucei and E. coli thioredoxin in 100 μl of 100 mM potassium phosphate, pH 7.0, were allowed to equilibrate at 25 °C in closed 1.5-ml reaction vessels purged with argon. After different times, 5-μl samples
were immediately analyzed by HPLC as described above. The column was run for 15 min in 39.2% acetonitrile in 0.1% trifluoroacetic acid followed by a 35-min gradient from 39.2 to 52.5% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.2 ml/min and 25 °C. The proteins were monitored at 214 nm. The redox equilibrium was reached after 2 h and was stable for at least 9 h.

RESULTS

T. brucei Thioredoxin Is Reduced by Different Thioredoxin Reductases—Because a specific thioredoxin reductase has not yet been detected in any kinetoplastid parasite, we studied thioredoxin reductases from different organisms for their ability to reduce T. brucei thioredoxin. To maintain a constant concentration of oxidized thioredoxin during the reaction, assays at low substrate concentrations contained an excess of insulin. As shown previously, T. brucei thioredoxin is readily reduced by human thioredoxin reductase (8). The E. coli enzyme accepts T. brucei thioredoxin with a $K_m$ value 8-fold higher than that for wild type E. coli thioredoxin 1 (1.84 μM) and comparable with that for a mutant where Asp-26 had been replaced by an alanine residue (9). The $k_{cat}$ value with the parasite thioredoxin is about 40-fold lower resulting in a 300-fold lower $k_{cat}/K_m$ value when compared with the homologous bacterial system (Table I) (22). This reflects the fact that, in contrast to the mammalian enzymes with very broad substrate specificity, bacterial thioredoxin reductases are much more specific for their disulfide substrate. T. brucei thioredoxin is also reduced by thioredoxin reductase from D. melanogaster with a $k_{cat}/K_m$ value 200-fold lower than that reported for the authentic substrate/enzyme couple (23). Taken together, thioredoxin reductases from different organisms accept T. brucei thioredoxin as substrate although with relatively low efficiency.

T. brucei Thioredoxin as Electron Donor for Ribonucleotide Reductase—Ribonucleotide reductase catalyzes the conversion of all four ribonucleoside diphosphates into the respective deoxyribonucleotides essential for DNA synthesis. Well known physiological electron donors in the reaction are the nearly ubiquitous dithiol proteins thioredoxin and glutaredoxin (24). In T. brucei, the trypanothione/tryparedoxin couple acts as an efficient thiol system for the parasite ribonucleotide reductase (4). T. brucei thioredoxin in concert with NADPH and human thioredoxin reductase is also able to deliver electrons to the enzyme. The apparent $K_m$ value of T. brucei ribonucleotide reductase for T. brucei thioredoxin derived from a Lineweaver-Burk plot was 29 ± 7 μM (data not shown). The value is about an order of magnitude higher than that for T. brucei tryparo-
doxin (3.7 μM) (4) and 20-fold higher than the $K_m$ value of E. coli ribonucleotide reductase for E. coli thioredoxin (1.25 (25) and 2.0 μM (9)). Interestingly, with the D26A mutant of E. coli thioredoxin the bacterial enzyme also yields a more than 10-fold increased $K_m$ value of 33 μM (9).

As shown previously, T. brucei thioredoxin is not a substrate of trypanothione reductase (8). Therefore we studied if thioredoxin is spontaneously reduced by trypanothione using the ribonucleotide reductase reaction as the first assay system. The reaction mixtures contained 100 μM trypanothione and varying concentrations of T. brucei thioredoxin and tryparo-
doxin, respectively. Addition of thioredoxin caused only a very slight increase in ribonucleotide reductase activity (Fig. 1) that may be because of the relatively low trypanothione concentration in the assay. Higher trypanothione concentrations could not be used because of the pronounced spontaneous reduction of ribo-
nucleotide reductase by the dithiol (4). As expected, trypa-
doxin strongly increased the trypanothione-dependent activity of ribonucleotide reductase under these conditions.

Reduction of T. brucei Thioredoxin by Trypanothione and Other Non-protein Thiols—Thioredoxins catalyze the reduction of protein disulfides such as those of insulin (8, 26). The reaction is followed by an increase in turbidity because of the precipitation of the insoluble B chain. Here we used the insulin assay to study different non-protein thiols for their ability to reduce T. brucei thioredoxin. Addition of E. coli thioredoxin as well as the parasite thioredoxin and tryparo-
doxin to a reaction mixture containing insulin and 515 μM trypanothione, strongly enhanced turbidity formation (Fig. 2). All three proteins are spontaneously reduced by TSH and then function as protein-disulfide reductases. Corresponding assays in the presence of 500 μM dihydrolipoamide and glutathionylspermidine, respectively, revealed that dihydrolipoamide but not the monothiol glutathionylspermidine also serves as an efficient electron donor for T. brucei thioredoxin (not shown). Thus, T. brucei thioredoxin is readily reduced by non-protein dithiols such as trypanothione. The marginal cellular concentration of free lipo-
peptide should rule it out as physiological reductant (27).

T. brucei Thioredoxin Delivers Electrons to C. fasciculata Tryparo-
doxin Peroxidase—C. fasciculata tryparo-
doxin is a 2 Cys-peroxiredoxin that obtains its reducing equiva-
ants from trypanothione via a tryparo-
doxin-mediated reaction (28). C. fasciculata tryparo-
doxin 1 shows a $k_{cat}$ of 6.5 s⁻¹ and a $K_m$ value of 2.2 μM for the peroxidase (29). Here we studied if T. brucei thioredoxin, kept reduced by human thiore-
doxin reductase, can replace the trypanothione/tryparo-
doxin couple. An apparent $K_m$ value of T. brucei thioredoxin for the peroxidase of 3 μM and a $k_{cat}$ of 0.8 s⁻¹ was obtained, which shows that C. fasciculata tryparo-
doxin peroxidase is also a substrate of T. brucei thioredoxin (data not shown).

Reduction of Non-protein Disulfides by T. brucei Thiore-
doxin—Reduction of different low molecular mass disulfides by T. brucei thioredoxin was followed using a coupled assay system. In the first reaction, T. brucei thioredoxin is reduced by
human thioredoxin reductase (hTrxR) and NADPH. After the reaction has run to completion, the disulfide (RSSR) is added, which is then reduced by thioredoxin in the second reaction.

\[

\text{NADPH} + H^+ + \text{Trx-S}_2 \xrightleftharpoons[k_{-2}]{k_2} \text{NADP}^+ + \text{Trx-(SH)}_2
\]

**REACTION 1**

\[

\text{Trx-(SH)}_2 + \text{RSSR} \xrightarrow{k_{-2}} \text{Trx-S}_2 + 2 \text{RSH}
\]

**REACTION 2**

Reaction 1 follows Michaelis-Menten type kinetics (8). Under steady state conditions, the rate of thioredoxin reduction by human thioredoxin reductase is given by

\[

v = \frac{V_{\text{max}}[\text{Trx-S}_2]}{K_{n} + [\text{Trx-S}_2]} = \frac{V_{\text{max}}}{K_{n} + [\text{Trx-S}_2]}
\]

(Eq. 1)

which is equal to the rate of the spontaneous reaction regenerating Trx-S\(_2\) from Trx-(SH)\(_2\) and the disulfide (Reaction 2),

\[

v = \frac{k_{2}[\text{Trx-(SH)}_2][\text{RSSR}]}{k_{2}[\text{Trx-(SH)}_2][\text{RSSR}]} = \frac{k_{2}[\text{Trx-(SH)}_2][\text{RSSR}]}{k_{2}[\text{Trx-(SH)}_2][\text{RSSR}]}
\]

(Eq. 2)

\(V_{\text{max}}, v, \text{ and } [\text{Trx-S}_2]\) were determined as described under “Experimental Procedures.” The second-order rate constant \((k_{2})\) for the reduction of trypanothione disulfide, glutathione disulfide, and lipoamide are given in Table II. The \(k_{2}\) value for the reduction of trypanothione disulfide by \(T. \text{ brucei}\) thioredoxin is 100 \(M^{-1} s^{-1}\), which corresponds to that for the reduction of glutathione disulfide by \(E. \text{ coli}\) thioredoxin (23, 30). Reduction of glutathione disulfide by \(T. \text{ brucei}\) thioredoxin is about 4- and 28-fold slower than that by \(E. \text{ coli}\) and \(P. \text{ falciparum}\) thioredoxin, respectively. Although lipoamide is a direct substrate of human thioredoxin reductase (31), the reaction rate is clearly enhanced in the presence of \(T. \text{ brucei}\) thioredoxin. In vivo, lipoamide occurs mainly as a protein-bound cofactor of mitochondrial multienzyme complexes and lipoamide dehydrogenase is selective for the \(R\)-isomer (32). When neglecting a possible stereoselectivity of human thioredoxin reductase toward lipoamide, the rate of lipoamide reduction by thioredoxin can be corrected for the activity of the reductase, which results in a \(k_{2}\) value of about 28 \(M^{-1} s^{-1}\).

With the \(k_{2}\) value for the reduction of trypanothione disulfide by \(T. \text{ brucei}\) Trx-(SH)\(_2\), the rate constant \((k_{-2})\) for the reverse reaction (Reaction 2) can be calculated. The Nernst equation

\[

\ln k_{2}/k_{-2} = (E_A^{\text{red}} - E_B^{\text{red}})(nF/RT),
\]

where \(E_A^{\text{red}}\) and \(E_B^{\text{red}}\) correspond
to the standard redox potentials of trypanothione (-242 mV (1)) and \(T. \text{ brucei}\) thioredoxin (-267 mV; see below), respectively, yields a \(k_{-2}\) value of 1.4 \(M^{-1} s^{-1}\). The turnover of oxidized thioredoxin by trypanothione \((v = k_{-2}[\text{Trx-S}_2]/[\text{Trx-S}_2])\) under physiological conditions (350 \(\mu M\) trypanothione (1, 33) and \(50 \text{ nM}\) thioredoxin) is then about 15 \(s^{-1}\), which should be sufficient to keep thioredoxin in the reduced state. The cellular concentration of thioredoxin was estimated from Western blots applying varying amounts of parasite extracts (1 \(\times 10^6\) to 4 \(\times 10^7\) procyclic cells) and 0.2 to 5 \(\mu M\) of recombinant His-tagged \(T. \text{ brucei}\) thioredoxin (data not shown).

Measurement of the Thiol Ionization State at 240 nm—The absorption spectrum of reduced \(T. \text{ brucei}\) thioredoxin was monitored between 200 and 400 nm in the pH range from 5 to 10 (Fig. 3). The thiolate anion has a significant absorption at 240 nm, whereas the thiol form does not (19). Because many other groups in a protein also absorb in this region, the spectra of oxidized wild type and C68S thioredoxin were recorded for comparison. In the mutant, the third cysteine was replaced by a serine to remove the additional titratable group. Cys-68 is not involved in catalysis because the behavior of the C68S mutant as substrate of human thioredoxin reductase and in the insulin assay was indistinguishable from that of wild type thioredoxin (data not shown). Unfortunately, reduced recombinant Trx C68S proved to be unstable and thus could not be used in the titration experiments. The spectra of the three protein species overlapped except for the region between 240 and 270 nm where the absorption of reduced thioredoxin was raised with increasing pH (Fig. 3). The \(\epsilon\) values at 295, 288, and 240 nm as a function of pH for reduced and oxidized wild type thioredoxin as well as for the oxidized mutant are presented in Fig. 4. Changes at 240 nm reflect ionization of the thiols, at 295 nm tyrosine ionization and at 288 nm unfolding of the protein (19). At 288 and 295 nm, the absorption coefficients of the three protein species were almost identical and up to pH 6 independent of the pH value. At 240 nm, reduced thioredoxin showed an increase of the absorption coefficient at pH values above 6.5, whereas the \(\epsilon_{240}\) values of oxidized thioredoxin and the C68S mutant remained constant up to pH 9.0. This demonstrates that in the oxidized wild type protein, cysteine 68 is not titrating between pH 7.0 and 9.0. Above pH 9.0, the extinction coefficients at 240 nm of reduced and oxidized wild type \(T. \text{ brucei}\) thioredoxin and the oxidized C68S mutant, which lacks any thiol, increased in parallel probably because of the titration of other groups in the protein.

Because of this pronounced absorption increase at high pH values, a sigmoidal dependence of \(\epsilon_{240}\) from the pH for reduced thioredoxin was not directly obvious. Subtracting the \(\epsilon_{240}\) values of the oxidized protein from those of the reduced thioredoxin revealed a maximum at pH 9.0 that was a function of the [Trx-S\(_2\)]. The maximum was smaller for reductase preparations containing a lower content of Trx-S\(_2\) (141 M, respectively) than for purified Trx-S\(_2\) (133 M NADPH, 10-30 M thioredoxin, 90-100 nm thioredoxin reductase, and 66 or 111 M trypanothione disulfide, 100 or 200 M lipoamide, and 66, 222, or 444 M glutathione disulfide, respectively, in 200 mM Tris-HCl, 2 mM EDTA, pH 7.5, at 25 °C. The standard deviations are based on five to nine measurements.

| Thioredoxin species | Disulfide | \(k_2\) | M\(^{-1}\) s\(^{-1}\) |
|---------------------|----------|-------|-----------------|
| T. brucei | Trypanothione disulfide | 100 ± 9.6 |
| T. brucei | Lipamide | 28 ± 5.8 |
| T. brucei | Glutathione disulfide | 23 ± 3.1 |
| E. coli | Glutathione disulfide | 100\(^{a}\) |
| P. falciparum | Glutathione disulfide | 650\(^b\) |

\(^a\) From Ref. 30.
\(^b\) From Ref. 23.

\(nF/RT\) and 0.5 values for the reaction rate is clearly enhanced in the presence of \(T. \text{ brucei}\) thioredoxin. In vivo, lipoamide occurs mainly as a protein-bound cofactor of mitochondrial multienzyme complexes and lipoamide dehydrogenase is selective for the \(R\)-isomer (32). When neglecting a possible stereoselectivity of human thioredoxin reductase toward lipoamide, the rate of lipoamide reduction by thioredoxin can be corrected for the activity of the reductase, which results in a \(k_{2}\) value of about 28 \(M^{-1} s^{-1}\).
din (34), however, resulted in a titration curve with a single inflection point at pH 7.6 (Fig. 4D). The pK value was derived from the Henderson-Hasselbalch equation: $A_{240}/A_{280} = A_{240}/A_{280}$, where $A_{240}/A_{280}$ corresponds to the product of two reactive cysteines having similar pK values. This approach was taken because the titration curves of the active site cysteines are similar (35).

**pH Dependence of the Reaction with Iodoacetamide—Cysteinyl groups are only alkylated in the deprotonated thiolate anion state. Following the alkylation rate as a function of pH has been used to determine the pK value of the reactive cysteine (21, 37, 38). In addition to the active site dithiol/disulfide, T. brucei thioredoxin possesses a third cysteine at position 68.

**Fig. 3. Absorption spectra of reduced T. brucei thioredoxin at different pH values.** The spectra were recorded at pH 5.1 (○), pH 7.3 (□), pH 8.3 (●), and pH 9.0 (+) as described under “Experimental Procedures.” Reduced thioredoxin in 1 mM each citrate, borate, phosphate, and 0.2 mM KCl was titrated with KOH, and the spectra were corrected for the resulting dilution.

**Fig. 4. Titration of T. brucei thioredoxin.** A–C, extinction coefficients of reduced (○) and oxidized (■) wild type T. brucei thioredoxin, and the oxidized C89S mutant (□) at 240 (A), 288 (B), and 295 nm (C) as a function of pH. D, difference curve of the extinction coefficients at 240 nm of reduced and oxidized thioredoxin. 10–18 μM Thioredoxin in 1 mM each citrate, borate, phosphate, and 0.2 mM KCl was titrated from pH 5 to 10 with KOH. The absorption values were corrected for the dilution.

To elucidate if this residue interferes with the specific alkylation of the active site cysteine(s), 100 μM oxidized thioredoxin was incubated with a 3-fold molar excess of iodoacetamide at pH 7.1 and 9.0. After 1 h, addition of 20 mM DTT stopped the alkylation and concomitantly reduced the active site disulfide bridge. Reduced wild type and reduced modified thioredoxin were separated by HPLC. The reduced protein alkylated at Cys-68 eluted about 2 min later than thioredoxin, which was carboxamidomethylated at the active site (data not shown). The peak did not appear when equimolar concentrations of reduced thioredoxin and iodoacetamide were incubated for short reaction times in accordance with Cys-68 not reacting.

50 μM Reduced thioredoxin was incubated with 50 μM iodoacetamide in the pH range from 5 to 9.7. After different time points, an aliquot was removed and the reaction was stopped by addition of 250 mM DTT. Reduced and carboxamidomethylated thioredoxin were separated by HPLC (Fig. 5) and the protein concentrations were calculated from the peak areas. ESI-MS of the reaction mixture at pH 9.0 confirmed that mono-carboxamidomethylated thioredoxin is formed (data not shown). The reaction between equal concentrations of reduced thioredoxin and iodoacetamide follows the equation: $1/[\text{Trx}] = [\text{Trx}_{\text{CAM}}]/k \times t + 1/[\text{Trx}_0]$, where $[\text{Trx}_0] = \text{initial thioredoxin concentration, [Trx}_{\text{CAM}}] = \text{concentration of carboxamidomethylated thioredoxin, k} = \text{apparent second-order rate constant, and t} = \text{time (37).}$ Plots of $1/[\text{Trx}] = [\text{Trx}_{\text{CAM}}]/k \times [\text{Trx}_0]$ versus time yielded straight lines consistent with a second-order reaction with a single rate constant (Fig. 6). The second-order rate constants were calculated according to the equation: $k = [\text{Trx}_{\text{CAM}}]/t \times [\text{Trx}_0] \times ([\text{Trx}_0] - [\text{Trx}_{\text{CAM}}])$ (37) and plotted against the pH value (Fig. 7). At pH 5 and 6, the rate constants are almost zero, whereas between pH 6.5 and 8 a strong dependence of the reaction rate from the pH is observed. The maximum rate constant is reached at pH 8.6. The pK value is calculated from the plateau rates at high and low pH values, $k_{hi}$, and $k_{lo}$, respectively: $k = k_{hi} + (k_{hi} - k_{lo})(1 + 10^{pK-pH})$. The resulting apparent pK value for T. brucei thioredoxin is 7.4, which is slightly lower than the value 7.6 ± 0.1 obtained from the absorption measurements at 240 nm. Most probably this reflects the pK value of Cys-31 but alkylation of Cys-34 cannot be excluded because the thiolate titration experiments indicate that in T. brucei thioredoxin both cysteines have very similar pK values. Carboxamidomethylation of the protein did not show a second inflection point at higher pH values in contrast to E. coli thioredoxin (37, 38) and T. brucei tryparredoxin (21).

**Redox Potential of T. brucei Thioredoxin—**The redox potential of T. brucei thioredoxin was determined by direct protein-protein redox equilibration (20). Different ratios of reduced

![Graph](http://example.com/graph.png)

**FIG. 5. Separation of reduced and carboxamidomethylated T. brucei thioredoxin by HPLC.** 50 μM Reduced thioredoxin was alkylated with 50 μM iodoacetamide in 10 mM each acetate, MOPS, Tris, and 0.2 mM KCl at 25 °C. After 3 min the reaction was stopped by addition of 250 mM DTT. The reduced and carboxamidomethylated proteins were separated by HPLC as described under “Experimental Procedures.”

The peak did not appear when equimolar concentrations of reduced thioredoxin and iodoacetamide were incubated for short reaction times in accordance with Cys-68 not reacting.

50 μM Reduced thioredoxin was incubated with 50 μM iodoacetamide in the pH range from 5 to 9.7. After different time points, an aliquot was removed and the reaction was stopped by addition of 250 mM DTT. Reduced and carboxamidomethylated thioredoxin were separated by HPLC (Fig. 5) and the protein concentrations were calculated from the peak areas. ESI-MS of the reaction mixture at pH 9.0 confirmed that mono-carboxamidomethylated thioredoxin is formed (data not shown). The reaction between equal concentrations of reduced thioredoxin and iodoacetamide follows the equation: $1/[\text{Trx}] = [\text{Trx}_{\text{CAM}}]/k \times t + 1/[\text{Trx}_0]$, where $[\text{Trx}_0] = \text{initial thioredoxin concentration, [Trx}_{\text{CAM}}] = \text{concentration of carboxamidomethylated thioredoxin, k} = \text{apparent second-order rate constant, and t} = \text{time (37).}$ Plots of $1/[\text{Trx}] = [\text{Trx}_{\text{CAM}}]/k \times [\text{Trx}_0]$ versus time yielded straight lines consistent with a second-order reaction with a single rate constant (Fig. 6). The second-order rate constants were calculated according to the equation: $k = [\text{Trx}_{\text{CAM}}]/t \times [\text{Trx}_0] \times ([\text{Trx}_0] - [\text{Trx}_{\text{CAM}}])$ (37) and plotted against the pH value (Fig. 7). At pH 5 and 6, the rate constants are almost zero, whereas between pH 6.5 and 8 a strong dependence of the reaction rate from the pH is observed. The maximum rate constant is reached at pH 8.6. The pK value is calculated from the plateau rates at high and low pH values, $k_{hi}$, and $k_{lo}$, respectively: $k = k_{hi} + (k_{hi} - k_{lo})(1 + 10^{pK-pH})$. The resulting apparent pK value for T. brucei thioredoxin is 7.4, which is slightly lower than the value 7.6 ± 0.1 obtained from the absorption measurements at 240 nm. Most probably this reflects the pK value of Cys-31 but alkylation of Cys-34 cannot be excluded because the thiolate titration experiments indicate that in T. brucei thioredoxin both cysteines have very similar pK values. Carboxamidomethylation of the protein did not show a second inflection point at higher pH values in contrast to E. coli thioredoxin (37, 38) and T. brucei tryparredoxin (21).
E. coli thioredoxin and oxidized T. brucei thioredoxin were incubated at 25 °C and pH 7.0. After allowing the system to equilibrate, the different protein species were separated and quantified by HPLC. A typical HPLC profile is shown in Fig. 8. The equilibrium was reached after 2 h and was stable for 9 h. The redox potential of T. brucei thioredoxin was calculated from the Nernst equation, $E^{0}_{\text{ox}} = E^{0}_{\text{red}} - (RT/nF) \ln ([B_{\text{red}}]/[B_{\text{ox}}] \times [C_{\text{red}}]/[C_{\text{ox}}])$. $E^{0}_{\text{ox}}$ and $E^{0}_{\text{red}}$ correspond to the standard redox potential of T. brucei and E. coli thioredoxin, respectively. $B_{\text{ox}}$ and $B_{\text{red}}$ are the concentrations of oxidized and reduced T. brucei thioredoxin and $C_{\text{ox}}$ and $C_{\text{red}}$ are the concentrations of oxidized and reduced E. coli thioredoxin. Using a standard redox potential of $E^{0}_{\text{ox}} = -270$ mV for E. coli thioredoxin (39), the redox potential $E^{0}_{\text{red}}$ of T. brucei thioredoxin is $-267 \pm 2$ mV. When reduced T. brucei thioredoxin was allowed to equilibrate with oxidized E. coli thioredoxin two small additional peaks (marked A and B in Fig. 8) became prominent. The peaks did not occur when reduced and oxidized T. brucei thioredoxin were mixed suggesting that both reduced T. brucei and oxidized E. coli thioredoxin are involved in the formation of the peaks. The nature of the protein species was not further analyzed.

**DISCUSSION**

A well known function of thioredoxins is the delivery of reducing equivalents for the synthesis of deoxyribonucleotides for DNA synthesis. As shown here, T. brucei thioredoxin serves as electron donor for ribonucleotide reductase whereby the $K_m$ value of the parasite enzyme for thioredoxin is an order of magnitude higher than that of E. coli ribonucleotide reductase for E. coli thioredoxin (9, 25). This may at least partially be because of the presence of a tryptophan residue instead of the highly conserved Asp-26 (E. coli thioredoxin). Mutation of this residue into an alanine in E. coli thioredoxin resulted in a protein that still served as reducing agent for ribonucleotide reductase but with only 6% of wild type activity mainly as a result of an increased $K_m$ value (9). In T. brucei, the trypanothione/tryparedoxin couple as well as trypanothione itself at millimolar concentrations are efficient reductants of ribonucleotide reductase (4). Tryparedoxin is a highly abundant protein with a cellular concentration of at least 100 μM (21). In contrast, the thioredoxin concentration of the parasite is more than 3 orders of magnitude lower, which probably indicates that thioredoxin does not play a significant role in the parasite synthesis of DNA precursors.

T. brucei thioredoxin also provides electrons for the parasite detoxication of hydroperoxides. Two different types of peroxidases, 2 Cys-peroxidoxins and glutathione peroxidase-related enzymes, occur in trypanosomatids (2, 5, 6, 28, 40). Both types of enzymes obtain their reducing equivalents from NADPH via a unique sequence of reactions involving trypanothione reductase, trypanothione, and tryparedoxin (Fig. 9). T. brucei thioredoxin can replace tryparedoxin in the reactions catalyzed by C. fasciculata tryparedoxin peroxidase I as well as by the T. brucei glutathione peroxidase-type enzyme as shown recently (6). In both cases, thioredoxin is less efficient than tryparedoxin.

Most of the physiological thioredoxin-catalyzed reactions are redox processes and the dithiol form of the protein must be regenerated for further catalysis. Because a thioredoxin reductase has not yet been described in any organism of the order of Kinetoplastida we studied if the trypanothione system can substitute for thioredoxin reductase activity. In the first assay system, reduction of T. brucei thioredoxin by trypanothione was coupled to the reaction catalyzed by ribonucleotide reductase. In the presence of 100 μM trypanothione, thioredoxin only slightly increases the activity of ribonucleotide reductase. Because higher concentrations of trypanothione cannot be used because of the pronounced direct reduction of ribonucleotide reductase (4) we measured the general disulfide reductase activity of thioredoxin in the so-called insulin assay. Different low molecular mass thiols were studied for their ability to reduce T. brucei thioredoxin. We could show that T. brucei thioredoxin is efficiently kept reduced by physiological concentrations of
trypanothione. Thus, a specific thioredoxin reductase seems not to be required. Under identical conditions, \( T. brucei \) trypanothione is a more efficient general disulfide reductase than \( T. brucei \) thioredoxin, which may indicate other more specific functions of the latter protein. Steinert et al. (41) observed only a marginal insulin reductase activity for \( F. fasciculata \) trypanothione 2. In their experiments, trypanothione was kept reduced by \( E. coli \) thioredoxin reductase. For \( E. coli \) thioredoxin it has been shown that the rate-limiting step in the insulin assay is the regeneration of the reduced protein (42). Thus the discrepancy between \( T. brucei \) trypanothione and \( F. fasciculata \) trypanothione 2 is probably because of the different reducing systems used. Alternatively, it represents specific catalytic properties of the individual proteins. With the insulin assay we could also observe reduction of \( T. brucei \) thioredoxin by dihydrolipoamide as reported earlier for \( E. coli \) thioredoxin (26, 43). However, almost all cellular lipoic acid is amide linked to protein components of mitochondrial multienzyme complexes. Recently AhpD, a thioredoxin-like protein of \( T. brucei \), has been characterized as substrate of dihydrolipoamide succinyltransferase, the E2 component of the \( \alpha \)-keto-glutarate dehydrogenase complex, which transfers electrons from a dihydrolipoyl residue of the E2 component to AhpC, a peroxiredoxin (44). This pathway may illustrate another possibility to reduce thioredoxins in the absence of a specific thioredoxin reductase. However, AhpD is only distantly related with thioredoxins and has been found in a few bacteria but not in any eukaryotic organism, where the system would also be restricted to the localization of the \( \alpha \)-ketoacid dehydrogenase complexes in the mitochondrion.

The physiological reactivity of thioredoxins is determined by the redox environment (45) as well as by the pK value of the nucleophilic cysteine and the redox potential of the protein. The thiolate absorption of reduced thioredoxin at 240 nm yielded a pK value of 7.6 ± 0.1 and the rate of carboxamidomethylation resulted in a pK of 7.4 ± 0.1. The values are comparable with those of the nucleophilic first redox active cysteine in \( E. coli \) thioredoxin (10, 38) and \( T. brucei \) trypanothione (21) (Table III). The difference titration curve (reduced versus oxidized thioredoxin) yielded a \( \Delta \text{p}K_{240} \) of 11.6 M⁻¹ cm⁻¹, which indicates that probably both active site cysteines are titrated and thus have very similar pK values. Therefore we cannot exclude that upon carboxamidomethylation, which clearly results in a mono-alkylated protein species, Cys-31 and Cys-34 are alternatively modified. On the other hand, as in other thioredoxins the crystal structure of \( T. brucei \) thioredoxin shows that Cys-31 is much more accessible (56).

The pK values of \( T. brucei \) thioredoxin, trypanothione, and trypanothione coincide with the intracellular pH of the parasites (46), which may contribute to their reactivity. As outlined by Gilbert (47), the second-order rate constants for thiol-disulfide exchange reactions exhibit an optimum when the thiol pK is equal to the surrounding pH.

\[ \text{Fig. 9. Flux of reducing equivalents from NADPH onto ribonucleotide reductase and peroxidases in trypanosomatids. A cascade composed of trypanothione reductase (TR), trypanothione, and trypanothione (Tpx) transfers electrons from NADPH onto ribonucleotide reductase (RiboR) and different trypanothione peroxidases (TpxPx), which then catalyze the reduction of nucleoside diphosphates (NDP) to deoxy nucleoside diphosphates (dNDP) and the detoxification of hydroperoxides (ROOH), respectively. As shown here, \( T. brucei \) thioredoxin (Trx) can replace trypanothione in these reaction sequences.} \]

\[ \begin{align*}
\text{NADPH} & \rightarrow \text{TR} \rightarrow \text{TSH} \rightarrow \text{TS} \rightarrow \text{TRx} \rightarrow \text{DNP} \rightarrow \text{ROD} \rightarrow \text{H}_2 \text{O} \\
\text{NADP} & \rightarrow \text{TR} \rightarrow \text{TSH} \rightarrow \text{TS} \rightarrow \text{TRx} \rightarrow \text{dNDP} \rightarrow \text{ROO} \rightarrow \text{H}_2 \text{O}
\end{align*} \]

\[ \text{TABLE III} \]

| Dithiol (protein) | pK value of the nucleophilic Cys | Redox potential |
|------------------|----------------------------------|----------------|
| \( T. brucei \) thioredoxin | 7.4-7.6 | -267 |
| \( E. coli \) thioredoxin | 6.7-7.4 | -270 |
| \( T. brucei \) trypanothione | 7.2-7.4 | -249 |
| Trypanothione | 7.4-7.4 | -242 |

\[ a \text{ From Refs. 38 and 37.} \]

\[ b \text{ From Ref. 39.} \]

\[ c \text{ From Ref. 21.} \]

\[ d \text{ From Ref. 55.} \]

\[ * \text{ From Ref. 1.} \]

Similar pK values for the active site cysteine residues have also been reported for \( E. coli \) thioredoxin (34) whereby these data were not in accordance with those obtained by others who showed pK values of 7.4 and 9.9 for Cys-32 and Cys-35, respectively (10). A remarkable difference between the \( T. brucei \) protein and other thioredoxins is the replacement of Asp-26 (\( E. coli \)) by Trp-25. One may speculate that this exchange alters the charge distribution in the active site resulting in similar pK values for both cysteines. UV and NMR measurements of the D26A \( E. coli \) thioredoxin mutant could also be interpreted in accordance with similar pK values for both thiols whereby the absorption coefficient was about 6.5 \( \text{mM}^{-1} \text{cm}^{-1} \) (10). In the crystal structure of oxidized thioredoxin 2 from \( A. nanae \) with a Tyr instead of Asp, the position of the disulfide ring formed by the active site cysteines is altered relative to the central pleated sheet (48). In addition, from the three-dimensional structure of \( F. fasciculata \) trypanothione, which also has a Tyr instead of Asp at the respective position, it has been suggested that this substitution influences the environment and reactivity of the buried cysteine (49).

\[ \text{C} \text{ys-68, the third thiol group in} \ T. \text{ brucei thioredoxin corresponds to} \text{Cys-69 in the human protein. Although in the crystal structure of human thioredoxin the residue is buried in the protein (50), Cys-69 is obviously accessible to S-nitrosylation (11), which is linked with the redox regulatory activity and anti-apoptotic function of the protein. In the parasite thioredoxin, Cys-68 is probably involved in the formation of covalent dimers (8) and may play a role for the stability of the protein because the recombinant C68S mutant was not stable in reduced form.} \]

In contrast to the measurements of the thiolate anion at 240 nm, which suggest titration of two (or three) residues, only one cysteine was modified when reduced thioredoxin was alkylated by equimolar concentrations of iodoacetamide, Cys-68 does not react under these conditions. A titration curve with a single transition at pH 7.4 was also obtained with several active site mutants of \( E. coli \) thioredoxin in contrast to wild type \( E. coli \) thioredoxin (38) and \( T. brucei \) trypanothione (21) where the pH dependence of the alkylation rate results in a second inflection point. In \( E. coli \) thioredoxin, this was attributed to an increased reactivity of the nucleophilic cysteine at higher pH values because of the titration of another residue influencing its reactivity (38).

At pH 5, reduced \( T. brucei \) thioredoxin does not react with iodoacetamide. A corresponding behavior has been reported for \( E. coli \) thioredoxin by Mössner et al. (38), whereby Kallis and Holmgren (37) found at pH 5.7 a \( k_{\text{App}} \) of 44.2 \( \text{mM}^{-1} \text{ s}^{-1} \). Also the reaction rates of \( T. brucei \) trypanothione and DsbA with iodoacetamide did not drop to zero at low pH values (19, 21). The maximum reaction rate of \( T. brucei \) thioredoxin with iodoacetamide is 60 \( \text{mM}^{-1} \text{ s}^{-1} \) at pH 8.5. This corresponds to the data for several active site mutants of \( E. coli \) thioredoxin (38) and is
about half of that observed for wild type E. coli thioredoxin. How the pKₐ value of the active site cysteines are lowered compared with model peptides with pKₐ values of about 8.9 (51) is not known. Most likely the localization of the cysteines at the N-terminal end of an α-helix leads to the stabilization of the thiolate anion by the positive partial charge of the helix dipole (52).

The redox potential of T. brucei thioredoxin is ~267 mV, which is nearly identical with that of E. coli thioredoxin. Although the redox potential of trypanothione (~242 mV) is significantly higher, the parasite thioredoxin can be kept reduced by the dithiol under physiological conditions because of the much higher cellular concentration of trypanothione. Thus a specific thioredoxin reductase is probably not required.

T. brucei possesses a single thioredoxin that catalyzes the transfer of electrons from trypanothione onto different peroxides as well as ribonucleotide reductase. Because these reactions are also fulfilled by the highly abundant trypanothione, the function of the parasite thioredoxin are still not known. Knockout and RNA interference experiments recently showed that thioredoxin is not essential for the viability of bloodstream and procyclic T. brucei at least under cell culture conditions (53). Mammalian thioredoxins regulate the activity of transcription factors, and different enzymes by heterodimer formation stimulate cell growth and inhibit apoptosis (13) and E. coli thioredoxin can act as chaperon (54). Thus there may be alternative specific functions for this single thioredoxin in the parasites.

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Heide Schmidt and R. Luise Krauth-Siegel

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