Identification and Functional Characterization of Thioredoxin from Trypanosoma brucei brucei*

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Trypanosomes and Leishmania, the causative agents of several tropical diseases, lack the glutathione/glutathione reductase system but have trypanothione/trypanothione reductase instead. The uniqueness of this thiol metabolism and the failure to detect thioredoxin reductases in these parasites have led to the suggestion that these protozoa lack a thioredoxin system. As presented here, this is not the case. A gene encoding thioredoxin has been cloned from Trypanosoma brucei, the causative agent of African sleeping sickness. The single-copy gene, which encodes a protein of 107 amino acid residues, is expressed in all developmental stages of the parasite. The deduced protein sequence is 56% identical with a putative thioredoxin revealed by the genome project of Leishmania major. The relationship to other thioredoxins is low. T. brucei thioredoxin is unusual in having a calculated pH value of 8.5. The gene has been overexpressed in Escherichia coli. The recombinant protein is a substrate of human thioredoxin reductase with a $K_{m}$ value of 6 $\mu$M but is not reduced by trypanothione reductase. T. brucei thioredoxin catalyzes the reduction of insulin by dithioerythritol, and functions as an electron donor for T. brucei ribonucleotide reductase. The parasite protein is the first classical thioredoxin of the order Kinetoplastida characterized so far.

Thioredoxins are small ubiquitous proteins with a molecular mass of about 12000 and a conserved redox active Cys-Gly-Pro-Cys motif. The proteins function in a wide variety of cellular processes (1). The first elucidated role was as donor of reducing equivalents for ribonucleotide reductase (2). In higher organisms, the thioredoxin system, composed of thioredoxin, NADPH, and thioredoxin reductase, seems to be a general dithiol-disulfide oxidoreductase. Thioredoxin also provides reducing equivalents for ribonucleotide reductase. This system, which catalyzes the reduction of hydrogen peroxide and organic peroxides, is widely distributed in nature (3).

Trypanosomes and Leishmania are the causative agents of severe tropical diseases, examples being African sleeping sickness (Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense), Nagana cattle disease (Trypanosoma congolense and Trypanosoma brucei brucei), Chagas’ disease (Trypanosoma cruzi), and the three manifestations of leishmaniasis (Leishmania donovani, Leishmania major, Leishmania mexicana). All these parasitic protozoa have a thiol metabolism that completely differs from that of other eukaryotes and prokaryotes. They lack the glutathione/glutathione reductase system as well as glutathione peroxidase and catalase. Trypanothione (N²,N⁶-bis(glutathionyl)spermidine) and monogluthathionylspermidine are the main low molecular mass thiols (4, 5). These glutathionylspermidine conjugates are kept in the reduced state by trypanothione reductase and NADPH. The dithiol trypanothione has been shown to be involved in the detoxification of hydroperoxides (6), homeostasis of ascorbate (7), as well as the synthesis of deoxyribonucleotides catalyzed by ribonucleotide reductase (8). Enzymes of the trypanothione metabolism are attractive target molecules for the rational development of new antiparasitic drugs (for a recent review see Ref. 5). The uniqueness of the trypanothione metabolism and the failure to detect thioredoxin reductases in trypanosomatids have led to the suggestion that these protozoa lack a thioredoxin system (4). Recently the genome sequencing project of L. major revealed a sequence that probably codes for a thioredoxin (9). Based on this observation we have cloned and overexpressed a gene encoding thioredoxin from Trypanosoma brucei. As shown here, the protein is a classical thioredoxin with several unusual properties.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli thioredoxin was purchased from Calbiochem, and bovine pancreas insulin was from Sigma. A sample of human thioredoxin reductase was a kind gift of Drs. Katja Becker and R. Heiner Schirmer, Heidelberg. The plasmids of the two genes of T. brucei ribonucleotide reductase were kindly provided by Drs. Anders Hofer and Lars Thelander, Umeå, Sweden. Recombinant T. brucei tryparedoxin (8), T. cruzi trypanothione reductase (10, 11), and T. brucei ribonucleotide reductase (12–14) were prepared as described. Polyclonal rabbit antibodies against the recombinant T. brucei thioredoxin were produced by Eurogentec.

Polymerase Chain Reaction Amplification of the T. brucei Thioredoxin Gene—Total RNA of T. brucei long slender bloodstream parasites was reverse transcribed into single-stranded cDNA as described (13). A degenerate reverse primer (5’-CTTCTCTCTAGGTTT/TTAA/

1 M. Dormeyer, and R. L. Krauth-Siegel, unpublished results.

2 The abbreviations used are: PCR, polymerase chain reaction; DTE, dithioerythritol; $trx$, thioredoxin gene.
The assay mixture contained in a total volume of 800 μl. Both strands were completely sequenced. DNA was sequenced by the dideoxynucleotide chain termination method using the T7 Sequencing Kit (Amersham Pharmacia Biotech). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG).

Expression of the Gene and Purification of the Recombinant Protein—Competent E. coli SG 13069 cells were transformed with the pQE 32 vector (Qiagen). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG).

Insulin Reduction Assay—A fresh solution of 1 mg/ml insulin was prepared in 100 mM potassium phosphate, 1 mM EDTA, pH 7.0 (16, 17). The assay mixture contained in a total volume of 100 μl of 10 mM potassium phosphate, 1 mM EDTA, pH 7.0, 50 mM sodium phosphate, pH 7.0, 70 mM mercaptoethanol containing 5 mM imidazole, 5 mM cysteine, 20 μM phenylmethylsulfonyl fluoride and disintegrated by sonication. After centrifugation the supernatant was applied onto a 13×1 ml nickel-nitrioltriacetic acid Superflow-Sepharose column (Qiagen) and ethanol) containing 5 mM imidazole, 150 nM pepstatin, 4 nM cystatin, (Life Technologies, Inc.) containing 100 μg/ml kanamycin, and the cells were grown at 30 °C to an A600 of 0.5. The gene encoding thioredoxin has been cloned from T. brucei genomic DNA with Pfu polymerase (94 °C, 2 min; 94 °C, 30 s; 50 °C, 30 s; 72 °C, 3 min; 30 cycles; 72 °C, 5 min) and cloned into the Sma I restriction site of the pQE-32 vector (Qiagen). DNA was sequenced by the dideoxynucleotide chain termination method using the T7 Sequencing Kit (Amersham Pharmacia Biotech). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG).

ribonucleotide reductase activity was determined from the rate of reduction of [3H]GDP to dGDP essentially as described for CDP reduction (18). The reaction mixture contained in a total volume of 200 μl of 50 μM Hepes, pH 7.6, 500 μM [3H]GDP, 100 μM dTTP, 100 mM KCl, 6.4 mM MgCl2, 1 mM DTE, and varying amounts of T. brucei ribonucleotide reductase, and E. coli thioredoxin, respectively. The reaction was started by adding T. brucei ribonucleotide reductase, and the assay mixture was incubated 20 min at 37 °C. After dephosphorylation educts and products were separated by high performance liquid chromatography (19).

Cultivation of T. brucei—Culture adapted bloodstream forms of T. brucei (cell line TC 221 (20)) were grown in Balfz medium supplemented with 16.7% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO2 (21). Procyclic T. brucei (cell line AnTat 1.1 (22)) were cultured in SDM-79 medium supplemented with 10% heat-inactivated fetal bovine serum at 27 °C (23) and harvested at a density of 3.5×106 cells/ml.

Cultivation of T. brucei in Mice—NMRI mice were infected intraperitoneally with the promastigote form of T. brucei clone AnTat 1.1 (22). After 3 days (long slender) and 5 days (short stumpy) trypanosomes were isolated from the blood by chromatography on DEAE-cellulose (24).

Isolation of Genomic DNA and Southern Blot—Genomic DNA from a culture of bloodstream TC221 T. brucei cells as well as from T. cruzi were prepared as described (25, 26). 10 μg of DNA was digested with Styl, and the fragments were separated on a 0.7% agarose gel. The DNA was blotted onto a Hybond N+ membrane (Amersham Pharmacia Biotech) by capillary transfer and hybridized with the digoxigenin-labeled trx gene. Southern blot analysis was performed as described (27) using the DIG High Prime DNA labeling and detection kit (Roche Molecular Biochemicals).

Isolation of T. brucei Total RNA and Reverse Transcriptase-PCR—Total RNA of procyclic, long slender, and short stumpy T. brucei was isolated using guanidinium isothiocyanate and phenol extraction as described (28). 1.5 μg of RNA was reverse transcribed and amplified using the 16s and 23s rRNA specific primers. 125 of the PCR reaction mixture was separated on a 1.5% agarose gel and hybridized in a Southern blot as described above.

RESULTS

Cloning and Sequencing of a Thioredoxin Gene from T. brucei—The gene encoding thioredoxin has been cloned from cDNA of long slender bloodstream forms of T. brucei brucei and sequenced. Both strands were completely sequenced. DNA was sequenced by the dideoxy chain termination method using the T7 Sequencing Kit (Amersham Pharmacia Biotech). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiating methionine (5′-TCGGTAGTGGATGTTTATAGCG). The spliced leader primer 5′-TAGAACCAGTTTCTGTAC-3′ and the very 5′-noncoding region is common in trypanosomes as no splicing. The spliced leader addition site is found 195 nucleotides upstream of the initiate codon. The complete coding region of the gene together with the 3′-untranslated region was amplified by PCR using a primer starting with the initial ATG together with a poly(T) primer. Two products of about 530 and 600 base pair length were obtained. The cDNA clones were identical in the coding sequence but differed in the length of the 3′-untranslated region (Fig. 1), the larger fragment containing an addition 7 and base pairs preceding the poly(A) stretch. Diversity in the 3′-noncoding region is common in trypanosomes as no specific polyadenylation signal is known (for a review see Ref. 29).
a 1-liter culture of recombinant E. coli proteins (37). Strictly hydrophobic residues at the respective positions are conserved throughout thioredoxin sequences (38). Despite the generally low sequence similarities within thioredoxins, mammalian thioredoxin reductases show broad specificities for their disulfide substrates and accept thioredoxins from other species. T. brucei thioredoxin is readily reduced by human thioredoxin reductase. In the spectrophotometric assay reduction of thioredoxin is followed in the presence of excess insulin ensuring the constant reoxidation of thioredoxin-(SH)₂ formed. In contrast to the bacterial enzymes, mammalian thioredoxin reductases show broad specificities for their disulfide substrates and accept thioredoxins from other species. T. brucei thioredoxin is readily reduced by human thioredoxin reductase. To test if trypanothione reductase is able to reduce thioredoxin, we replaced human thioredoxin reductase by T. cruzi trypanothione reductase in the NADPH/thioredoxin/insulin assay (data not shown). No activity could be detected in agreement with thioredoxin not being a substrate for trypanothione reductase.

**T. brucei Thioredoxin Catalyzes Reduction of Insulin**—The two interchain disulfides of insulin are substrates of thioredoxins. Reduction of the disulfide bonds generates the free A and B chains of insulin, and precipitation of the insoluble B chain is measured by the increase in turbidity (16). The reduction of insulin by DTE was followed at pH 7.0 in the absence and presence of T. brucei and E. coli thioredoxin (Fig. 5). The maximal rates of precipitation measured as ΔA₆₅₀/min were very similar for both thioredoxins yielding a relative specific activity of 3.6 D₆₅₀ × min⁻¹ mg⁻¹ of protein. The activities measured here are twice as high as those for E. coli and human thioredoxin reported previously (17), which may be because of slightly different assay conditions. Taken together, the data show that the three thioredoxins behave very similar in their insulin reduction capacity.

**T. brucei Thioredoxin Is an Electron Donor for Ribonucleotide Reductase**—Ribonucleotide reductase catalyzes the NADPH-dependent reduction of thioredoxin disulfide to the dithiol. In the spectrophotometric assay reduction of thioredoxin is followed in the presence of excess insulin ensuring the constant reoxidation of thioredoxin-(SH)₂ formed. In contrast to the bacterial enzymes, mammalian thioredoxin reductases show broad specificities for their disulfide substrates and accept thioredoxins from other species. T. brucei thioredoxin is readily reduced by human thioredoxin reductase. To test if trypanothione reductase is able to reduce thioredoxin, we replaced human thioredoxin reductase by T. cruzi trypanothione reductase in the NADPH/thioredoxin/insulin assay (data not shown). No activity could be detected in agreement with thioredoxin not being a substrate for trypanothione reductase.

**T. brucei Thioredoxin Is a Substrate of Human Thioredoxin Reductase**—Thioredoxin reductases catalyze the NADPH-dependent reduction of thioredoxin disulfide to the dithiol. In the spectrophotometric assay reduction of thioredoxin is followed in the presence of excess insulin ensuring the constant reoxidation of thioredoxin-(SH)₂ formed. In contrast to the bacterial enzymes, mammalian thioredoxin reductases show broad specificities for their disulfide substrates and accept thioredoxins from other species. T. brucei thioredoxin is readily reduced by human thioredoxin reductase. To test if trypanothione reductase is able to reduce thioredoxin, we replaced human thioredoxin reductase by T. cruzi trypanothione reductase in the NADPH/thioredoxin/insulin assay (data not shown). No activity could be detected in agreement with thioredoxin not being a substrate for trypanothione reductase.
The thioredoxin eluted from the nickel chelator column; in the sample buffer; thioredoxin after prolonged storage in the absence of thiols and no thiols thiolated in the reaction is then reduced by NADPH and thioredoxin glutaredoxins. The disulfide form of thioredoxin generated is subsequently reduced by glutathione reductase at the expense of NADPH (1). The replacement of glutathione reductase by trypanothione reductase in trypanosomatids raised the question as to the donors of reducing equivalents for the parasite ribonucleotide reductase. As shown here, both T. brucei thioredoxin and trypararedoxin (8) catalyze the reduction of T. brucei ribonucleotide reductase by DTE as efficiently as E. coli thioredoxin (Fig. 6). Thus, most probably trypanosomes have developed two systems that provide electrons for the synthesis of DNA precursors as it is the case in other organisms (1).

Interestingly, T. brucei and L. major thioredoxins lack the highly conserved Asp-26, which in E. coli thioredoxin has been shown to play a crucial role for catalytic activity. It is the only acidic residue not localized on the surface of the protein (41), and mutation to an Ala increased the \( K_m \) value for thioredoxin reductase by a factor of 10. In addition, the mutant E. coli protein had a drastically lowered ability to serve as a hydrogen donor for ribonucleotide reductase (38). T. brucei thioredoxin is an excellent substrate of human thioredoxin reductase, and like E. coli, thioredoxin is able to deliver the electrons for T. brucei ribonucleotide reductase. These findings indicate that in the parasite thioredoxins an acidic residue at this position is not essential for catalysis.

AFRERICAN TRYPANOSOMES change between three main life stages. In the blood of the mammalian host the parasites occur as dividing long slender and non-dividing short stumpy forms. Upon a blood meal on an infected animal the tsetse fly takes up parasites and the short stumpy cells differentiate to procyclics, which multiply in the insect vector. The thioredoxin gene is expressed in all three developmental stages of T. brucei. The occurrence of the mRNA in the non-dividing short stumpy parasites may indicate that the protein is not only involved in deoxyribonucleotide synthesis but serves additional purposes. Of course, it cannot be excluded that the mRNA synthesized in the parasite thioredoxins an acidic residue at this position is not essential for catalysis.

**DISCUSSION**

African trypanosomes possess a classical thioredoxin. The T. brucei protein is the first thioredoxin of an organism belonging to the order Kinetoplastida that has been characterized to date. Phylogenetically the thioredoxins of T. brucei and L. major form a new branch distinct from all other eukaryotic lineages, whereby the parasite proteins are more closely related to mammalian thioredoxins than those of yeasts and plants (not shown).

One of the best studied functions of thioredoxin is the delivery of reducing equivalents for the synthesis of deoxyribonucleotides catalyzed by ribonucleotide reductase. In many organisms the physiological electron donors are the thioredoxin and glutaredoxin systems. The disulfide form of thioredoxin generated in the reaction is then reduced by NADPH and thioredoxin reductase. In the case of glutaredoxin the dithiol is regenerated spontaneously by glutathione, and the glutathione disulfide formed is subsequently reduced by glutathione reductase at the expense of NADPH (1). The replacement of glutathione reductase by trypanothione reductase in trypanosomatids raised the question as to the donors of reducing equivalents for the parasite ribonucleotide reductase. As shown here, both T. brucei thioredoxin and trypararedoxin (8) catalyze the reduction of T. brucei ribonucleotide reductase by DTE as efficiently as E. coli thioredoxin (Fig. 6). Thus, most probably trypanosomes have developed two systems that provide electrons for the synthesis of DNA precursors as it is the case in other organisms (1).

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Another important function of thioredoxins is to provide reducing equivalents for the detoxification of hydroperoxides by thioredoxin peroxidases. The thioredoxin peroxidases of yeast (3) and mammals (45) and the alkyl hydroperoxide reductases of bacteria (46) form the large family of peroxiredoxins found in all phyla. In trypanosomatids a unique cascade composed of trypanothione/trypanothione reductase/trypararedoxin/trypararedoxin peroxidase has been shown to detoxify hy-

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3 N. Reckenfelderbäumer, and R. L. Krauth-Siegel, unpublished results.
droperoxides (6, 47–49). The parasite peroxidase is a member of the peroxiredoxin family of proteins. Thus future work will show if tryparedoxin peroxidase also accepts electrons from the parasite thioredoxin and how the dithiol form of thioredoxin is subsequently regenerated.

*T. brucei* thioredoxin is rather unique in having a calculated pI value of 8.5. The protein contains several arginine residues resulting in an overall positive charge. Nearly all thioredoxins studied so far are acidic proteins with pI values between 4.5 and 5.0. Very recently another highly basic thioredoxin III has been described in mitochondria of *Saccharomyces cerevisiae* (42). The sequence of *T. brucei* thioredoxin is slightly more similar to this thioredoxin than to the cytosolic thioredoxins I and II of yeast. So far it is not known if the pronounced charge differences correlates with distinct functions or the localization of the proteins. The *T. brucei* sequence does not show an N-terminal extension that could serve as a mitochondrial import signal. In the putative *L. major* thioredoxin, most of the basic residues found in the *T. brucei* protein are conserved, but the protein has a theoretical pI value of 5.4 (9).

*T. brucei* thioredoxin is a substrate of human thioredoxin reductase but is not reduced by trypanothione reductase, which strongly suggests the presence of a thioredoxin reductase. From a phylogenetic point of view it will be highly interesting which kind of thioredoxin reductase *Kinetoplastida* have because two completely different types of enzymes have been realized in nature. Procaryotes, yeast, and the protozoan parasite *Giardia lamblia* (50) possess small homodimeric proteins with a subunit molecular mass of about 35,000 (51). Mammalian thioredoxin reductases (52) are homodimeric selenoproteins consisting of two subunits of about 55,000. These enzymes carry a selenocysteine in their C-terminal dipeptide, which is involved in the enzymes’ catalytic activity (53). The malarial parasite *Plasmodium falciparum* also possesses a large homodimeric enzyme but with two redox active cysteine residues in the C-terminal region (54), and as shown recently, the nematode *Caenorhabditis elegans* obviously possesses both types of large enzymes (55). The accessibility of recombinant *T. brucei* thioredoxin should now allow the characterization of the first trypanosomatid thioredoxin reductase. Disruption of the *trx* gene in different life stages of *T. brucei*. 1.5 μg of total RNA was reverse transcribed and amplified by reverse transcriptase-PCR using a spliced leader primer and a gene-specific primer of the *trx* 3′-end. The product was applied onto a 1.5% agarose gel, blotted, and visualized by hybridization with the digoxigenin-labeled *trx* gene. Lane 1, digoxigenin-labeled DNA size marker; lane 2, procyclic culture form; lane 3, long slender bloodstream parasites isolated from mice; lane 4, short stumpy bloodstream parasites isolated from mice. bp, base pairs.
gene in *T. brucei* is in progress to reveal if thioredoxin is essential for the viability and virulence of the parasite.

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