Trypanosoma brucei tryparedoxin, a thioredoxin-like protein in African trypanosomes

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Abstract A gene has been cloned from *Trypanosoma brucei* which encodes a protein of 144 amino acid residues containing the thioredoxin-like motif WCPPCR. Overexpression of the gene in *E. coli* resulted in 4 mg pure protein from 100 ml bacterial cell culture. Recombinant *T. brucei* tryparedoxin acts as a thiol-disulfide oxidoreductase. It is spontaneously reduced by trypanothione. This dithiol, exclusively found in parasitic protozoa, also reduces *E. coli* glutaredoxin but not thioredoxin. The trypanothione/tryparedoxin couple is an effective reductant of *T. brucei* ribonucleotide reductase. Like thioredoxins it has a poor GSH:disulfide transhydrogenase activity. The catalytic properties of tryparedoxin are intermediate between those of classical thioredoxins and glutaredoxins which indicates that these parasite proteins may form a new class of thiol-disulfide oxidoreductases.

Key words: Tryparedoxin; Thioredoxin; CPPC motif; Trypanothione; *Trypanosoma brucei*

1. Introduction

Thiol-disulfide oxidoreductases with the active site motif CXXC are found in all living cells. Well known representatives of this large protein family are the thioredoxins (CGPC), glutaredoxins (CPYC), eukaryotic protein-disulfide isomerases (CGHC) and the bacterial periplasmatic protein thiol:disulfide oxidoreductases DsbA (CPHC) [1]. An important function of thioredoxins and glutaredoxins – small proteins with an M_r of 12 000 and 10 000, respectively – is the delivery of reducing equivalents to ribonucleotide reductase. Oxidized thioredoxin formed in the reaction is then reduced by NADPH catalyzed by thioredoxin reductase. Oxidized glutaredoxin reacts spontaneously with glutathione and the resulting glutathione disulfide is subsequently reduced by glutathione reductase [2]. Trypanosomes, leishmania and amoebas – the causative agents of severe tropical diseases – lack the nearly ubiquitous glutathione/glutathione reductase system. Glutathionyl spermidine conjugates such as trypanothione [N^1,N^8-bis(glutathionyl)spermidine] are their main low molecular weight thiols which are kept reduced by the enzyme trypanothione reductase [3–5].

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Abbreviations: *tpx*, gene encoding tryparedoxin; TR, trypanothione reductase (EC 1.6.4.8); $T(SH)_2$, trypanothione [N^1,N^8-bis(glutathionyl)spermidine]; TS_2, trypanothione disulfide; GSH, glutathione; GSSG, glutathione disulfide; HEDS, β-hydroxyethyl disulfide

In all eukaryotes studied the synthesis of deoxyribonucleotides is a thiol-dependent reaction. Recently, the genes of ribonucleotide reductase from *Trypanosoma brucei* have been cloned and overexpressed [6,7]. The uniqueness of the trypanothione metabolism raises the question if this dithiol is the donor of reducing equivalents for the parasite synthesis of deoxyribonucleotides and if the reaction is catalyzed by a specific protein. One interesting candidate is 'tryparedoxin'. This protein – which has an M_r of 16 000 and contains a CPPC motif – has recently been isolated from *Crithidia fasciculata* as a component of a trypanothione-dependent peroxidase system [8,9]. Here we report the cloning and overexpression of the gene of a tryparedoxin from the African parasite *Trypanosoma brucei brucei*. The catalytic properties of the recombinant protein are compared with those of *Escherichia coli* thioredoxin and glutaredoxin.

2. Materials and methods

2.1. Materials

Trypanothione disulfide was purchased from Bachem, Switzerland. *E. coli* glutaredoxin was obtained from IMCO, Sweden. *E. coli* thioredoxin was from Calbiochem and HEDS from Aldrich.

Recombinant *T. cruzi* trypanothione reductase [10,11], human glutathione reductase [12] and *T. brucei* ribonucleotide reductase [6,7] were purified as described. The plasmids of the two genes of *T. brucei* ribonucleotide reductase were kindly provided by Drs. A. Hofer and L. Thelander, Umeå University, Umeå, Sweden. *E. coli* thioredoxin reductase was a kind gift of Dr. Charles Williams, University of Michigan, Ann Arbor, MI, USA. The *E. coli* strain A179 (*garB10, fhuA22, ompF627(T_2^R), fadL701(T_2^R), relA1, pit-10, spoT1, trxA14::kan, mcrB1, creC510*) [13] was obtained from Dr. Mary Berlyn, *E. coli* Stock Center, Yale University, USA.

2.2. PCR amplification and sequencing

Total RNA of long slender bloodstream *T. brucei* was reverse transcribed into single-strand cDNA as described [7,14]. Two degenerate primers (*tpx1*, 5′-TGGTG(CT)CCICCITG(CT)(AC)GIGGITT(CT) and *tpx2*, 5′-GT(AG)AAICCIC(GT)(AG)CAIGGIGG(AG)CACCA) which covered the active site motif were derived from the partial protein sequence of *C. fasciculata* tryparedoxin [8]. The 5′ end of the gene was amplified from the cDNA using *tpx2* and a spliced leader primer (5′-TAGAACCAGTTTCTGTACTATATTG) (94°C, 2 min; 94°C, 30 s; 60°C, 30 s; 72°C, 2 min; 30 cycles; 72°C, 5 min). For cloning the 3′ end *tpx1* and an oligo(T) primer were used (94°C, 2 min; 94°C, 30 s; 50°C, 30 s; 72°C, 3 min; 30 cycles; 72°C, 3 min). Both fragments were cloned into the pBluescript SK(+) vector (Stratagene) for sequencing.

The complete gene was obtained from the cDNA using a 3′ primer which contained an additional *Bgl*II cleavage site (5′-AGATCTTCACAGACAGCATGGCATCTC) and a 5′ primer (5′-ATGTCTGGCCTCGCCAAGTATCTTCC). PCR was carried out with *Pfu* polymerase (94°C, 2 min; 94°C, 30 s; 55°C, 30 s; 72°C, 2 min; 30 cycles; 72°C, 5 min). The PCR product was cloned into the pQE-60 vector (Qiagen) predigested by *Nco*I and mung bean nuclease I.

DNA was sequenced by the dideoxynucleotide chain termination

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GTTCAAGACAAGCGCACTGCTAAACCGAAAGAACAAATCCAAAAGAACAAACAAGACATTCAACTCATATTGTAATT   -5

GACGATGTCTGGCCTCGCCAAGTATCTTCCTGGCGCAACCAACCTGCTGTCCAAGTCGGGTGAAGTTTCACTGGGAT   73
     M  S  G  L  A  K  Y  L  P  G  A  T  N  L  L  S  K  S  G  E  V  S  L  G    24

CCCTCGTTGGGAAAACTGTGTTTCTTTACTTTTCTGCCTCCTGGTGCCCCCCATGCCGGGGTTTTACACCGGTCCTC  150
S  L  V  G  K  T  V  F  L  Y  F  S  A  S  W  C  P  P  C  R  G  F  T  P  V  L    50

GCCGAGTTCTACGAGAAGCATCATGTGGCGAAAAACTTCGAAGTCGTGCTGATTTCCTGGGATGAAAACGAGAGCGA  227
 A  E  F  Y  E  K  H  H  V  A  K  N  F  E  V  V  L  I  S  W  D  E  N  E  S  D   76

CTTCCATGATTACTACGGCAAGATGCCATGGCTCGCTCTCCCATTTGACCAACGCTCGACAGTTTCGGAATTGGGCA  304
   F  H  D  Y  Y  G  K  M  P  W  L  A  L  P  F  D  Q  R  S  T  V  S  E  L  G    101

AGACATTTGGCGTGGAATCCATTCCGACTCTTATCACAATCAATGCTGATACCGGTGCCATCATTGGCACTCAGGCC  381
K  T  F  G  V  E  S  I  P  T  L  I  T  I  N  A  D  T  G  A  I  I  A  T  Q  A   127

CGTACCCGTGTCATTGAGGATCCCGATGGTGCCAACTTTCCGTGGCCCAACTGAGATGCCATGCTGTCTGTGAAGGA  458
 R  T  R  V  I  E  D  P  D  G  A  N  F  P  W  P  N  *                         144

AGTGATAAGTATTGATGAAGTACGGTATGGTGTGGGGCATCTGGTGTGGAAGCACTAGTTATTCGGGAGCAAGAAAA  535

TAAGTAAGTGAAAAGTACAGAGCGAACAGGAGGGAGGGAATGAAAGTGAAAATGCTACGGAACTGAGCAGTAAGCGA  612

AAACGGAAAATATGCTTTTGTGCCAGGGGAACATTTGTCATGCGGTCCTGCGTGTGTTTTTGTCGATATCTCGTCAT  689

CCATCTTGCGGAGTTTTGTTTATTGCTCTTTGTTTCGGGGTTGGATTCCTGTTGTGCAGGGGTCCTGAACCTCACAC  766

TTTTGTCATTACATATGTTCTGACCGGCACGCGCCATAATCTTCTGTGGCGTGGTTTGAATCGGTGTGGGATATGAG  843

CAGGAGCGGTAAGATATTGAGATATGACGAAATGTTGCCTTTACGAAAAAAAAAAAAAAAAAA                 906
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Fig. 1. Nucleotide sequence and deduced protein sequence of *T. brucei* tryparedoxin. The ATG start codon, the TGA stop codon, and the poly(A) stretch are underlined.

method using the T7 Sequencing Kit (Pharmacia Biotech). Both strands were completely sequenced.

2.3. Expression of the gene and purification of the recombinant tryparedoxin

The *E. coli* strain A179 [13] which is deficient in thioredoxin was used for the expression of the *T. brucei* tryparedoxin gene. Competent cells were transfected with the pQE-60/*tpx* plasmid. A 100 ml culture of transformed cells in LB medium containing 100 μg/ml carbenicillin and 100 μg/ml kanamycin was grown at 37°C to an OD_{600} of 0.4. Expression was induced with 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside) overnight at 37°C. The cells were harvested and disintegrated by sonication. The cell debris was removed by centrifugation and ammonium sulfate was added to the supernatant to a saturation of 45%. After centrifugation the pellet was discarded and the ammonium sulfate concentration in the supernatant was increased to 68%. After centrifugation the protein pellet was dissolved in 100 mM potassium phosphate, 1 mM EDTA, pH 7.0 and chromatographed on a Superdex 75 HiLoad 16/60 FPLC column (Pharmacia). This step resulted in a fraction of pure *T. brucei* tryparedoxin as judged by PAGE on an 18% SDS gel. The protein concentration was determined using the bicinchoninic acid kit (Pierce). For pure tryparedoxin a protein concentration of 1 mg/ml corresponds to a $\Delta A_{280} = 1.7$. From a one liter culture of recombinant *E. coli* cells 40 mg of pure protein was obtained.

2.4. Glutathione:HEDS transhydrogenase assay

1 ml assay mixture contained 200 μM NADPH, 150 mU glutathione reductase, 1 mM GSH, 750 μM HEDS in 100 mM Tris-HCl, 1 mM EDTA, pH 8.0. The reaction was started by adding a sample of *T. brucei* tryparedoxin, *E. coli* glutaredoxin or thioredoxin. The GSSG formed is reduced by NADPH in the coupled glutathione reductase reaction. The absorbance decrease at 340 nm due to NADPH oxidation is followed at 25°C [15]. The activities were corrected for the rate of the spontaneous reduction of HEDS by GSH.

2.5. Thiol-disulfide exchange with trypanothione

A 90 μl assay mixture contained 100 μM NADPH, 1.34 mU TR, 50 μM $T(SH)_2$ and 10–30 μM of the respective CXXC protein (*T. brucei* tryparedoxin, *E. coli* thioredoxin or glutaredoxin). Trypanothione disulfide generated in the reaction is reduced by trypanothione reductase (TR). The absorbance decrease at 340 nm due to NADPH oxidation is followed at 25°C.

An apparent K_m value of *T. brucei* tryparedoxin for $T(SH)_2$ was obtained using GSSG as a final electron acceptor [9]. The reaction mixture contained 130 μM NADPH, 400 mU TR, 20–110 μM $T(SH)_2$ and 4.4 mM GSSG. The reaction was started by adding 1 μM tryparedoxin. The NADPH consumption was followed at 340 nm and corrected for the spontaneous reaction between $T(SH)_2$ and GSSG.

2.6. Thioredoxin reductase assay

90 μl reaction mixture contained 130 μM NADPH and *E. coli* thioredoxin reductase in 100 mM potassium phosphate, 2 mM EDTA, pH 7.4. The reaction was started by adding 14 μM *T. brucei* tryparedoxin, *E. coli* thioredoxin, or glutaredoxin. The NADPH oxidation was followed at 340 nm.

2.7. Ribonucleotide reductase assay

Ribonucleotide reductase activity was determined from the rate of reduction of [^{3}H]GDP to dGDP as described for CDP reduction [16]. The reaction products were separated by HPLC [17].

3. Results and discussion

3.1. Cloning and sequencing of the T. brucei tryparedoxin gene

Recently a protein, isolated from the insect parasite *Crithidia fasciculata*, was reported to be involved in a peroxidase cascade and to contain the unusual thioredoxin-like sequence WCPPCRGF [8]. From this peptide sequence a sense and an antisense primer were derived. PCR amplifications on *T. brucei* cDNA were performed with either a poly(T) primer or a spliced leader specifying primer in combination with the respective gene specific primer. The spliced leader which is added by *trans*-splicing is the very 5′ end of all trypanosomal mRNAs. The PCR amplifications yielded two fragments (244

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T. brucei   ..........  ..........  ..........  .........M SGLAKYLPGA  11
C. elegans  ..........  ..........  ..........  .......... ...MSLLAGV   7
A. thaliana ..........  ..........  ..........  .......... MAAEEGQVIG  10
P. denitr.  MARFSPMMLL  PVAIFAGFAG  LSGWALLPND  PDALPSAMIG REAPSVGEAT  50
Mouse Nucl  RVSNIPSLIF  LDATTGKVVC  RNGLLVIRDD  PEGLEFPWGP KPFREVIAGP 177
E. coli     ..........  ..........  ..........  .......... SDKIIHLTDD  10
                                                    #  #
                      * *             **** *   ** ***   *
T. brucei   TNLLSKSGEV  L...SGSLVG  KTVFLYFSAS  WCPPCRGFTP VLAEFYEK.H  57
C. fascic.                    SLAG  KLVFFYFSAS  WCPPCRGFTP QLIEFYDKFH
C. elegans  ..KLEKRDKT  LVDATEALAG  KAVGFYFSAH  WCPPCRGFTP ILKDFY...E  52
A. thaliana CHTNDVWTVQ  LDKAKESN..  KLIVIDFTAS  WCPPCRMIAP IFNDL.....  53
P. denitr.  ....LPGKVQ  LTDEIVRQPG  .VKLVNFWAS  WCPPCRAEHP TLTEL.....  90
Mouse Nucl  ..LLRNNGQS  L..ESSSLEG  SHVGVYFSAH  WCPPCRSLTR VLVESYRKIK 223
E. coli     ....SFDTDV  L......KAD  GAILVDFWAE  WCGPCKMIAP ILDEIAD...  47

                                    *             *
T. brucei   HVAKNFEVVL  ISGMKTRADF  HDYYGKMP.W  LALPFDQPST VSELGKTFGV 106
C. fascic.  ESK                          MP.W  LAVPFAQSWA VQK...HFNV
C. elegans  EVEDEFEVVF  VSFDRSESDL  KMYMSEHGDW  YHIPYGNDAI KE.LSTKYGV 101
A. thaliana ..AKKFMSSA  I.........  .........F  FKVDVDE...  LQSVAKEFGV  80
P. denitr.. ..SARLPVYG  VDLKDPEGAA  LGFLSEHGD.  PFHALAADP.  RGRVAIDWGV 136
Mouse Nucl  EAGQEFEIIF  VSADRSEESF  KQYFSEMP.W  LAVPYTDEAR  RSRLNRLYGI 272
E. coli     ..........  ..........  .EYQGKLT.V  AKLNIDQNP.  ..GTAPKYGI  72

              ***
T. brucei   ESIPTLITIN  ADTVAIIGTQ  ARTRVIEDPD  GANFPWPN              144
C. fascic.  ESIPTLIGVD  ADSGDVVTTR  ARATLVKDPE  GEQFPSKDA
C. elegans  SGIPALIIVK  PDG.TEVTKD  GRNDVQNGKD  .......PKA TVAKWKA    140
A. thaliana EAMPTFVFIK  AGEVVD...K  LVGANKEDLQ  AKIVKHTGVT TVVNQFEA   125
P. denitr.  TAPPETFIMH  GSGRI.LHRH  AGPLVREDYT  NRFLPELEKA LAAE       179
Mouse Nucl  QGIPTLIVLD  PQG.EVITRQ  GRVEVLNDED  CREFPWHPKP VLELSDSNAV 321
E. coli     RGIPTLLLFK  NGEVA..ATK  VGALSKGQLK  EFLDANLA              108
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Fig. 2. Alignment of *T. brucei* tryparedoxin with other WCPPCR-containing proteins as well as *E. coli* thioredoxin. *C. fascic.*, *Crithidia fasciculata* tryparedoxin (fragments) [8]; *C. elegans, Caenorhabditis elegans* thioredoxin-like protein (clone) ([19]; accession number U23511); *A. thaliana, Arabidopsis thaliana* thioredoxin *h* ([18]; accession number Z35473); *P. denitr., Paracoccus denitrificans* periplasmatic protein-disulfide oxidoreductase ([21]; accession number Z71971); Mouse Nucl, mouse nucleoredoxin ([20]; accession number X92750); *E. coli, Escherichia coli* thioredoxin ([22]; accession number M54881). Of the 435 residues long sequence of mouse nucleoredoxin only residues 128–321 are depicted. Residues which are found in at least five of the seven aligned sequences are given in bold letters. Redox-active Cys pair. *Residues which are highly conserved in thioredoxins of different species and which are also found in *T. brucei* tryparedoxin.

bp and 750 bp, respectively) which covered the whole sequence of the gene. The complete gene was also amplified from the *T. brucei* cDNA. The spliced leader addition site is found 80 nucleotides upstream of the start codon. The following open reading frame of 432 bp corresponds to a deduced protein sequence of 144 amino acid residues (Fig. 1). The 3′ non-coding region comprises 350 bp followed by a poly-A stretch.

3.2. Structural comparison of tryparedoxin with classical thioredoxins and other proteins containing a CPPC motif

The deduced protein sequence of *T. brucei* tryparedoxin is 144 residues long and exhibits a WCPPCR motif. Several proteins containing this motif have been identified in the last 3 years. Most of them are of plant origin like three thioredoxins *h* from *Arabidopsis thaliana* [18]. Others have been identified as genes in invertebrates, for instance a thioredoxin-like protein of *Caenorhabditis elegans* [19]. Mouse nucleoredoxin [20], tryparedoxin from *Crithidia fasciculata* [9] and a periplasmatic protein-disulfide oxidoreductase from *Paracoccus denitrificans* [21] also contain this motif. The CPPC motif is clearly distinct from the classical sequences of thioredoxins (CGPC), glutaredoxins (CPYC) or protein disulfide oxidoreductases (CGHC). Fig. 2 gives an alignment of six proteins with the WCPPCR motif together with the sequence of *E. coli* thioredoxin [22]. Except for the common motif, the proteins show relatively little sequence similarity. When comparing *T. brucei* tryparedoxin with the *C. elegans* protein, thioredoxin *h* of *A. thaliana*, the protein-disulfide oxidoreductase of *P. denitrificans*, mouse nucleoredoxin and *E. coli* thioredoxin 32, 24, 16, 38 and 26% of all compared positions are found to be occupied by identical residues. An alignment of *T. brucei* tryparedoxin with human glutaredoxin revealed only 15% residues in common (data not shown). The primary structures of *T. brucei* and *C. fasciculata* tryparedoxins show 57% identity reflecting a much closer relationship between the two parasitic proteins. For none of the proteins containing a WCPPC motif structural information is so far available. On the other hand, valuable 3-dimensional models for different thioredoxins – despite often low sequence similarities – have been constructed based on the high resolution structure of *E. coli* thioredoxin [23]. In the following the primary structure of

Table 1
Thiol-disulfide reactions involving CXXC proteins

	Reduction by $T(SH)_2$ k_{app} (M^{-1} s^{-1})[a]	GSH:HEDS transhydrogenation (U/mg)
Tryparedoxin	$>2.0\times10^2$	≤0.15
Thioredoxin	8.6	≤0.05
Glutaredoxin	$>2.7\times10^2$	50–60

The assays were carried out as described in Section 2.
[a]Because of the very high rates with tryparedoxin and glutaredoxin, non-saturating trypanothione reductase concentrations were used; the second order rate constants are highly underestimated values under these conditions.

T. brucei tryparedoxin will be discussed in light of the 3-dimensional structure of *E. coli* thioredoxin [24,25]. Tryparedoxin is 35 residues longer than *E. coli* thioredoxin. The sequence alignment indicates that most of the additional residues appear as one long insertion starting around position 47 of *E. coli* thioredoxin. In light of the 3-dimensional structure of thioredoxin this stretch is supposed to protrude from the protein surface. Besides the active site motif, a second cluster of conserved residues starts at Gly-105 of *T. brucei* tryparedoxin (Gly-71 of *E. coli* thioredoxin; Fig. 2). In the structure of thioredoxin this region – which includes the conserved only *cis* proline residue – is spatially adjacent to the redox-active site [24,25] and is involved in major conformational changes upon reduction of the active site disulfide [26].

About one third of all residues are conserved within the thioredoxin family [23]. These residues form the active site, are essential for the high thermal stability of the proteins, or are involved in the interaction with other proteins. Many of them are also found in the sequence of tryparedoxin (residues marked with an asterisk in Fig. 2) but there are a few interesting exceptions. The most obvious exchanges occur at the active site where Ser-36 and Pro-41 replace Trp-28 and Gly-33 of *E. coli* thioredoxin. The internal salt bridge between Asp-26 and Lys-57 in *E. coli* thioredoxin which is generally found in disulfide oxidoreductases [27] is obviously missing in tryparedoxins, nucleoredoxin and the *C. elegans* protein since the acidic residue at this position is replaced by a tyrosine. In *E. coli* thioredoxin, mutation of Asp-26 to an alanine increases the stability of the protein; the mutant protein still serves as substrate of ribonucleotide reductase but the K_m value is 10-fold increased [28].

3.3. Overexpression and purification of recombinant T. brucei tryparedoxin

The *T. brucei tpx* gene was overexpressed in the thioredoxin-deficient *E. coli* strain A179 [13]. The recombinant protein was purified by fractionated ammonium sulfate precipitation and subsequent gel chromatography on Superdex 75. In order to exclude any contamination with a bacterial glutaredoxin only the very first fractions of the elution peak which had been shown by SDS-PAGE to be homogenous were used for the kinetic studies described below. From 100 ml *E. coli* culture 4 mg of recombinant protein was purified.

3.4. Catalytic properties of T. brucei tryparedoxin

Recombinant *T. brucei* tryparedoxin was studied for its ability to catalyze classical reactions of thiol-disulfide oxidoreductases. Glutaredoxins have high activities as general GSH-disulfide oxidoreductases [15]. As shown in Table 1, in the GSH:HEDS transhydrogenase reaction tryparedoxin behaves like thioredoxin being a very poor catalyst of the glutathione-dependent disulfide reduction. This finding is in agreement with the primary structure of the protein (Fig. 1) which does not give any indication for a specific glutathione binding site [29].

Regeneration of thioredoxin and glutaredoxin from their respective disulfide forms follows different mechanisms. In thioredoxins the dithiol form is restored in an NADPH-dependent reaction catalyzed by thioredoxin reductase. In contrast, glutaredoxin disulfides are reduced spontaneously by glutathione and the GSSG formed is then reduced by NADPH and glutathione reductase [2]. In trypanosomatids, glutathione reductase is replaced by trypanothione reductase which catalyzes the reduction of trypanothione disulfide by NADPH [3,4,30]. In *C. fasciculata* the trypanothione system has been shown to be involved in a unique cascade catalyzing the detoxication of hydroperoxides [8,31]. A thioredoxin-like protein with an M_r of 16 000 and an active site WCPPC motif is a component of this peroxidase system. This CXXC protein – named tryparedoxin – transfers the reducing equivalents from trypanothione to a peroxiredoxin-type peroxidase which then reduces hydroperoxides [8,9]. This mechanism comprises the spontaneous reduction of tryparedoxin by trypanothione. The *T. brucei* protein described here shares 57% of all residues with the crithidial tryparedoxin. We therefore studied the reduction of *T. brucei* tryparedoxin by trypanothione in comparison to other CXXC proteins. As shown in Table 1, reduction of tryparedoxin by trypanothione is very fast (Table 1). *E. coli* glutaredoxin is also readily reduced by the dithiol but the respective rate of thioredoxin reduction is only 5% that for tryparedoxin. The values given in Table 1 are apparent rates since trypanothione reductase was limiting in the assay system; in the presence of saturating concentrations of trypanothione reductase the reactions were too fast to be measured. The results may indicate that in vivo tryparedoxin – like glutaredoxin, but in contrast to thioredoxin – is non-enzymically regenerated by thiol/disulfide exchange. On the other hand, *T. brucei* tryparedoxin is readily reduced by human thioredoxin reductase (S. Gromer and R.H. Schirmer, personal communication) which is known for its broad substrate specificity [32]. So far nothing is known about the specificity of the enzyme from low eukaryotes.

Tryparedoxin is not a substrate of *E. coli* thioredoxin reductase (data not shown) in accordance with the high specificity of the bacterial enzymes for their respective thioredoxin.

A K_m value of *T. brucei* tryparedoxin for trypanothione was estimated in an assay system which measures the tryparedoxin-catalyzed reduction of GSSG by trypanothione. An apparent K_m value of ≥ 150 μM was obtained which is in good agreement with the value of 130 μM reported for *C. fasciculata* tryparedoxin using the peroxidase/hydroperoxide system as final electron acceptor [9].

A classical function of thioredoxins and glutaredoxins is the delivery of reducing equivalents for ribonucleotide reductase [2]. Preliminary studies on *T. brucei* ribonucleotide reductase show that trypanothione serves as effective reductant of the enzyme and tryparedoxin strongly stimulates the overall reduction of GDP (M. Dormeyer and R.L. Krauth-Siegel, in preparation).

In conclusion, *T. brucei* tryparedoxin containing a CPPC

active site motif effectively catalyzes certain thiol-disulfide exchanges. The nature of the two residues between the active site cysteines strongly affects the redox properties of thioredoxin-like thiol:disulfide oxidoreductases [1,33]. In the periplasmic protein thiol:disulfide oxidoreductase DsbA a mutation that results in two central proline residues increases the redox equilibrium constant by more than 1000-fold which results in a more reducing protein species [33]. Recently a protein-disulfide oxidoreductase has been discovered in the periplasma of *P. denitrificans* which contains a CPPC motif and has been proposed to reduce protein-disulfide bonds in vivo rather than to form them [21]. In some aspects tryparedoxin resembles a classical thioredoxin, in others it behaves like a glutaredoxin indicating that the parasite tryparedoxins may form a new class of thiol-disulfide oxidoreductases.

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