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

Heike Lüdemann a, Matthias Dormeyer a, Christian Sticherling a, Dirk Stallmann b, Hartmut Follmann b, R. Luise Krauth-Siegel b, * a Biochemie-Zentrum Heidelberg, Ruprecht-Karls-Universität, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany b Fachbereich Biologie/Chemie, Universität Gesamthochschule Kassel, D-34132 Kassel, Germany

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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 WCPPC. 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 (CGHC), glutaredoxins (CPYC), eukaryotic protein-disulfide isomerases (CGHC) and the bacterial periplasmatic protein thioldisulfide oxidoreductases DsbA (CPHC) [1]. An important function of thioredoxins and glutaredoxins – small proteins with an Mr of 12000 and 10000, 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 enzyme [3]. Trypanothione 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. Thelanguages, 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, fhuA2, ompF67/Tys, fadl704/Tys, relA1, pits-10, spoT1, trxA14::kan, mcrB1, creC510) [13] was obtained from Dr. Mary Berlyn, E. coli Stock Center, Yale University, USA.

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 kindly provided by Drs. A. Hofer and L. Thelanguages, 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, fhuA2, ompF67/Tys, fadl704/Tys, relA1, pits-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 (tps1, 5'-TGGTGTGTT(C)CCICCTGTC(AG)CaGIGGT(C)G(T)TGTGTT(C)G and tps2, 5'-GT(AG)AAICCIC(AG)CaGIGGIGG(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 the tps1 and a spiked leader primer (5'-TAGACGACGTTCGATGACTATAATGG) (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 tps1 and an oligo(T) primer were used (94°C, 2 min; 94°C, 30 s; 50°C, 30 s; 72°C, 2 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 BglII cleavage site (5'-AGATCTTCGAGACGAGATCCATGCTTTC) resulting 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.
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 \( \mu \)g/ml carbenicillin and 100 \( \mu \)g/ml kanamycin was grown at 37\(^\circ\)C to an OD \( \text{OD}_{600} \) of 0.4. Expression was induced with 100 \( \mu \)M IPTG (isopropyl-\( \beta \)-D-thiogalactopyranoside) overnight at 37\(^\circ\)C. The cells were harvested and disrupted 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 \( A_{280} \) = 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 \( \mu \)M NADPH, 150 \( \mu \)M glutathione reductase, 1 mM GSH, 750 \( \mu \)M HEDS in 100 mM Tris-HCl, 1 mM EDTA, pH 7.0. 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\(^\circ\)C [15]. The activities were corrected for the rate of the spontaneous reaction between HEDS and GSSG.

2.5. Thiolsulfide exchange with trypanothione

A 90 \( \mu \)l assay mixture contained 100 \( \mu \)M NADPH, 1.34 \( \mu \)M TR, 50 \( \mu \)M Th(II) and 10–30 \( \mu \)M 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\(^\circ\)C.

2.6. Thioredoxin reductase assay

90 \( \mu \)l reaction mixture contained 130 \( \mu \)M NADPH and *E. coli* thioredoxin reductase in 100 mM potassium phosphate, 2 mM EDTA, pH 7.4. The reaction was started by adding 14 \( \mu \)M *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 \([^3H]GDP\) 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 *Cricthidia 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

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.
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 *T. brucei* 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 in common with several proteins containing this motif that 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-disulphide 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 disulphide oxidoreductases (CGHC). Fig. 2 gives an alignment of six proteins with the WCPPCR motif (Fig. 2). 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*, *Paracoccus* denitrificans periplasmatic protein-disulphide oxidoreductase (accession number Z71971), mouse nucleoredoxin [20] (accession number X92750); *E. coli*, *Escherichia coli* thioredoxin (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.*
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 tryparedoxin, 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 diethyl form is restored in an NADPH-dependent reaction catalyzed by thioredoxin reductase. In contrast, glutaredoxins 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 WCPC 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-enzymatically regenerated by thiol/disulfide exchange. On the other hand, T. brucei tryparedoxin is readily reduced by human thioredoxin reductase (S. Gomer 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 \( \geq 150 \mu M \) was obtained which is in good agreement with the value of 130 \( \mu M \) 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 CPC

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**Table 1**

<table>
<thead>
<tr>
<th>Reduction by</th>
<th>GSH-HEDS transhydrogenation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{T(SH)}_2 )</td>
<td>( k_{\text{app}} ) (M(^{-1}) s(^{-1}))</td>
</tr>
<tr>
<td>Tryparedoxin</td>
<td>( &gt; 2.0 \times 10^2 ) ( \leq 0.15 )</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>8.6</td>
</tr>
<tr>
<td>Glutaredoxin</td>
<td>( &gt; 2.7 \times 10^2 ) ( 50-60 )</td>
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The assays were carried out as described in Section 2.

*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.*
active site motif effectively catalyzes certain thiol-disulfide exchange reactions. 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 periplasm 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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References