Structure of thioredoxin from *Trypanosoma brucei brucei*

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Abstract The three-dimensional structure of thioredoxin from *Trypanosoma brucei brucei* has been determined at 1.4 Å resolution. The overall structure is more similar to that of human thioredoxin than to any other thioredoxin structure. The most striking difference to other thioredoxins is the absence of a buried carboxylate behind the active site cysteines. Instead of the common Asp, there is a Trp that binds an ordered water molecule probably involved in the protonation/deprotonation of the more buried cysteine during catalysis. The conserved Trp in the WCGPC sequence motif has an exposed position that can interact with target proteins.

Key words: Thioredoxin; Redox active disulfide; X-ray structure; *Trypanosoma brucei brucei*

1. Introduction

Thioredoxins are ubiquitous redox active thiol proteins with a variety of functions, most well known are their roles in deoxyribonucleotide synthesis, in sulfoxide and sulfate reduction [1]. Currently, thioredoxin activity has been found outside the cell (cell growth stimulation and chemotaxis), in the cytoplasm (as an antioxidant and a reductant cofactor), in the nucleus (regulation of transcription factor activity), and in the mitochondria [2]. In plants, thioredoxins regulate enzymatic activities in the chloroplasts [3]. Thioredoxin also provides reducing equivalents for thioredoxin peroxidase [4]. This system, which catalyzes the reduction of hydrogen peroxide and organic hydroperoxides, is widely distributed in nature.

Trypanosomatids – the causative agents of severe tropical diseases such as African sleeping sickness – possess an unparalleled thiol metabolism in which the ubiquitous glutathione/glutathione reductase system is replaced by the trypanothione/trypanothione reductase couple [5–7]. The uniqueness of the trypanothione metabolism and the failure to detect thioredoxin reductase in trypanosomatids led to the suggestion that the parasites lack a thioredoxin system [5]. This is not the case. We have cloned a gene encoding thioredoxin from cDNA of bloodstream *Trypanosoma brucei* and overexpressed it in *Escherichia coli* [8].

The protein sequence of *T. brucei* thioredoxin (Trx) displays 56% identity with a putative thioredoxin from *Leishmania major*, another trypanosomatid parasite. To other thioredoxins the overall similarities range from 21% (*E. coli*) to 32% (human) and 33% (yeast I). *T. brucei* Trx is clearly distinct from tryparedoxin, another small dithiol protein with WCPPC motif also found in the parasite [9].

The crystal structures of several thioredoxins have been solved, from *E. coli* [10,11], human [12], *Anabaena* [13], *Chlamydomonas reinhardtii* [14,15] and spinach [16]. In this paper, we report the three-dimensional structure of *T. brucei* Trx at 1.4 Å resolution. This is the first thioredoxin structure from a kinetoplastid organism, which branched off very early from the common eukaryotic lineage. The structure is overall similar to other thioredoxins but has a few unusual features that give new functional aspects for thioredoxins.z

2. Materials and methods

2.1. Expression, purification, crystallization

T. brucei Trx was expressed [8] and purified as described elsewhere [17]. Bipyramidal crystals of recombinant His-tagged *T. brucei* Trx were obtained by the hanging drop vapor-diffusion method at 293 K employing a sparse matrix screening method (Crystal Screen I, Hampton Research, CA, USA). Three μl protein solution (6,8 mg/ml in 250 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate, pH 7.2) was mixed with 3 μl reservoir solution (2 M sodium formate in 0.1 M sodium acetate pH 4.6) and allowed to equilibrate with 900 μl reservoir solution. Crystals of $350\times350\times400$ μm^3 were obtained within 4–7 days.

2.2. Data collection

Data collection was performed on beamline ID29 at ESRF in Grenoble, France, using an ADSC detector. Data were collected at 100 K on a crystal flashed-cooled in liquid nitrogen after a short dip in a cryo solution consisting of the crystallization solution with an addition of 30% glycerol. The crystals diffract to 1.4 Å and belong to the primitive orthorhombic space group $P2_12_12_1$. The data were indexed, integrated, scaled and merged using *dTREK [18]. The crystals have one protein molecule in the asymmetric unit, corresponding to a solvent content of 41%.

2.3. Structure determination and refinement

The structure was determined by molecular replacement using the program AMoRe [19], using a polyalanine model generated from reduced human Trx [12] (PDB entry: 1ERT) as a search model. This protein shows the highest degree of homology to the *Trypanosoma* protein with a 32% sequence identity. Initially, a round of simulated annealing was performed using the program CNS [20]. From the resulting maps, we were able to trace the polypeptide chain. Further refinement was carried out using the program Refmac5 [21]. The positions for all amino acids were checked using a symmetry omit map generated with CNS [20]. The final model contains 113 of the

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125 amino acids and 126 water molecules. Model geometry was checked with PROCHECK [22]. Model building was performed with O [23] and general crystallographic calculations with CCP4/CCP4i [24]. Coordinates have been deposited at the Protein Data Bank, and have the accession number 1R26.

3. Results

3.1. Quality of the structure

The structure of *T. brucei brucei* Trx carrying a 19-residue-long N-terminal extension with six His residues was solved by molecular replacement using the coordinates of reduced human Trx [12]. The structure has been refined at 1.4 Å resolution to an *R*-value of 16.8% (R_{free} 19.8%) with good stereochemistry (Table 1). There are no Ramachandran plot outliers [25]. All residues except for the first 11 of the N-terminal extension and the last residue at the C-terminus could be located in the electron density map. The side chains of Lys34, Glu37, Arg38, Lys42, Lys82 and Lys104, all located at the surface, have undefined electron density[1]. The side chains of Ser16, Ser65 and Ser80 occupy two different conformations.

3.2. Overall structure

The overall structure of *T. brucei* Trx is shown in Fig. 1. The general fold consists of a five-stranded β-sheet surrounded by four α-helices as it is the case in thioredoxins from other sources [26]. The loops between the secondary elements are short as in other thioredoxins. As expected from a sequence alignment (Fig. 2), the *T. brucei* Trx structure is similar to that of human Trx and 103 Cα-atoms can be superimposed with a root mean square deviation (RMSD) of 1.15 Å. It is also similar to the structure of *E. coli* Trx and in this case, 104 Cα atoms can be superimposed with an RMSD of 1.52 Å.

The second helix is broken into two halves (α2A and α2B) as in most other thioredoxins due to the presence of a Pro. However, in *T. brucei* Trx, the Pro is not at the position observed in most other thioredoxins, but occurs one residue later. The effect of the presence of Pro39 in breaking the helix is, however, the same in both cases. From mutant studies, it was concluded that Pro40 in *E. coli* is not essential for maintaining the redox function of thioredoxin but rather is required for the stability of the protein [27].

3.3. Active site

The active site of thioredoxins contains a redox active cysteine pair, which can be in oxidized disulfide form or in the reduced dithiol form. Conformational changes between the two forms are usually small and can mainly be described as a movement of the cysteine sulfur atoms away from each other [12,28]. The electron density of the *T. brucei* Trx structure shows that the cysteines do not form a disulfide (Fig. 3) but are in the reduced form in spite of the fact that no reductant was present in the crystallization solution. The disulfide bridge might have been reduced by the X-ray beam during data collection, as seen in other experiments using third generation synchrotron sources [29].

[1] The residue numbering in the paper refers to the sequences without the initial Met residue [8].

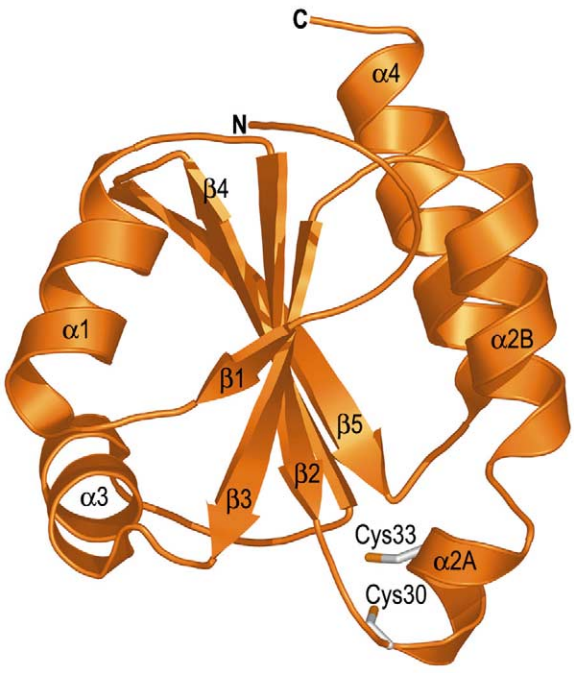

Fig. 1. The three-dimensional structure of *T. brucei* Trx. Schematic drawing showing the central pleated β sheet, with both parallel and antiparallel strands, surrounded by α helices. The redox active disulfide is located in a small cleft between the main body of the molecule and a protrusion in the protein at the N-terminus of the α2A helix. The figure was made using PyMOL [54].

Cys30 is exposed at the surface whereas Cys33 is in a more buried position. Thioredoxins normally contain a buried Asp behind the less exposed cysteine but in *T. brucei* Trx the corresponding position is occupied by Trp24. A water molecule is hydrogen bonded to the side-chain nitrogen of this Trp and the water molecule is further hydrogen bonded to the sulfur of Cys33, which is also hydrogen bonded to Thr26. Among the thioredoxin crystal structures containing a buried Asp behind the less exposed Cys, most structures have a water molecule hydrogen bonded to one of the oxygens on the carboxylate. *Anabaena* sp. 7120 thioredoxin also lacks the buried Asp and has a Tyr at the corresponding position [13]. Super-

Table 1

Data collection and refinement statistics	
Space group	$P2_12_12_1$
Resolution range (Å)	30–1.4 (1.45–1.4)
Unit cell (Å)	$a = 37.88$, $b = 51.72$, $c = 57.39$
Total observations	232 960
Unique reflections	22 807
R_{merge}[a] (%)	7.8 (51.5)
Redundancy	10 (10.4)
Data completeness (%)	99.6 (99.6)
$I/\sigma(I)$	5.8 (1.0)
R factor/R_{free}[b] (%)	16.8/19.8
RMSD bond (Å)	0.012
RMSD angle (°)	1.3
Average *B* factor (Å^2)	
Protein	16.0
Solvent	30.8

Values in parentheses are for the highest resolution shell.
[a] $R_{merge} = \Sigma_h\Sigma_i |I_{hi} - \langle I_h \rangle| / \Sigma_h\Sigma_i |I_{h,i}|$.
[b] *R* factor $= \Sigma ||F_o| - |F_c|| / \Sigma |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

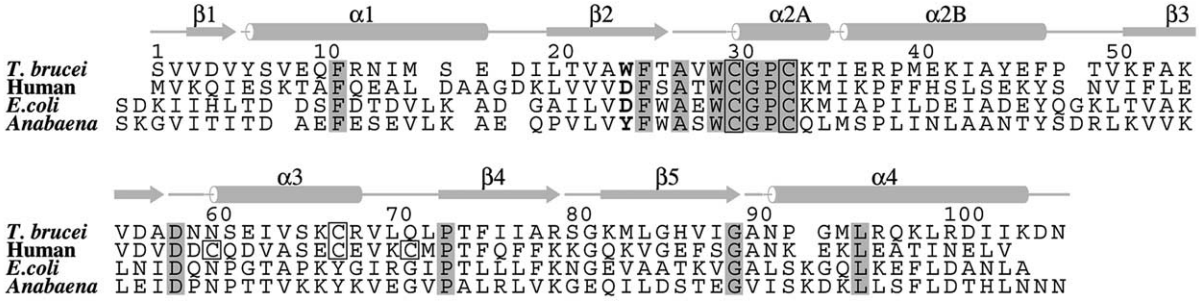

Fig. 2. Structure based sequence alignment of four different thioredoxins. Sequence numbering refers to the *T. brucei* Trx sequence without the initial Met residue. Residues conserved in all sequences are shown against gray background. Boxes enclose Cys residues. Trp24 and its aligned residues are shown in bold. The sequence identities between the *T. brucei* sequence (EMBL accession no. Q9NG23) and the sequences of thioredoxins from human (SwissProt: P10599), *E. coli* (SwissProt: P00274) and *Anabaena* (SwissProt: P20857) are 27%, 21% and 18%, respectively. The secondary structure for *T. brucei* Trx is given on top. The figure was made using Alscript [55].

position of the *Anabaena*, human and *T. brucei* Trx structures shows that the position of the water molecule bound to Trp in the parasite Trx and the water molecule bound to Asp in *E. coli* Trx correspond very closely to the position of the side-chain hydroxyl of the tyrosine in *Anabaena* Trx (Fig. 4).

3.4. Interaction area for redox partners

The active site contains a conserved Trp, which is present in the characteristic thioredoxin WCGPC sequence motif. This residue is at the surface of the active site and appears to interact with thioredoxin's redox partners. In most thioredoxin structures, this Trp has a similar position as in the original *E. coli* Trx structure forming a flat surface close to the active site [11]. In *T. brucei* Trx, the Trp is flipped out and interacts with a neighboring protein molecule in the crystals (Fig. 5). Exposed but slightly different positions of the Trp have been reported for one crystal form of the spinach thioredoxin-f [16] and for two mutant *E. coli* Trx [30,31]. Even if the unusual position is influenced by crystal interactions, the *T. brucei* Trx structure demonstrates that this Trp can adopt different positions to allow optimal interactions with thioredoxin's redox partners.

Other parts of the structure close to the active site are similar to those in other thioredoxins. The parasite protein has the *cis*-Pro73 implemented in binding of redox partners, and the neighboring conserved Gly89 that allows close interactions with other proteins [32]. The hydrophobic area suggested to be involved in binding thioredoxin to other proteins is hydrophobic also in *T. brucei* Trx with Gly31, Pro32, Leu70, Pro73, and Ile88, Gly89, and Ala90. At the outer rim of the hydrophobic area, the exposed parts of the structure are different and change the charge distribution.

The calculated isoelectric point of *T. brucei* Trx is unusually high, 8.5 [8]. All other known thioredoxins have p*I* values between 4.5 and 5.0 except the mitochondrial thioredoxin III of *Saccharomyces cerevisiae*, which is also a highly basic protein [33]. The basic residues of *T. brucei* Trx are distributed all over the surface of the molecule with the exception of the area around the active site that has been implemented in interactions with the thioredoxin redox partners. This area is devoid of positively charged residues as for other thioredoxins.

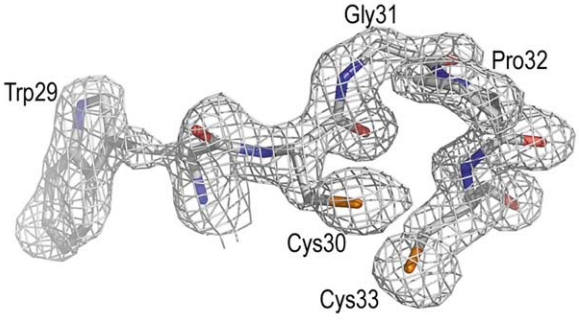

Fig. 3. $2F_o-F_c$ map, contoured at 1σ of the redox active WCGPC motif of *T. brucei* Trx. Although no reductant was present in the crystallization solution, the cysteines do not form a disulfide but are present in the reduced form. The distance between the cysteine sulfurs measures 2.66 Å. The figure was made using PyMOL [54].

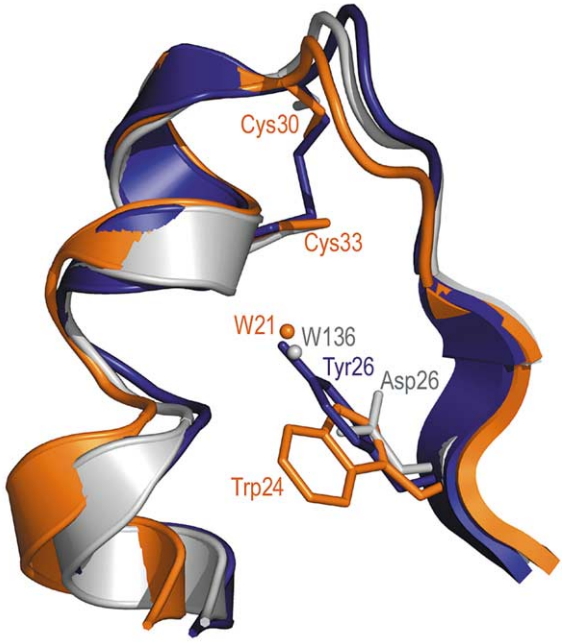

Fig. 4. Superposition of the active sites from *T. brucei* (orange), *Anabaena* (blue) (pdb entry: 1THX) and human (gray) (pdb entry: 1ERT) Trx. The overlay shows that the position of a water molecule bound to Trp in *T. brucei* Trx, Asp26 in *E. coli* Trx corresponds to the position of the side-chain hydroxyl of the tyrosine in *Anabaena* Trx. The figure was made using PyMOL [54]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

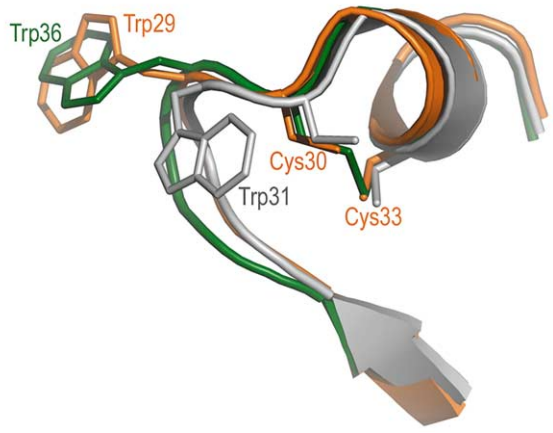

Fig. 5. Superposition of the active site tryptophans from *T.brucei* (orange), spinach (green) (pdb entry: 1F9M) and human (gray) (pdb entry: 1ERT). Trp29 is flipped out compared to the position in most thioredoxins, exemplified by Trp31 in human Trx. The figure was made using PyMOL [54]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Dimer formation

Prolonged storage of recombinant *T. brucei* Trx can lead to the formation of covalent dimers. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the stored protein sample without thiols in the sample buffer shows an additional band with a molecular mass of about 28 000, whereas under reducing conditions a single protein band of 13 000 is obtained [8]. In *T. brucei* Trx Cys67 is the only cysteine in addition to the active site couple. Mammalian thioredoxins possess three additional conserved cysteine residues corresponding to cysteine 62, cysteine 69 and cysteine 73 in the human protein. Oxidized and reduced human Trx form inactive dimers, which contain an intermolecular disulfide bridge between Cys73 from each monomer [34].

Cys67 in *T. brucei* and *L. major* Trx corresponds to Cys69 in the human protein. Whereas Cys73 in the human Trx is exposed at the surface of the protein and thus suitable for forming an intermolecular disulfide, Cys69 in the human protein and Cys67 in *T. brucei* are rather buried in the protein structure. It is located on the side of an α-helix (α3) partly exposed to a shallow cleft along the side of the molecule, about 17 Å from the active site cysteines. It appears that formation of an intermolecular disulfide should not be favorable and require some rearrangement like turning around of the helix. Yeast thioredoxin III also contains a cysteine residue at the equivalent position but dimer formation has not been observed in that case [33]. Although not available for protein dimer formation, Cys69 in human Trx is accessible to small reagents and has recently been shown to be *S*-nitrosylated [35]. It is thus not clear if Cys67 or possibly Cys30 of the active site, which is more exposed, is involved in intermolecular dimer formation in the parasite protein. Even though in the crystal structure, Cys67 of *T. brucei* Trx is only partly exposed to the solvent the residue may be involved in disulfide formations. Western blot analysis of the recombinant wild type enzyme in the absence of any reducing agent results in a prominent additional protein band that probably represents a dimer and weak higher bands. In contrast, the Cys67Ser mutant shows only a faint dimer band and no signals at higher masses. It is, however, not clear if these intermolecular protein species occur physiologically or are only formed when the recombinant protein gets slightly denatured.

4. Discussion

T. brucei Trx is functionally similar to other thioredoxins [8]. It is reduced by human Trx reductase as are human and *E. coli* Trx and the three proteins behave very similar in their insulin reduction capacity. Interestingly, *T. brucei* and *L. major* Trx lack the highly conserved Asp26, which in *E. coli* Trx has been shown to play a crucial role for catalytic activity. Instead, there is a Trp at the equivalent position with a bound water molecule pointing towards the active site cysteines. The function of this buried Asp has been thoroughly investigated [36–53]. Mutation to an Ala in *E. coli* Trx decreased the k_{cat}/K_m value by a factor of 10 in the reaction with thioredoxin reductase and a similar decrease in the activity as a disulfide reductase [41]. Furthermore, the mutant *E. coli* protein had a drastically lowered ability to serve as a hydrogen donor for ribonucleotide reductase [49]. *T. brucei* Trx is an excellent substrate of human Trx reductase, and like *E. coli* Trx is able to deliver the electrons for *T. brucei* ribonucleotide reductase [8,17]. These findings indicate that an acidic residue at this position is not essential for catalysis.

Thioredoxins participate in redox reactions by catalyzing thiol-disulfide exchanges. In the reductive reaction, the first, more exposed cysteine of the WCGPC motif acts as nucleophile attacking the disulfide of the target protein. The resulting thioredoxin-target mixed disulfide is finally attacked by the more buried second cysteine resulting in a thioredoxin disulfide. In *E. coli* and human Trx, the pK_a of the buried cysteine is high, values of 9.9 and higher have been reported [40–42]. In the second part of the reaction, the nucleophilicity of this cysteine is thought to be increased by the Asp, which has been suggested to take up a proton from the thiol [38,39,42]. Most probably, the buried Asp acts via a water molecule since the Cys–Asp distance is 6.5 Å [42]. The crystal structure of *T. brucei* Trx shows that the ordered water molecule bound to Trp is in roughly the same position as water molecules in human and *E. coli* Trx (Fig. 4).

The water molecules in *T. brucei* Trx are not polarized to the same extent and should not have the same possibilities to act as part of a protonation/deprotonation system. Lys57 in *E. coli* Trx, which is close to Asp26, influences the effect of the D26A mutation on catalytic efficiency [41]. The combination of a D26A mutation with a mutation of Lys57 to a Met reduces the catalytic activity even further. *T. brucei* Trx has a Lys at the equivalent position as in *E. coli* Trx and in the parasite protein, this residues may influence the pK_a of the water bound to Trp which is about 6 Å away. The Lys side chain may swing down and come closer to the active site during catalysis. In *Anabaena* sp. 7120 Trx, the residue corresponding to the buried Asp is a tyrosine with the side-chain hydroxyl in a similar position as the water molecule in *T. brucei* Trx. The pK_a of the Tyr hydroxyl group in *Anabaena* Trx may be modified as the water molecule in *T. brucei* Trx by the Lys in equivalent position.

From the different thioredoxin structures that are now available, the common theme appears to be a water molecule or an OH group of a tyrosine side chain located close to the

more buried cysteine of the active site and that neighboring residues assist in modulating the pK_a:s to optimize catalysis.

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