Tryparedoxins from *Crithidia fasciculata* and *Trypanosoma brucei*

PHOTOREDUCTION OF THE REDOX DISULFIDE USING SYNCHROTRON RADIATION AND EVIDENCE FOR A CONFORMATIONAL SWITCH IMPLICATED IN FUNCTION*

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Tryparedoxin (TryX) is a member of the thioredoxin (TrX) fold family involved in the regulation of oxidative stress in parasitic trypanosomatids. Like TrX, TryX carries a characteristic Trp-Cys-Xaa-Xaa-Cys motif, which positions a redox-active disulfide underneath a tryptophan lid. We report the structure of a *Crithidia fasciculata* tryparedoxin isoform (CfTryX2) in two crystal forms and compare them with structures determined previously. Efforts to chemically generate crystals of reduced TryX1 were unsuccessful, and we carried out a novel experiment to break the redox-active disulfide, formed between Cys-40 and Cys-43, utilizing the intense x-radiation from a third generation synchrotron undulator beamline. A time course study of the S–S bond cleavage is reported with the structure of a TryX1 C43A mutant as the control. When freed from the constraints of a disulfide link to Cys-43, Cys-40 pivots to become slightly more solvent-accessible. In addition, we have determined the structure of *Trypanosoma brucei* TryX, which, influenced by the molecular packing in the crystal lattice, displays a significantly different orientation of the active site tryptophan lid. This structural change may be of functional significance when TryX interacts with tryparedoxin peroxidase, the final protein in the trypanothione-dependent peroxidase pathway. Comparisons with chloroplast TrX and its substrate fructose 1,6-bisphosphate phosphatase suggest that this movement may represent a general feature of redox regulation in the trypanothione and thioredoxin peroxidase pathways.

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Tryparedoxin (TryX) is a thiol-disulfide oxidoreductase found in parasitic trypanosomatids belonging to the order Kin-...
**Structure and Reactivity of Tryparedoxin**

![Image](https://example.com/figure1.png)

**FIG. 1.** The trypanothione peroxidase pathway. NADPH supports reduction of the disulfide oxidoreductant TryX, which processes the polyamine-peptide conjugate trypanothione disulfide (TSH) to maintain high levels of the dithiol form (TSH$_2$). TryX is reduced by TSH$_2$ and in turn reduces TryP, the enzyme that catalyzes the reduction of peroxides.

TryX1 and structures of the disulfide form and chemically reduced TryX2 recently published (9). Unable to crystallize TryX as a homogeneous free dithiol form, we sought to generate this structure by photoconversion of the redox-active disulfide of CFTRYX1 using the intense x-ray beam available from an undulator beamline at the European Synchrotron Radiation Facility (ESRF). As a control for this novel experiment and to study the effect of removing the disulfide linkage, Cys-43 was mutated to alanine, and the structure (CfTryX-C43A) was determined to 1.30-Å resolution. We also determined the structure of Trypanosoma brucei tryparedoxin (TbTryX) at 2.3-Å resolution, which presents an active site significantly different from any other TryX structure. Analysis of the interactions between TbTryX and TbTryX-A and a symmetry-related molecule suggests structural alterations that may be relevant to the interaction between TryX and the partner peroxidase, TryP. Based on crystal structures of a chloroplast Trx (10), one of its redox partners, fructose-1,6-bisphosphate phosphatase (11), and our own sequence analyses, we propose that the conformational lability of the tryptophan lid may contribute to specific redox events.

**MATERIALS AND METHODS**

**Cloning, Expression, and Purification of Recombinant Tryparedoxins**—The gene coding for CfTryX2 was obtained by PCR amplification of genomic DNA of *C. fasciculata* H6b TryX2 open reading frame (GenBank accession number AP056880) using the oligonucleotides 5′-CAT ATT TAT CAC ACC CTT CTC TAC-3′ for the sense strand and 5′-CAT GGA TCG TTA CTT CAC GTC CAC GGT GGG-3′ for the antisense strand. The sense strand oligonucleotide contains an NdeI cloning site (underlined) incorporating an initiation codon (bold), whereas the antisense oligonucleotide contains a BamHI restriction site (underlined) downstream of the antisense stop codon (bold). The PCR products were blunt-end ligated into the SmaI site of pUC18 (SureClone, Amersham Biosciences), and then the inserts were excised by restriction enzyme digestion and ligated into the pET-15b vector (Novagen), creating plasmids pET-TbTryX and pET-CfTryX2. The mutagenesis of cysteine to alanine at residue 43 in *C. fasciculata* tryparedoxin-I (CfTryX-C43A) was performed using the method described by Deng and Nickoloff (12) with the Chameleon kit (Stratagene). The plasmid pET-CfTryX1 (13) provided the matrix, and the oligonucleotide was 5′-TGG GCC CGC GCC GCC TTC ACG-3′ for the antisense strand. The sense strand oligonucleotide carried an NdeI cloning site (underlined) incorporating an initiation codon (bold), whereas the antisense strand oligonucleotide carried an NdeI restriction site (underlined) just downstream of the antisense stop codon (bold). The integrity of the cloned genes was confirmed by sequencing.

All recombinant proteins were expressed in *Escherichia coli* strain BL21 (DE3). Expression and purification protocols followed those published by Alphey et al. (13) and involved the use of metal ion affinity chromatography to exploit the presence of the N-terminal histidine tag, which was introduced by using the pET-15b vector and which was subsequently removed by cleavage with thrombin (Amersham Biosciences). Protein concentration was determined spectrophotometrically at 280 nm using a theoretical extinction coefficient of 38030 M$^{-1}$ cm$^{-1}$ (14), and purity was evaluated using SDS-PAGE and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Crystallization, Data Collection, and Data Processing—Crystals were grown using the hanging drop vapor diffusion setup, and diffraction data were processed, reduced, and scaled using the HKL (15) and CCP4 suite of programs (see Table I) (16). Crystals of CfTryX1 and CfTryX-C43A were obtained using the published conditions (13). Two tetragonal crystal forms (A and B) of CfTryX2 were obtained. Form A precipitates as reds and orange crystals in 0.1 M sodium HEPES, pH 7.5, 5 mM dithiothreitol, and 60 mM ammonium sulfate. These crystals display space group P4$_2$.2$_2$ with unit cell dimensions of $a = b = 111.7, c = 56.5$ Å and are isomorphous to samples studied by Hofmann et al. (9). Form B crystals displayed a biphasic morphology and grew from solutions of ~10 mg ml$^{-1}$ protein, 500 mM sodium citrate, 30 mM sodium HEPES, pH 7.5, 5 mM dithiothreitol. They are in space group P4$_2$.2$_2$ with unit cell dimensions of $a = b = 114.3, c = 102.0$ Å. Single crystals of both forms were cryo-protected by soaking in crystallization mother liquor containing either 15% (form A) or 10% (form B) of glycerol prior to transfer in a nitrogen gas stream at ~170 °C. A single crystal was used on the bending magnet H5C monomethyl ether 2000 and then flash-cooled at 100 K for form A and 170 K for form B. The crystal data collection was carried out at $\lambda = 0.977$ Å to $d_{min} = 1.5$ Å with a MARCCD133 detector in a single sweep totaling 90° of oscillation in 0.5° steps. For form B, a single crystal was mounted on the ESRF undulator beamline ID14-EH2, and data were collected at $\lambda = 0.933$ Å using an ADSC QUANTUM4 detector. Despite the relatively large size of the crystal used for data collection (~250 × 150 × 150 μm$^3$), diffraction maxima were only visible to ~2.2-Å resolution, and these were only apparent after a relatively long exposure time of 45 s/0.5° oscillation. Radiation damage was evident after 75 images, and the crystal was translated such that a fresh section was exposed to the x-ray beam and a further 44 0.5° images were collected. Both batches of data were processed and scaled together and are presented complete to 2.35 Å.

For the time course experiment on CfTryX1 and the analysis of CfTryX-C43A, crystals were cryo-protected with 40% polyethylene glycol mononal monomer ether 2000 and then flash-cooled at ~170 °C. Data were collected on ID14-EH2 (λ = 0.933 Å) using the STRATEGY program (17) to determine the angular range for collection. For the time course experiment, a series of data sets were measured over the same oscillation range. Data sets A and B were consecutive and measured to a resolution of 1.5 Å. An intermediate exposure of 780 s, which corresponds to the total exposure time for measurement of a data set, was made while rotating the crystal, although no data were actually recorded. Data set C was then measured, and it was noted that the sample had been diffracted to 1.7-Å resolution. Data were collected on ID14-EH2, and the crystal after data set CfTryX-C was judged too vague to warrant further useful data collection. The three data sets and the models derived from each are labeled CfTryX-A, -B, and -C, respectively.

Clumps of small monoclinic plate-like crystals of TbTryX grew over a period of weeks in drops made by mixing a solution of 10 mg ml$^{-1}$ protein, 50 mM HEPES, pH 7.5, with the reservoir solution of 30% polyethylene glycol 4000, 100 mM sodium acetate, pH 4.6, 200 mM ammonium acetate. The crystals display space group $P_2_1$, with unit cell dimensions of $a = 30.6, b = 31.5, c = 56.9$ Å, $\beta = 93.4$°. The asymmetric unit comprises a monomer with ~30% solvent content and $V_m$ of 1.8 Å$^3$ Da$^{-1}$. A small fragment (~200 × 50 × 10 μm$^3$) was removed from a crystal to obtain a radiographic determination of the reservoir solution adjusted to include 20% 2-methyl-2,4-pentanediol, and then transferred into a stream of nitrogen gas at ~170 °C. Data were measured to 2.3-Å resolution on a Rigaku rotating anode ( copper Ka $\lambda = 1.5418$ Å)-Raxius IV image plate system.

**Structure Solution and Refinement**—The initial phases for both CfTryX and CfTryX-I structures were calculated using the molecular replacement (MR) technique as implemented in the CNS software package (18) and data in the resolution ranges 15–3 Å for form A and 20–4 Å for form B. The structure of CfTryX1 (Protein Data Bank code 1QK8) (5) was tripped of all solvent molecules was used as the search model. After this procedure, it was clear that both crystal forms contain two molecules/asymmetric units, which results in a calculated Matthews coefficient (19) of 2.3 and 4.4 Å$^3$ Da$^{-1}$ for forms A and B, respectively. The unit cell of form A has a much lower bulk solvent volume, 46%, than the 72% observed for form B, and this helps to explain the different diffraction limits of the two forms. For crystal form A, the initial MR phases
obtained were extended to the resolution limit of the data set, 1.5 Å, using a combination of non-crystallographic symmetry averaging, solvent flattening, and histogram matching as implemented in the program DM (20) after first calculating reliable \( \sigma_A \)-weighted figures-of-merit (FOM) (21) for the MR phase set. The resulting electron density map (Fobs, \( \sigma_{
abla{obs}} \), FOMreal) was of excellent quality, and a model was constructed using the program ARP/wARP (22). Refinement was then carried out using CNS interspersed with rounds of rebuilding in QUANTA (Accelrys) during which solvent molecules were included. To complete the refinement, a final round was performed using the program REFMAC (23) in which the two sulfur atoms in the active site were refined with anisotropic temperature factors. For crystal form B, a similar protocol to that described for form A was used to extend the MR phases to the diffraction limit of the data set. A model was built manually with QUANTA, and refinement carried out in a similar manner to that for form A.

The CTryX-A, -B, and -C structures are isomorphous with the disulfide form of CTryX1 (5), which provided the starting model for refinements using REFMAC. Following rigid body refinement, additional rounds of positional and B-factor refinement combined with graphics fitting (O) (24) were carried out. Water molecules were added using ARP/wARP. Once the R-factor and R-free had dropped from about 40 to 25%, anisotropic B-factor refinement was introduced.

The structure of BTryX was solved by MR (AMoRe) (25) using a poly-Ala structure of CTryX1 as the search model. A clear solution was obtained that, after rigid body refinement, gave an R-factor of 48% and a correlation coefficient of 0.56 for data in the range of 30–2.3-Å resolution. Density modification (DM) improved the electron density map that was then used for model building. Simulated annealing molecular dynamics (to reduce model bias), least-squares refinement with CNS, together with the placement of water molecules completed the analysis. Approximately 5% of each data set was set aside to provide an R-free to monitor the progress of all refinements (26), whereas PROCHECK (27) and OOPS (28) were used to assess model geometry. Further experimental details are provided (see Table I) and in the Protein Data Bank depositions.

RESULTS AND DISCUSSION

Overall Structures—The tryparedoxin structure is constructed around a seven-stranded twisted β-sheet with parallel and antiparallel alignments. This sheet starts with a β-hairpin formed by β1 and β2, and thereafter a β3-α1-β4-α2-β5 combination. A final β5-hairpin between β6 and β7 completes the structure. The active site Try-Cys-Pro-Pro-Cys motif is located between strand β3 and the N terminus of helix α1 (Fig. 2a). A structure-based sequence alignment of the three highly conserved tryparedoxins used in this study is shown in Fig. 2b.

The Second Tryparedoxin Isoform of C. fasciculata (CTryX2)—The structure of CTryX2 was determined independently in two crystal forms, each of which presents two molecules/asymmetric unit. A pairwise least-squares superposition of all Ca atoms for the four molecules gave root mean square deviation values that ranged from 0.2 to 0.5 Å, and the results are similar whether we used a MR protocol or the anomalous dispersion from sulfur atoms (29) to provide the initial phase information. When comparing our MR-derived structures with those of CTryX2 determined by Hofmann et al. (9), least-squares superposition values of between 0.2 and 0.6 Å were observed. These values indicate close agreement of the second isoform structures irrespective of how or where they were determined or the redox state of the protein (see below). The structures reported here confirm that, when compared with the structure of CTryX1, the helices α1 and α2 are closer to each other in the structure of CTryX2, allowing the formation of a hydrogen bonding network around the less solvent-exposed sulfur atom in the active site S–S bridge (9). Both crystal forms of CTryX2 were grown from solutions containing dithiothreitol. The electron and difference density maps were suggestive of a time and space average of S–S bridge oxidized and reduced states. The refined S–S distances are 2.9 and 2.8 Å for the two molecules in form A and 3.2 and 3.0 Å for the two molecules in form B.

During the time course experiment, from CTryX-A to CTryX-C (Table I), we noted general symptoms of radiation damage to the sample; resolution decreased, the B factors for
structure and Reactivity of Tryparedoxin

Table I
Data collection, refinement, and model geometry statistics

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<tr>
<th>Structure</th>
<th>$Tb$TryX</th>
<th>$Cf$TryX2A</th>
<th>$Cf$TryX2B</th>
<th>$Cf$TryX-A</th>
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<th>$Cf$TryX-C</th>
<th>$Cf$TryX-C43A</th>
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<td>1.7</td>
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<td>29.8 (9.6)</td>
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<td>4.5 (35.4)</td>
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<td>5.2 (53.8)</td>
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<td>6.1 (60.5)</td>
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<td>20.7/23.5</td>
<td>19.6/21.3</td>
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<td>20.3/26.6</td>
<td>21.0/27.6</td>
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<td>0.013/1.4</td>
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a) Denotes the two molecules per asymmetric unit.

Minor Structural Perturbation Results from Disulfide Breakage—In contrast to previous observations of radiation-induced damage to structural disulfides (31), we do not see deterioration in electron density for the S-S atoms of Cys-40 or Cys-43 (Fig. 3). As the disulfide breaks, the Cys-40 side chain moves toward solvent, and the flanking residues Trp-39 and Pro-41 move slightly up and out (not shown). Structures of TrX in the reduced form have been determined (Ref. 10 and references therein) and also $Cf$TryX2 (9), and similar observations have been made.

In addition to breaking the disulfide using synchrotron radiation, it was anticipated that the C43A mutant would allow Cys-40 to adopt a position similar to that occupied when the protein is reduced. The mutant structure correlates well with $Cf$TryX-C (Figs. 4 and 5). The C43A mutation also produced small shifts within the active site involving Ser-36 and Tyr-80. In $Cf$TryX1, the side chain of Ser-36 is held in position through interactions with the immobile Cys-43, but in the mutant, Ala-43 no longer has a stabilizing effect on Ser-36. The side chain of Ser-36 adopts a different position, forming a hydrogen bond with the hydroxyl of Tyr-80, which has moved some 1.2 Å from the native structure to stabilize the new arrangement (Fig. 4).

The overall conclusion from the radiation-induced disulfide

breakage and mutant structure analyses is that the active site of TryX appears to be relatively unperturbed by the redox state. Minor structural changes occur that serve to make Cys-40 S-S slightly more reactive to interact with the cognate partners. This is similar to what has been observed in structures of the dithiol form of TrX by itself (10) or in complex with thioredoxin reductase (34). Capitanii et al. (10) studied the variation in oxidation state of the disulfide in a chloroplast TrX, but in contrast to our study, they first measured data on the reduced form of TrX, and then over a period of almost 2 days, using an in-house x-ray source and crystals at 4 °C, were able to isolate a data set that indicated that the disulfide had reformed without any large perturbation to the active site.

Structure of $Tb$TryX and a Model for the Interaction with Try—The high degree of sequence conservation (Figs. 2 and 5) and similar biophysical properties (35, 36) of $Tb$TryX as compared with the C. fuscata tryparedoxins suggest that the three-dimensional structures should be similar. An overlap of $Cf$TryX1, $Cf$TryX2, and $Tb$TryX highlights the structural homology of tryparedoxins (Fig. 5). The root mean square deviation for 139 Ca atoms in common between $Tb$TryX and $Cf$TryX1 is 0.8 Å. The largest differences are observed in the N-terminal region, in particular at the turn between β1 and β2. This is on the opposite side of the molecule from the redox-active site.

The similarities extend beyond the overlay of Ca atoms to the residues that constitute the hydrophobic core of the protein. Thirteen aromatic residues in $Cf$TryX1 (tyrosines 34, 54, and 80, tryptophans 70 and 86, phenylalanines 32, 35, 46, 53, 63, 77, 91, 104) are strictly conserved in the three tryparedoxins (Fig. 2b). In addition, phenylalanines at positions 33, 57, 67, and 81 of $Cf$TryX1 are replaced by Leu-33, His-57, Leu-67, and Tyr-81 of $Tb$TryX. The residues that cluster around the redox-active site are also highly conserved between $Cf$TryX and $Tb$TryX. Indeed those residues that were first implicated in $Cf$TryX1 binding tripanothione (5), namely Trp-39, Pro-41, Pro-42, Arg-44, Trp-70, Asp-71, Glu-72, Lys-83, Ile-109, Pro-
110, and Arg-128, are strictly conserved. Hofmann et al. (9) were able to confirm that the last three residues did in fact interact with the ligand in a mutant CfTryX2 glutathionylspermidine complex. This suggests that similar molecular features determine the association with the TryX redox partners in both *Crithidia* and *Trypanosoma*.

An overlay of residues that comprise the active site of TbTryX and CfTryX1 reveals Trp-39 in a different position in TbTryX than in the CfTryX structures (Fig. 6). In CfTryX1, Trp-39 is placed over the redox disulfide, and Ne1 donates a hydrogen bond to the carbonyl of Trp-70 (5). In TbTryX, Trp-39 adopts a different rotamer and is flipped out at the surface of *Trypanosoma*.
the protein with Nε1 forming a hydrogen bond with Asp-76 Oε2 from a symmetry-related molecule (not shown). In the TbTryX crystal structure, a symmetry-related molecule is positioned such that the Val-59-Ala-60-Lys-61 segment is placed in the cleft at the redox-active site. The valine side chain fills the site, which in CfTryX is occupied by the Trp-39 lid, apparently forcing the tryptophan to adopt a different conformation. The alanine methyl group points toward the redox-active disulfide, and the lysine side chain is directed away from the disulfide.

This alternative conformation of the tryptophan lid has been noted previously in TrX and attributed to crystal packing effects, in the case of a mutant E. coli TrX (37), or due to sequence effects in a chloroplast TrX (10). The adjustment serves to expose the N-terminal cysteine of the redox-active disulfide; therefore, we decided to investigate whether such conformational pliability is relevant to the function of TryX or TrX when they interact with their cognate peroxidases and other proteins. We first considered TryP, a 2Cys-peroxiredoxin, well characterized biochemically (3) and for which a crystal structure of the reduced form has been determined (2).

All peroxiredoxins carry an essential N-terminal cysteine, often in a tetrapeptide Val-Cys-Pro-Thr motif. The 2Cys-peroxiredoxins have, in addition, a conserved C-terminal cysteine in a Val-Cys-Pro tripeptide motif, which interacts with the TryX Trp-Cys-Pro-Pro-Cys redox center. Reduced TryX interacts with oxidized TryP, but the only structure available for TryP is that of the reduced form (2). However, the structure of the homologous and oxidized form of the 2Cys-peroxiredoxin HBP23 has been determined (38). Since TryP and HBP23 share almost 60% sequence identity and are closely related in three-dimensional structure (2), we superimposed the C-terminal Val-Cys-Pro motif of one monomer of the dimeric HBP23 onto the symmetry-related Val-Ala-Lys tripeptide of TbTryX. The
Structure and Reactivity of Tryparedoxin

Ca fit was with an root mean square deviation of 0.3 Å. Although this can only be a crude model, we note that the side chain of HBP23 Val-172 adopts a different orientation as compared with the TbaTryX Val-59 but that HBP23 Cys-173 is turned directly toward the redox-active Cys-40 of the tryparedoxin (Fig. 6b) with the Sزي atoms 3.3 Å apart. The model suggests that when TryX associates with TryP, a repositioning of the trypanothion lid might occur in conjunction with other molecular features such as the electrostatic interactions discussed by Hofmann et al. (3) to facilitate interaction of the redox components.

If the combination of a pliable tryptophan and a valine-cysteine dipeptide is indeed important for the tryparedoxin-peroxidase interaction, we reasoned that it might also contribute to thioredoxin-protein associations. Thioredoxin peroxidases are homologous to TryP, and the Val-Cys-Pro motif is strictly conserved (3), which would be consistent with our hypothesis. Also, the truncated form of chloroplast TrX shows the partner for this TrX is chloroplast FBPase, for which a structure is available (11). Chloroplast TrX regulates the activity of FBPase by reduction of the disulfide formed between Cys-153 and Cys-173 (pea FBPase numbering). Cys-153 occurs in the sequence Val-Cys-Gln-Pro-Gly located on a flexible loop, whereas Cys-173 occurs in an α-helix. A search of the EXPASY data base (ca.expasy.org) indicated that the pentapeptide segment, with valine preceding the redox-active cysteine, is strictly conserved in chloroplast FBPase. The observations hint at a role for a Val-Cys combination to interact with a conformationally labile tryptophan to assist TryX and Trx pass on their reducing equivalents. Definitive proof would require a structure of the functional complexes, and we are trying to obtain this for TryX-TryP.

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