Structural insights into the catalytic mechanism of *Trypanosoma cruzi* GPXI (glutathione peroxidase-like enzyme I)

Shreenal PATEL*, Syeed HUSSAIN*, Richard HARRIS*, Sunita SARDIWAL*, John M. KELLY†, Shane R. WILKINSON‡, Paul C. DRISCOLL*‡§ and Snezana DJORDJEVIC*†

*Institute of Structural and Molecular Biology, Division of Biosciences, University College London, London WC1E 6BT, U.K., †Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, U.K., ‡School of Biological and Chemical Sciences, Queen Mary University of London, London E1 4NS, U.K., and §Division of Molecular Structure, Medical Research Council National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

Current drug therapies against *Trypanosoma cruzi*, the causative agent of Chagas disease, have limited effectiveness and are highly toxic. *T. cruzi*-specific metabolic pathways that utilize trypanothione for the reduction of peroxides are being explored as potential novel therapeutic targets. In the present study we solved the X-ray crystal structure of one of the *T. cruzi* enzymes involved in peroxide reduction, the glutathione peroxidase-like enzyme TcGPXI (*T. cruzi* glutathione peroxidase-like enzyme I). We also characterized the wild-type, C48G and C96G variants of TcGPXI by NMR spectroscopy and biochemical assays. Our results show that residues Cys48 and Cys96 are required for catalytic activity. In solution, the TcGPXI molecule readily forms a Cys48–Cys96 disulfide bridge and the polypeptide segment containing Cys96 lacks regular secondary structure. NMR spectra of the reduced TcGPXI are indicative of a protein that undergoes widespread conformational exchange on an intermediate time scale. Despite the absence of the disulfide bond, the active site mutant proteins acquired an oxidized-like conformation as judged from their NMR spectra. The protein that was used for crystallization was pre-oxidized by tert-butyl hydroperoxide; however, the electron density maps clearly showed that the active site cysteine residues are in the reduced thiol form, indicative of X-ray-induced reduction. Our crystallographic and solution studies suggest a level of structural plasticity in TcGPXI consistent with the requirement of the atypical two-cysteine (2-Cys) peroxiredoxin-like mechanism implied by the behaviour of the Cys48 and Cys96 mutant proteins.

Key words: Chagas disease, enzyme mechanism, NMR spectroscopy, peroxidase, X-ray crystallography.

INTRODUCTION

Across the tropics over 20 million people are infected by the protozoan parasites *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania*, the causative agents of African sleeping sickness, Chagas disease and leishmaniasis respectively [1]. With little prospect of protective vaccines for these diseases, drugs are the only viable option to ameliorate the effects of these infections. For Chagas disease, treatment is based on the nitroheterocyclic drugs nifurtimox and benznidazole, which require enzyme-mediated activation to render them trypanocidal. However, use of these compounds is problematic; they can cause serious side effects and parasites refractory to drug treatment are commonly encountered [2,3]. The search for new therapeutics and potential therapeutic targets in *T. cruzi* is underpinned by extensive research on basic metabolic systems that are essential for parasite infectivity and viability, focusing predominantly on those pathways that are unique to *T. cruzi*. One of the major differences between trypanosomes and their mammalian hosts is that the former utilize trypanothione (*N*8-*N*8-bisglutathionylspermidine) as their low-molecular-mass cytoplasmic redox agent. In *T. cruzi*, trypanothione is maintained in its reduced form (dihydrotrypanothione) by the NADPH-dependent flavoenzyme trypanothione reductase. Dihydrotrypanothione subsequently provides reducing equivalents to hydroperoxides via several possible routes, including via GSH and GPXs (glutathione-dependent peroxidases), tryparedoxin (a thiol-peroxidin-like protein) and peroxidoxins, or ascorbate and ascorbate-dependent haemoperoxidase (see Supplementary Figure S1 available at http://www.BiochemJ.org/bj/425/bj4250513add.htm) [4]. The crystal structures of some of the components of the *T. cruzi* antioxidant pathway have been determined, including that of trypanothione reductase (PDB code 1AOG/1BZL) [5,6] and the typical two-cysteine (2-Cys) cytosolic peroxiredoxin (PDB code 1UUL) [7].

The peroxiredoxin and GPX family of enzymes have been extensively characterized in various organisms including in mammals and *Trypanosoma* [4,7–14]. However, the discovery of peroxidases in trypanosomes with sequence similarities to mammalian glutathione-dependent phospholipid hydroperoxidases was unexpected, as no glutathione-reductase activity is present in these organisms [9]. Furthermore, GSH concentrations in trypanosomes are reportedly rather low [17]. Considering the low apparent affinity of trypanosomal GPXs for GSH (the *Km* is in the millimolar range), the physiological function of these enzymes was difficult to ascertain [15]. Furthermore, it was demonstrated that one of these enzymes, TcGPXI (*T. cruzi* glutathione peroxidase-like enzyme I), can be reduced by *T. cruzi* tryparedoxin I, resulting in an 8–15-fold higher peroxidase activity compared with that obtained in a GSH-dependent reaction [16]. An even higher enhancement in activity with these different electron donors was observed.
for the orthologous enzyme TbPxIII (Trypanosoma brucei peroxidase III), consistent with an emerging view that many of the cysteine-dependent GPX-like molecules utilize thioredoxin-like proteins for regeneration of their reduced state [17]. RNAi (RNA interference) experiments identified TbPxIII as essential for the viability of this parasite [18,19]. Although similar RNAi experiments could not be performed in T. cruzi, as this organism lacks components of the RNAi pathway, increasing the level of TcGPXI through overexpression led to an enhanced resistance to H₂O₂ and t-butyl hydroperoxide confirming the important role of these enzymes in maintaining pathogen viability [15–16,20].

In the present paper we report a combined NMR and X-ray crystallographic analysis of TcGPXI. Structural studies and enzymatic assays of the wild-type enzyme, as well as TcGPXI proteins where residues Cys⁴⁸, Cys⁷⁷ and Cys⁸⁶ have been separately mutated, provide strong evidence for the requirement of both Cys⁴⁸ and Cys⁸⁶ for enzymatic activity. The results suggest that TcGPXI adopts an intramolecular disulfide bond in the oxidized state thus utilizing a mechanism resembling that of the atypical 2-Cys peroxiredoxin enzymes [21]. The results also further reinforce the functional similarities of TcGPXI to thioredoxin-dependent peroxiredoxins despite the significant sequence similarity to glutathione-dependent peroxidases that utilize a single selenocysteine mechanism [9]. Comparative analysis of the crystal structure of TcGPXI and the NMR spectroscopic results with the recently reported structures for the homologous T. brucei proteins [22,23] suggests that this family of enzymes exhibits significant structural plasticity that is intrinsic to the catalytic mechanism. In addition, our structural analysis allowed us to consider the putative ligand-binding sites in TcGPXI, thereby identifying surfaces that could be targeted with drug-like molecules to block the protein function.

**EXPERIMENTAL**

**Expression and purification of TcGPXI**

PCR-amplified DNA coding sequence for the wild-type TcGPXI residues 14–177 (EMBL accession number CAC58914) was ligated into NarI- and HindIII-digested pProEX expression vector (Invitrogen). The resultant recombinant protein contained an N-terminal His tag, and a TEV (tobacco etch virus) protease-cleavage site. Following cleavage with TEV protease, additional plasmid-derived glycine and alanine residues remained at the N-terminus of the TcGPXI construct. Single cysteine residue mutations were introduced by site-directed mutagenesis using the QuikChange™ site-directed mutagenesis kit (Stratagene). Wild-type TcGPXI and TcGPXI mutant proteins were overexpressed in Escherichia coli Rosetta (DE3) pLysS cells at 30°C. For NMR studies uniform [¹⁵N]- and [¹⁵N/¹³C]-labelling was obtained by growing cells in M9 media containing ([¹⁵N]NH₄)₂SO₄ and [¹³C₆]glucose (Cambridge Isotope Laboratories) as the sole nitrogen and carbon sources respectively. Soluble TcGPXI protein was purified by immobilized metal ion-affinity chromatography on Ni-NTA (Ni²⁺-nitrilotriacetate) agarose resin (Novagen). For crystallization experiments the proteins were further purified by size-exclusion chromatography (using a Superdex 100 column; GE Healthcare) and dialysed into buffer (10 mM Tris/HCl, pH 7.4, containing 200 mM NaCl). For NMR experiments, dialysed was carried out in 50 mM sodium phosphate buffer, pH 6.5, containing 300 mM NaCl. Samples of the reduced protein were obtained by addition of 10 mM DTT (dithiothreitol).

**NMR spectroscopy**

Two-dimensional ¹H,¹³N-HSQC (heteronuclear single-quantum coherence) and three-dimensional triple-resonance NMR [HNCO, HNCA, HN(CO)CA, CBCA(CO)NH, HN(CA)CO, HA(CA)NH and HA(CA(CO))NH] spectra were recorded at a proton frequency of 500, 600 and 800 MHz on Varian Unityplus, Varian INOVA or Bruker Avance III spectrometers at 25°C using protein samples at a concentration of 0.3–1 mM. NMR data were processed using NMRPipe [24] and Azara software packages and the spectra were analysed using the program ANSIG [25]. Secondary structure prediction on the basis of Cα, Cβ, CO and Hα chemical shifts of oxidized wild-type TcGPXI was obtained using the CSI (chemical shift index) [26] program.

**Enzyme assays**

Wild-type TcGPXI, and the mutant C48G, C77G and C96G TcGPXI proteins, were freshly prepared under reducing conditions. The enzymatic activity was measured in duplicate reactions by following the oxidation of NADPH spectrophotometrically (ΔA₅₅₀) at 30°C as described previously [9,16]. For the glutathione-dependent assay, the standard reaction mixture in 100 mM Tris/HCl, pH 7.4, contained 0.5 mM EDTA, 0.2 mM β-NADPH, 1 mM NaN₃, 3 mM reduced glutathione, 0.1% (v/v) Triton X-100, 1.4 units of Saccharomyces cerevisiae glutathione reductase and 2.2 μM TcGPXI. This reaction was initiated by the addition of 1-butyldihydroperoxide substrate (3.6–50 μM) and followed for 2 min. The basal ΔA₅₅₀ level was obtained in the absence of substrate. Tryparedoxin-dependent enzymatic assays were carried out in 50 mM Hepes, pH 8.0, containing 0.5 mM EDTA, 0.2 mM β-NADPH, 0.5 μM Crithidia fasciculata trypanothione reductase, 20 μM tryparedoxin. 2 μM TcGPXI, 1 μM recombinant T. cruzi tryaredoxin I and the substrate 1-butyldihydroperoxide (0.5–100 μM). All components except NADPH, tryparedoxine reductase and the TcGPXI were added to create an assay blank. NADPH was added and the absorbance monitored for one minute prior to the addition of tryparedoxine reductase to obtain a background reading. The enzyme reaction was then initiated by addition of TcGPXI and the ΔA₅₅₀ followed for 2 mins. Kinetic parameters were obtained by linear regression to the Michaelis–Menten equation [27].

**Structure determination of oxidized TcGPXI**

A 5-fold excess of 1-butyldihydroperoxide was added to the purified wild-type TcGPXI protein and left at room temperature (22°C) for 2 h. The solution was then applied to a Superdex 100 size-exclusion chromatography column for further purification. Crystallization was performed using the hanging-drop vapour diffusion method at 20°C, with protein concentrated to 15–20 mg/ml. TcGPXI crystals grown from unbuffered 1.8–2.0 M ammonium sulphate were submerged into cryoprotectant solution containing 20% (v/v) glycerol prior to data collection. X-ray diffraction data were collected in-house (using rotating anode Rigaku RU-H3R) at 100 K to 2.3 Å (1 Å = 0.1 nm) resolution using a R-Axis IV image plate detector system and Osmic mirrors. Data were collected for 180° over 1° oscillations and diffraction images were scaled, and the data merged and indexed using the program d*TREK [28] in the CrystalClear software suite (http://www.rigaku.com/software/crystalclear.html). Structure determination was carried out by molecular replacement using MOLREP (CCP4 package) [30] with an edited homology
model of TcGPXI based on the template structure of oxidized poplar PtGPX5 (Populus trichocarpa x deltoides) thioredoxin peroxidase 5; 42% sequence identity to Tc-GPX; PDB code 2P5R). The initial model did not include any surface loops or poorly structured regions.

Structure refinement was carried out using the maximum-likelihood restrained method with simple scaling in the REFMAC/CCP4 program [31] and simulated annealing torsion angle dynamics with the maximum likelihood function in CNS (Crystallography & NMR System) [32]. Real-space refinement/manual-fitting of the electron density map and model-building were carried out using the COOT tool [33].

For the prediction of potential ligand- or protein-partner-binding sites the TcGPXI crystal co-ordinates (edited to remove solvent water and other ligands present in the refined model) were submitted to the Q-SiteFinder server (http://www.modelling.leeds.ac.uk/qsitefinder/) [34].

RESULTS

NMR spectroscopy of oxidized TcGPXI

The two-dimensional $^1$H,$^1$H-HSQC spectrum of purified wild-type TcGPXI showed cross-peaks that were well-dispersed, of approximately uniform line-width and relatively homogenous intensity. The spectrum contains in total approx. 170 cross-peaks, with a maximum of 154 peaks from backbone amide groups (i.e. a total of 164 residues including ten proline residues). Complete analysis of the triple-resonance spectra recorded for a $^{13}$C,$^{15}$N-labelled sample yielded the definitive assignment of 148 (96% completed amide resonance assignment) amino acid residues in the $^1$H,$^1$H-HSQC spectrum of wild-type TcGPXI [Figure 1, Supplementary Figure S2 (available at http://www.BiochemJ.org/bj/425/bj4250513add.htm)]. We were unable to assign peak positions for the amide groups of the Cys48, Asn87, Thr97, G1y113, Ser114 and Lys134 residues in the two-dimensional $^1$H,$^1$H-HSQC spectrum, but overall a total of 99% of the $^{13}$Cα chemical shifts, 91% of the $^{13}$Cβ chemical shifts, 97% of the $^1$Hα chemical shifts and 99% of the $^1$Hβ chemical shifts were assigned.

Detection of disulfide bonds in wild-type TcGPXI

It has been shown previously that the chemical shift values of Cα and Cβ resonances can be used to predict the redox state of cysteine residues [35,36]. When surveyed over NMR resonance assignments for proteins with known three-dimensional structure the chemical shift ranges for the Cα and Cβ of a reduced cysteine were found to be 59.3 ± 3.2 p.p.m. and 28.4 ± 2.4 p.p.m. respectively, whereas the chemical shift ranges for Cα and Cβ of an oxidized cysteine were 55.5 ± 2.5 p.p.m. and 40.7 ± 3.8 p.p.m. respectively. Wild-type TcGPXI has three cysteine residues: Cys48, designated the catalytic cysteine [9], Cys77 and Cys96. As no amide resonance could be identified for Cys48 in the $^1$H,$^1$H-HSQC spectrum, the i-1 correlations connected to the NH cross peak of Gly26 were examined. For Cys77 the Cα (58.1 p.p.m.) and Cβ (29.9 p.p.m.) chemical shifts were consistent with that of a reduced, thiol state. However, for Cys48 (Cα 56.4 p.p.m.; Cβ 42.0 p.p.m.) and Cys96 (Cα 56.3 p.p.m.; Cβ 43.3 p.p.m.) the chemical shifts were consistent with that of the oxidized state, suggesting the presence of a disulfide bond between Cys48 and Cys96. We surmise that the TcGPXI protein became oxidized during the purification and remained in that state throughout the NMR data acquisition.

NMR-based secondary structure prediction for oxidized TcGPXI

The individual chemical shifts for the Cα, Cβ, CO and Hα atoms of the oxidized sample of TcGPXI were analysed using the CSI program [26] to assess the secondary structure composition. The CSI prediction for oxidized wild-type TcGPXI contains five β-strands and four α-helices (see Supplementary Figure S3 available at http://www.BiochemJ.org/bj/425/bj4250513add.htm). The following regions were identified as β-strands: Gln20–Ala23, Leu26–Ala29, Phe70–Phe75, Ser142–Ile145 and Val150–Phe155. The regions interpreted as α-helical are: Ser52–His54, Gly56–Gly57, Pro143–Thr146 and Val161–Lys166. Thus the CSI prediction for TcGPXI yields a β−α−β−α−β−β−α−β−α composition that is consistent with a three-dimensional thioredoxin fold. Interestingly, the region Ala88–Phe99, which is identified as helical in the three-dimensional structures of the reduced forms of homologous enzymes (PtGPX5 and TbPxII), but as an extended loop in the oxidized state of the same proteins [22,23,37], is predicted by the CSI analysis to adopt a non-regular loop conformation in the solution structure of TcGPXI.
out by comparison of the two-dimensional $^1$H,$^{15}$N-HSQC and three-dimensional HNCO spectra with those obtained for the oxidized protein. Assignments were transferred by inspection for those NH cross-peaks that were well resolved from other amide cross-peaks, and that overlaid with assigned cross-peaks in the oxidized $^1$H,$^{15}$N-HSQC spectrum. For cross-peaks where there was ambiguity in the assignment through the direct comparison with the oxidized TcGPXI spectrum, the closest match of the corresponding carbonyl carbon chemical shifts was chosen, unless more than one candidate cross-peak lay within 1 ppm of the carbonyl carbon shift. This protocol resulted in tentative sequence-specific backbone assignment of the reduced wild-type spectrum for 86 out of the 148 residues assigned in the oxidized spectrum. Mapping assigned amide resonances in the reduced TcGPXI $^1$H,$^{15}$N-HSQC spectrum upon the secondary structure prediction of the oxidized protein reveals that those residues that were not assigned were located in the predicted loop regions surrounding the active site cysteine residues. The appearance of the reduced TcGPXI spectrum is thus consistent with a picture in which the overall folding topology of TcGPXI is maintained in the reduced state, but with an active site region demonstrating dynamic flexibility and chemical exchange on the intermediate ($\mu$s–ms) timescale. A similar observation was recently reported for the reduced state of the closely homologous TbPxIII protein [22].

NMR spectroscopy of TcGPXI cysteine mutants

In an attempt to produce a model of the reduced TcGPXI structure that would be more generally tractable to NMR spectroscopic studies, site-directed mutagenesis to replace Cys$^{48}$ or Cys$^{66}$ with glycine residues was performed. We expected that in the absence of the disulfide bridge these mutant proteins would adopt a more reduced-like conformation. In contrast, superposition of the two-dimensional $^1$H,$^{15}$N-HSQC spectra of the C96G and C48G mutants with reduced and oxidized wild-type TcGPXI spectra shows that the mutants appear more similar to the oxidized wild-type TcGPXI (see Supplementary Figure S4 available at http://www.BiochemJ.org/bj/425/bj4250513add.htm). Considering that the reduced protein showed clear indications of increased flexibility from conformational-exchange-induced line-broadening it appears that the mutation of the TcGPXI active site cysteine residues distinctly favours an oxidized-like conformation of the active site. Moreover this observation indicates that the oxidized-like conformation might be accessible to the reduced wild-type TcGPXI.

Enzyme assays

The catalytic activity of the recombinant wild-type TcGPXI protein, which was used for the crystallization and NMR spectroscopic studies, was confirmed by enzyme assays utilizing both tryparedoxin- and glutathione-dependent reactions (see Supplementary Figure S5 available at http://www.BiochemJ.org/bj/425/bj4250513add.htm). In the tryparedoxin-linked assay, using t-butyl hydroperoxide as the substrate in a concentration range of 3–100 $\mu$M, the Eadie–Hofstee plot for the wild-type TcGPXI reaction yielded values of $V_{\text{max}} = 1300 \pm 160$ nmol min$^{-1}$ mg$^{-1}$, corresponding to a $k_{\text{cat}} = 24 \pm 3$ min$^{-1}$ and $K_m = 51 \pm 10$ $\mu$M, with $k_{\text{cat}}/K_m = 0.5 \pm 0.2$ min$^{-1}$ $\mu$M$^{-1}$. These values are essentially equivalent to those reported for GPXI and cumene hydroperoxide under similar conditions [16]. As expected, activity in the glutathione-dependent assay for the reaction of wild-type TcGPXI with t-butyl hydroperoxide was much lower with $V_{\text{max}} = 360 \pm 20$ nmol min$^{-1}$ mg$^{-1}$, corresponding to $k_{\text{cat}} = 6.6 \pm 0.3$ min$^{-1}$ and $K_m = 52 \pm 4$ $\mu$M, with $k_{\text{cat}}/K_m = 0.13 \pm 0.02$ min$^{-1}$ $\mu$M$^{-1}$. Although the assay was carried out at conditions identical with those in Wilkinson et al. [9] the apparent reaction rate and $K_m$ were both higher than reported previously. Considering the possible experimental errors when measuring such a low activity and taking into account that the estimated $K_m$ value corresponds well with that reported for the T. brucei orthologue, TbPxIII (47 $\mu$M) [17], the discrepancy with the parameters determined previously was not pursued further.

In contrast with the wild-type TcGPXI protein the C48G and C96G mutant enzymes exhibited no activity in either assay format. On the other hand, conservative mutation of Cys$^{77}$ (C77G and C77S) did not significantly perturb the catalytic function (results not shown).

Protein crystallization

Wild-type TcGPXI was initially crystallized from the unbuffered ammonium sulphate solution. These crystals took a long time to grow, sometimes up to 2 months, and despite testing of a range of additives it proved impossible to find conditions for reproducible crystal growth for the protein in this form. The parallel NMR investigation revealed that TcGPXI has a pronounced tendency to oxidize by forming the Cys$^{48}$–Cys$^{66}$ disulfide. This observation suggested that the crystals obtained probably contained the oxidized form even though the protein had been purified under nominally reducing conditions. Most of the crystals obtained at this stage grew in clusters. X-ray diffraction was performed using one of the individual crystals; however, the data were of poor quality and only to 3 Å resolution. In order to more carefully control the oxidation process a t-butyl hydroperoxide-treated protein sample was used for crystallization. Under these conditions, large, prism-shaped crystals (~0.2 mm) grew reproducibly within 2 days.

Description of the X-ray crystal structure of substrate-treated TcGPXI

The X-ray structure of substrate-oxidized TcGPXI was determined by molecular replacement using data at a resolution of 2.3 Å and refined to a crystallographic $R_{\text{factor}}$ and $R_{\text{free}}$ of 20.9 % and 25.4 % respectively (Table 1). Following iterative refinement all residues were clearly identified in the difference electron density map except for Gln$^{83}$–Glu$^{93}$, which were not included in the final model (Figure 2A). Additionally, two molecules of glyceral and an ammonium ion were identified in the electron density map. The final model contains 75 water molecules.

The overall fold type of the TcGPXI tertiary structure is consistent with a modified thioredoxin fold, characteristic of the GPX family (Figure 2B), including four $\beta$-strands (of which $\beta3$ is antiparallel) flanked by $\alpha$-helices. Additional secondary structure elements, analogous to those typically observed in other GPX structures, are located at the N-terminus and, as an insertion to a thioredoxin fold, between $\beta$-strand 2 and $\alpha$-helix 2. For clarity we name all thioredoxin-like secondary structure elements in the standard mode, such that the central $\beta$-sheet, strands $\beta1$–$\beta4$, is sandwiched between helices $\alpha1$ and $\alpha3$ on one side and helix $\alpha2$ on the other. The main GPX family insertion to the thioredoxin fold lies between strand $\beta2$ and the helix $\alpha2$, and in the structure of substrate-treated TcGPXI this region has an extended loop conformation. However, in some structures of reduced forms of homologous proteins this region adopts a helical conformation that we refer to as helix $\alphaX$. Normally helix $\alphaX$ is presented
Figure 2 Crystal structure of TcGPXI

(A) The crystal structure of TcGPXI is represented as a ribbon. The structure shows the presence of a thioredoxin fold comprising an α–β–α sandwich, where α-helices (red) flank a central core of β-strands (yellow) with additional shorter secondary structural elements at the N-terminus. The catalytic residues Trp138, Asn139 and Cys48 are shown as sticks. Although the catalytic residue Gln83 was not identified in the electron density and not built in the model, the position of the adjacent residue Gly82 is indicated. The resolving cysteine residue of TcGPXI (Cys96) occurs in a loop structure that is present in the oxidized-type fold of GPX-type enzymes exhibiting an atypical 2-Cys peroxiredoxin mechanism. Two glycerol molecules are shown as surface spheres (purple) and an ammonium ion from the crystallization solution is represented as a green sphere. (B) Schematic diagram of TcGPXI topology. Structural elements coloured orange and grey represent embellishments to the consensus minimal thioredoxin fold (blue). The grey-coloured helix αX is not present in the TcGPXI structure reported in (A).

Table 1 Data collection and refinement statistics

A summary of the data collection and refinement statistics for substrate-treated TcGPXI crystals (with t-butyl hydroperoxide as the substrate). Values in parentheses refer to the highest resolution shell. $R_{\text{free}} = \Sigma |F_o - F_c| / \Sigma F_o; R_{\text{free}}$ was calculated with 5 % of data withheld from refinement; RMSD, root mean square deviation.

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on the same side of the major β-sheet as helix α2. At the N-terminus of TcGPXI there are two 3_10-helical turns labelled Nα1 and Nα2 and a β-hairpin motif designated Nβ1–Nβ2. Again this β-hairpin structure has been found in homologous proteins such as PtGPX5 [37] and the T. brucei tryparedoxin-dependent peroxidases [22,23].

Analysis of the oxidized PtGPX5 structure previously led to the prediction of a thioredoxin-recognition site motif with the amino acid sequence ‘EPGXX(D/E)XIXX(F/M)(V/A)CT(R/K)FK’ (X denotes any amino acid residue), which includes the C-terminal active site cysteine residue [37]. The primary sequence of TcGPXI exhibits a similar sequence motif, EPGTALEVKEFACTRFK, containing Cys96, which could represent a potential recognition site for tryparedoxin. In the electron density map for the TcGPXI structure, only the seven residues at the C-terminal end of this sequence motif are clearly discernible, whereas the N-terminal portion appears disordered. Nevertheless, the visible part of the structure suggests that surface-exposed hydrophobic residues, such as Phe94 and Tyr50, in the neighbourhood of the active site Cys48, could become sequestered from the solvent by interaction of tryparedoxin with TcGPXI.

Active site region

In the first step of the consensus peroxidase reaction, a redox-active cysteine (called the peroxidatic cysteine) targets the substrate peroxide and is oxidized to a sulfenic acid intermediate (Scheme 1). In the GPX proteins, thioredoxins and topologically similar peroxiredoxins, the location of the peroxidatic cysteine residue (sometimes a selenocysteine) is in a conserved location in the loop connecting the first β-strand (strand β1) to helix α1 (Figure 2B). Site-directed mutagenesis and sequence comparison studies identified specific tryptophan and glutamine residue side chains as being required for lowering the pKa of the corresponding peroxidatic selenocysteine/cysteine, leading to the suggestion that all members of the GPX-like family of proteins utilize a catalytic mechanism involving this catalytic triad. More recently it has been postulated that a fourth catalytic residue, an asparagine, also plays a role in promoting the activity of the peroxidatic cysteine [38,39].
The peroxidatic and resolving cysteine residues are labelled with (P) and (R) respectively. The product of the first step of reaction is sulfenic acid and the second step of the reaction leads to formation of a disulfide bond. In the atypical 2-Cys mechanism (P) and (R) are on the same polypeptide chain. In the context of the present paper the reductant is tryparedoxin or glutathione.

In the crystal structure of TcGPXI Cys48, which clearly represents the peroxidatic cysteine, Trp138 and Asn139 are all well-resolved in the electron density map. However, the putative catalytic glutamine residue is localized in the loop region that is only partially built due to the disorder reflected in the diffuse electron density and thus Gln83 is absent in the model. The position of Gln83 can be estimated from the position of the adjacent surface-exposed residue Gly82, which is separated by 9.2 Å (Ca–Ca distance) from the surface-exposed residue Trp138 (Figure 3). Overall, the active site appears open and solvent exposed. Crucially, in the conformation of the active site we observe that the Cys48 residue is significantly (>9 Å) removed from the residues proposed to be participating in its activation, Trp138, Gln83 and Asn139.

The second step of the consensus peroxidase reaction mechanism involves an attack by a resolving cysteine residue side chain on the sulfenic acid intermediate of the peroxidatic cysteine residue, and hence formation of a disulfide bond, which is subsequently reduced by an appropriate electron donor. Our mutagenesis results suggest that in TcGPXI Cys96 adopts the role of the resolving cysteine residue. In the crystal structure this residue is located in an extended loop region following the strand β2. The conformation of the loop containing Cys96 places this residue in close proximity to Cys48, in a position apparently competent to form the disulfide bond. This interaction is reminiscent of the proposed catalytic mechanism of atypical 2-Cys peroxiredoxins where the peroxidatic and resolving cysteines are found on the same polypeptide chain.

In the observed TcGPXI crystal structure the active site cysteine residues are reduced

As the TcGPXI protein was revealed by our NMR investigation to be readily oxidized to the Cys48–Cys96 disulfide-bonded form, and the protein formed well-ordered crystals following oxidation with a peroxide substrate, we expected the electron density to be consistent with the presence of a disulfide bond between the two cysteine side chains. Indeed, the absence of the helix αX in our structure, consistent with the secondary structure analysis based upon chemical shifts, together with the similarity of the general active site conformation to oxidized states of homologous structures, suggest that it is the oxidized conformation of TcGPXI that is amenable to crystallization.

Although the structure was solved by the molecular replacement method using a structure of the oxidized form of PtGPX5, to minimize model bias all the loop regions were deleted from the search model and the loops were subsequently built through the iterative process of interpretation of the electron density map and refinement. Any model of the TcGPXI structure that contained disulfide-linked Cys48–Cys96 showed an increase in the $R_{free}$ value and a relatively poor fit to the map, with clear negative electron density between the two sulfur atoms in the difference map. Refinement of the TcGPXI structure without the explicit disulfide bond resulted in improved refinement statistics and a model that fitted well into the electron density. The structure of the whole of the active site was additionally refined by several rounds of calculation of the omit map for the separate regions of the two loops containing the active site cysteine residues. We conclude that even though the crystallized protein was pre-oxidized by treatment with substrate, and hence overall the resulting model is indicative of a typical oxidized conformation, it appears that subsequently to crystal formation the disulfide bond was reduced due to the effect of X-ray radiation. In the active site conformation observed in the TcGPXI electron density (Figure 3), the peroxidatic cysteine residue, Cys48, and resolving cysteine

**Figure 3** Structure of the TcGPXI active site

(A) Electron density for the weighted 2Fo–Fc map in the region of the active site residues is shown, viewed at 1.0σ. Catalytic residues Cys48, Cys96, Trp138 and the Gly82 residue adjacent to catalytic residue Gln83 are highlighted. Both cysteine residues are shown to exist in their reduced thiol form. (B) Close-up view of the redox-active cysteine residues of TcGPXI.
Structure and mechanism of TcGPXI

Figure 4  Glycerol molecule-binding sites in the active site region of TcGPXI

(A) Ribbon diagram of TcGPXI molecule with the two glycerol molecules shown explicitly as yellow stick models. The top-four-ranked potential ligand-binding sites from the Q-site finder analysis are represented as clusters of methyl probes (spheres). (B) Detailed view of ligand probe clusters and the residues surrounding them.

residue, Cys96, are in a close proximity; the distance between the sulfur atoms of the Cys48 and Cys96 side chains is 4.7 Å and only small changes in side-chain torsion angles would be sufficient for the two residues to form a disulfide bond. The crystal packing arrangement apparently constrains the two cysteine residues to remain in close proximity to one another. The phenomenon of X-ray induced reduction has been observed for other protein crystals, for example in the case of DsbG disulfide isomerase [40] and in the radiation-cleaved structures of the N-terminal domain of the AhpF protein [41] and tryparedoxin [42]. Therefore although the active site cysteine residues of TcGPXI are clearly in the reduced thiol state according to the electron density, the close proximity of these residues and the overall active site structure are more representative of the oxidized protein conformation.

Glycerol molecules and putative ligand-binding sites

Two glycerol molecules associated with the surface of TcGPXI were identified from the difference electron density map. One of the glycerol molecules is located between the peroxidatic and resolving cysteine residues near residue Tyr50, which flanks the active site. The position of this glycerol molecule, so close to the catalytic cysteine residues, is indicative of a crevice on the molecular surface that could otherwise be engaged in interaction with tryparedoxin or another electron donor. The second glycerol molecule is positioned on the surface region near residue Trp138 (Figure 4). The binding of this glycerol molecule is stabilized through interaction with the side chains of Trp138 and Arg154, as well as the backbone amide of Lys137. Trp138 is implicated in catalysis; the face of the indole that is opposite the glycerol-binding site lines the region postulated to comprise the active site in the reduced conformation of TcGPXI, together with the peroxidatic Cys48, Asn139 and the putative catalytic residue Gln83. Interestingly, the side chain of Trp138 adopts a different rotamer conformation in the structures of the homologous T. brucei [22] and poplar proteins [37]. Specifically, in an NMR solution structure of TbPxIII [22] the corresponding tryptophan side chain occupies the space equivalent to the second glycerol-binding site in TcGPXI.

Prediction of potential ligand-binding regions on the structure of oxidized TcGPXI was carried out with Q-SiteFinder [34]. Q-SiteFinder uses the interaction energy between the protein and a simple van der Waals probe to locate energetically favourable binding sites. Clusters of favourable probe sites are ranked based on their likelihood of identifying a binding site according to the sum total binding energies for each cluster. When applied to TcGPXI, the top three ranked regions for potential ligand interaction were found in surface cavities around the peroxidatic Cys48 and resolving Cys96 residues. Two out of the top three potential ligand-interaction sites correspond to regions of the crystal structure populated by glycerol molecules. Figure 4 illustrates the location of putative ligand probes placed within the top four most probable ligand-binding regions. The potential ligand probe clusters designated I, II and III have a volume of 222 Å³, 174 Å³ and 123 Å³ respectively. Potential binding site III is the most distant from the redox-active cysteine residues, it is within the space adjacent to Trp138 where a glycerol molecule is also observed in the crystal structure of TcGPXI. Ligand probe site II is located near Cys48, in an extended cavity delineated by Trp138 and Asn139 on one side and Tyr50, Ala52 and Gly53 on the other. This region extends further and connects to the predicted fourth site (174 Å³), which reaches towards the missing loop in the structure. The ‘missing’ part of the loop region between Gly46 and Phe99 might further contribute to the distinctive appearance of this pocket. The top-ranked putative binding site I partially overlaps with the second glycerol molecule in the TcGPXI crystal structure. This site comprises the surface pocket lined by Cys48 and Cys96, polar residues Thr51 and Thr97, hydrophobic residues Tyr50, Tyr55, Phe99, and charged residues Arg98 and Lys100. Interestingly, the
corresponding charged residues from this pocket (Lys\textsuperscript{97} and Lys\textsuperscript{99}) were identified, through mutagenesis studies, as contributing to the tryparedoxin interaction with TbPxIII [22].

Each of the top-ranked putative binding sites occupies a relatively small volume corresponding to potential ligands comprised of approximately ten non-hydrogen atoms. Close examination of the identified sites, however, reveals that the top two sites connect and extend towards site four, thus greatly enlarging the potential space for binding of a drug-like molecule. The proximity of the predicted ligand-binding sites to the redox-active cysteine residues legitimizes targeting of these surfaces in the design of potential inhibitors that would either stabilize the oxidized conformation of TcGPXI or interfere with tryparedoxin binding.

DISCUSSION

Conformational changes associated with oxidation of GPX-like enzymes utilizing the atypical 2-Cys mechanism

Over the last two years it has been demonstrated that thioredoxin-dependent poplar PtGPX5 and TbPxIII, which are closely related to glutathione peroxidase-type enzymes, utilize a catalytic mechanism that involves formation of an intramolecular disulfide bridge, commonly associated with atypical 2-Cys peroxiredoxin proteins [43]. X-ray crystallographic investigation of PtGPX5 revealed extensive conformational changes that accompany the transition between the reduced and the oxidized states of the protein. The reduced conformation of PtGPX5 exhibited a topology previously observed in other members of this structural family, such as bovine GPX1 ([44]; PDB 2F8A) and human GPX1–4 (PDB 2F8A, 2HE3, 2R37, 2GS3 and 2OBI respectively), which includes helix \( \alpha \)X between the strand \( \beta2 \) and helix \( \alpha2 \) of a canonical thioredoxin fold. Unlike the selenocysteine members of the family, the PtGPX5 sequence contains an additional cysteine residue within the helix \( \alpha \)X region and it is this residue that provides the role of the resolving cysteine in the 2-Cys catalytic mechanism. In the reduced PtGPX5 structure, the two cysteine residues are \( >15 \) Å apart; in the oxidized structure the two residues are brought closer by a change in conformation that includes unwinding of the C-terminal end of helix \( \alpha \)X [37]. These results point to the capacity for proteins in this family to undergo significant conformational plasticity and dynamic exchange that must underpin the 2-Cys mechanism and ‘Ping Pong’ interactions with the peroxide substrate and upstream reductant.

Recently there have been extensive investigations into the functional and structural properties of both TbPxIII and its close homologue TbPxII (Trypanosoma brucei peroxidase II). Melchers et al. [22] obtained NMR solution structures of the oxidized and reduced form of TbPxIII. Although the NMR spectrum of the reduced form of TbPxIII is characterized by significant chemical shift differences with respect to the oxidized protein and disappearance of several cross-peaks due to exchange broadening, the derived three-dimensional solution structures of the oxidized and reduced forms of the protein are essentially the same. An X-ray crystal structure of the oxidized form reveals a structure that correlates well with that obtained by solution NMR methods. Similar to the X-ray crystal structure of TcGPXI we obtained in the present study, even the reduced form of TbPxIII shows that the two active site cysteine residues of TbPxIII are in close proximity and the reduced GPX-type helix \( \alpha \)X is clearly absent. In addition, in the reduced active site the peroxidatic cysteine residue is not found to interact with the residues that were previously suggested to be involved in stabilizing the thiolate nucleophile [38,39]. Owing to a lack of unambiguous distance restraints to the surrounding residues, the exact orientation of several residues in the active site was not well-determined by the NMR data, although it was postulated that the active site cysteine residue activation might occur through interaction of the side chain with the backbone [22]. It was therefore proposed that the catalytic mechanism of TbPxIII differs fundamentally from that of any of the previously characterized enzymes and that the T. brucei enzyme does not undergo such a large conformational change as described for PtGPX5. Independent work by Alphey et al. [23] provided an alternative view into the structure of the T. brucei enzymes when they described an X-ray crystal structure of the reduced form of the TbPxII, an enzyme whose amino acid sequence differs from TbPxIII only at a single residue (threonine compared with asparagine) at a position remote from the active site. Whereas the cores of the TbPxII and TbPxIII structures superpose closely, the reduced TbPxII active site conformation differs significantly from that of the reduced TbPxIII NMR structure. Specifically, in the reduced form of TbPxII the resolving cysteine residue is positioned very distantly from the peroxidatic cysteine residue, and the consensus helix \( \alpha \)X is present. Furthermore, the peroxidatic cysteine is engaged in a network of interactions with the tryptophan, glutamine and asparagine residues that were previously identified as forming a catalytic tetrad [38,39]. Thus on the basis of the crystal structure of reduced TbPxII, Alphey et al. [23] declared that the catalytic mechanism of the T. brucei enzyme(s) is similar to that described for PtGPX5 [37].

Structural plasticity of T. cruzi GPXI

The apparently contradictory reports either provide support for, or explicitly dismiss, the occurrence of a large conformational change in an atypical 2-Cys peroxidase mechanism for the TbPxII/III enzymes. It is highly unlikely that the discrepancy can be attributed to the single amino acid difference between the two proteins. The present study on TcGPXI, which exhibits 72 % sequence identity with TbPxIII, suggests that both Cys\textsuperscript{97} and Cys\textsuperscript{99} are required for the reaction mechanism. The NMR spectrum of the oxidized state is consistent with a stable fold in which the loops containing these residues are tethered by the disulfide cross-link. The NMR spectrum of reduced TcGPXI displays significant line-broadening and loss of resonances for the majority of residues within the regions surrounding the active site. These results demonstrate that the reduced state of TcGPXI can adopt multiple conformations that exchange on the \( \mu \)-s–ms timescale indicating the presence of an equilibrium between conformational sub-states separated by one or more thermal activation barriers. In their solution NMR studies, Melchers et al. [22] reported that the reduced form of TbPxIII also displayed a spectrum consistent with a \( \mu \)–ms timescale exchange. It is tempting to argue that in both cases the spectra reveal that the reduced states of these proteins can sample a range of conformations that spans the type of arrangements observed in the crystal structures of reduced PtGPX5 and TbPxII, in which the helix \( \alpha \)X is formed and the active site cysteine residues are separated by \( >10 \) Å, as well as conformations in which the resolving cysteine residue can approach the peroxidatic cysteine residue, as it must when acting to resolve the sulfenic acid intermediate state in the reaction pathway. In this context, the conformation reported in the present paper for the crystal structure of TcGPXI, which we attribute to X-ray-induced modification of crystals of ‘oxidized’ protein, represents a snapshot of an extreme of the conformational landscape for the reduced state of the protein. Notably, in the TcGPXI structure the crystallographic two-fold axis is very close to the active site and interactions between
symmetry-related molecules presumably provide constraints on the conformation of the extended sequence region containing the resolving cysteine, limiting the potential of the protein to undergo more significant rearrangement upon reduction of the disulfide bond. Moreover, the finding that the conformational exchange-broadening of the NMR spectrum of reduced TcGPXI is largely relieved by mutation of either Cys₄₈ or Cys₉₆ to glycine is further evidence that the ‘untethered’ protein has an intrinsic capacity to explore different configurations. The fact that the spectrum of each of the mutants appears rather similar to that of the oxidized wild-type protein perhaps suggests that in the conformational equilibrium, which must exist for the reduced protein, the energy landscape is biased in favour of conformations that resemble the oxidized protein, a conclusion that would be consistent with the observation that the NMR-derived solution structure of the reduced TbPxIII protein (despite the presence of conformational exchange) emerges with an overall conformation similar to that of the oxidized state [22]. Extrapolation of this analysis leads to the prediction that the conformations of reduced PtGPX5 and TbPxII “trapped” by crystallization represent the opposite extremes of the underlying conformational equilibrium, possibly stabilized by specific crystal packing forces.

Concluding remarks
The present results provide a platform upon which to build a more extensive study of TcGPXI. To this end, we have found that in titrations of the Cys→Gly mutant forms of TcGPXI with t-butyl hydroperoxide, no changes in the ¹H,¹⁵N chemical shifts occur even at a 1.5 mM concentration (results not shown). Although further exploration of this observation is needed, it appears that the protein conformations favoured by the Cys→Gly mutants, which might be closer to that of the oxidized protein, exhibit a very poor affinity for the peroxide substrate. It is anticipated that a truly reduced-type conformation would bind the substrate with an affinity comparable with that of the Kₘ (approx. 50 μM). Poor affinity for the substrate of an oxidized-type conformation is consistent with the Ping Pong reaction mechanism that is displayed by TcGPXI and the T. brucei homologues [16,17]. Moreover, we analysed the molecular surface of the oxidized-type conformation in the TcGPXI crystal structure and identified surface cavities that could be targeted for therapeutic intervention. Structure-led design of small molecules that preferentially bind the specific conformation might provide a novel avenue for inhibiting the peroxidase activity of this enzyme by interfering with the interaction of the upstream reducing agent.

AUTHOR CONTRIBUTION
Shreenal Patel carried out main body of the experimental work as a part of her Ph.D. thesis. Syed Hussain was closely involved in enzymatic assays and analysis of kinetic parameters; he also obtained initial crystals of the wild-type GPXI. Richard Harris was involved in NMR spectroscopy data acquisition and processing. Sunila Sardwai assisted in production of mutant proteins and protein purification. John Kelly and Shane Wilkinson originally cloned and characterized GPXI and provided assistance with activity assays and with their expertise in T. cruzi biology. Paul Driscoll provided leadership in the NMR aspect of the project, including in-depth interpretation of data. Snezana Djordjevic was the principal investigator on the project and provided X-ray crystallographic expertise.

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SUPPLEMENTARY ONLINE DATA

Structural insights into the catalytic mechanism of *Trypanosoma cruzi* GPXI (glutathione peroxidase-like enzyme I)

Shreenal PATEL*, Syeed HUSSAIN*, Richard HARRIS*, Sunita SARDIWAL*, John M. KELLY†, Shane R. WILKINSON‡, Paul C. DRISCOLL*§ and Snezana DJORDJEVIC*1

*Institute of Structural and Molecular Biology, Division of Biosciences, University College London, London WC1E 6BT, U.K., †Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, U.K., ‡School of Biological and Chemical Sciences, Queen Mary University of London, London E1 4NS, U.K., and §Division of Molecular Structure, Medical Research Council National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

Figure S1  The trypanosomal oxidative defence mechanism

In the oxidative defence system of *T. cruzi* reduction of hydroperoxides occurs via the transfer of reducing equivalents from NADPH and dihydrotrypanothione ([TSH]₂) to either: (i) GSH and GPX enzymes; (ii) ascorbate (ASC) and ascorbate-dependent peroxidase (APX); (iii) tryparedoxin (TXN) and cytosolic/mitochondrial peroxiredoxins (CPX/MPX) or GPX enzymes (present in cytosol, glycosomes or endoplasmic reticulum).

1 To whom correspondence should be addressed (email s.djordjevic@ucl.ac.uk).

The chemical shift assignments for oxidized TcGPXI will appear in the BioMagResBank under accession number 16518. The atomic co-ordinates for TcGPXI structure will appear in the PDB under ID 3E0U.
Figure S2  NMR sequence specific assignments of oxidised TcGPXI

Two-dimensional $^1$H,$^{15}$N- HSQC spectrum of $^{13}$C,$^{15}$N-labelled TcGPXI in 50 mM sodium phosphate, pH 6.5, containing 300 mM NaCl. Sequence-specific backbone assignments were made for all residues except Cys$^{48}$, Asn$^{78}$, Thr$^{97}$, Gly$^{113}$, Ser$^{114}$ and Lys$^{134}$. The side chain NH group of the lone tryptophan is denoted W$^{138}$. 
Structure and mechanism of TcGPXI

Figure S3  The chemical shift index for the secondary structure prediction of oxidised TcGPXI

Secondary chemical shift values for: (A) $\Delta \Delta C_{\alpha} - \Delta C_{\beta}$, (B) $\Delta \Delta CO$ and (C) $\Delta \Delta H_{\alpha}$. (D) CSI secondary structure prediction for each residue based on $C_{\alpha}$, $C_{\beta}$, CO and $H_{\alpha}$. $\beta$-strand regions are: Gln 20–Ala23 ($\beta_1$); Leu39–Ala45 ($\beta_2$); Phe70–Phe75 ($\beta_3$); Ser142–Ile145 ($\beta_4$); and Val150–Phe155 ($\beta_5$). Regions assigned $\alpha$-helical are Ser 32–His34 ($\alpha_1$); Gly 53–Gly67 ($\alpha_2$); Pro118–Thr126 ($\alpha_3$); Val161–Lys166 ($\alpha_4$). Blank regions indicate the absence of a cross-peak or a residue that was not assigned.

Figure S4 Superposition of two-dimensional $^1H,^{15}N$-HSQC spectra of the C48G and C96G mutant and wild-type TcGPXI

Superposition of two-dimensional $^1H,^{15}N$-HSQC spectra of the C48G mutant TcGPXI (magenta) with the C96G mutant (blue) and wild-type protein (black) in 50 mM sodium phosphate, pH 6.5, containing 300 mM NaCl. All three spectra overlap significantly.

Figure S5 Kinetic investigation of wild-type TcGPXI using the glutathione-dependent and tryptaredoxin-dependent antioxidant pathway

Eadie–Hofstee plot constructed from values obtained for the kinetic investigation of wild-type TcGPXI in (A) glutathione-dependent and (B) tryptaredoxin-dependent assay format.