

# Biochemical characterization of a trypanosome enzyme with glutathione-dependent peroxidase activity

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In most eukaryotes, glutathione-dependent peroxidases play a key role in the metabolism of peroxides. Numerous studies have reported that trypanosomatids lack this activity. Here we show that this is not the case, at least for the American trypanosome *Trypanosoma cruzi*. We have isolated a single-copy gene from *T. cruzi* with the potential to encode an 18 kDa enzyme, the sequence of which has highest similarity with glutathione peroxidases from plants. A recombinant form of the protein was purified following expression in *Escherichia coli*. The enzyme was shown to have peroxidase activity in the presence of glutathione/glutathione reductase but not in the presence of trypanothione/trypanothione reductase. It could metabolize a wide range of hydroperoxides (linoleic acid hydroperoxide and phosphatidyl-

cholinehydroperoxide > cumenehydroperoxide > t-butylhydroperoxide), but no activity towards hydrogen peroxide was detected. Enzyme activity could be saturated by glutathione when both fatty acid and short-chain organic hydroperoxides were used as substrate. For linoleic acid hydroperoxide, the rate-limiting step of this reaction is the reduction of the peroxidase by glutathione. With lower-affinity substrates such as t-butyl hydroperoxide, the rate-limiting step is the reduction of the oxidant. The data presented here identify a new arm of the *T. cruzi* oxidative defence system.

**Key words:** hydroperoxide metabolism, *Trypanosoma cruzi*, trypanothione.

## INTRODUCTION

Organisms that live within an aerobic environment are exposed to reactive oxygen species. For an intracellular parasite such as *Trypanosoma cruzi*, the causative agent of Chagas' disease, reactive oxygen species may be generated by a number of mechanisms, including drug metabolism. The drugs currently used in the treatment of Chagas' disease, nifurtimox and benznidazole, are unsatisfactory as they often have toxic side effects and can fail to eradicate parasitaemia. Their mode of action is unknown, although both have been shown to undergo redox cycling within the parasite [1,2]. Thus the identification and functional analysis of parasite enzymes involved in the oxidative defence system may be of importance in the context of improved chemotherapy.

In most eukaryotes, glutathione peroxidases (GPXs) and catalases are among the front-line enzymes in peroxide metabolism. Both types of enzyme have been reported to be absent from trypanosomes [3–7]. Until recently it was unclear how *T. cruzi* metabolized peroxides and it has been proposed that this occurred via non-enzymic interactions involving the trypanosome-specific thiol trypanothione (*N*<sup>1</sup>,*N*<sup>8</sup>-bisglutathionyl-spermidine) [8]. This situation was clarified by the identification of a trypanothione-dependent, peroxide-metabolizing redox cycle [9]. The pathway, first identified in the cytosol of *Crithidia fasciculata* [10,11], involves the reduction of trypanothione by the NADPH-dependent flavoprotein trypanothione reductase. This drives a two-step oxidoreductase cascade involving the thioredoxin-like molecule, tryparedoxin, and peroxiredoxin enzymes [12–14]. In addition to the cytosolic pathway, a mitochondrial peroxiredoxin has also been identified in *T. cruzi*. The

activity of this enzyme has been shown to be trypanothione-dependent, but the tryparedoxin/peroxiredoxin interaction has yet to be confirmed [9]. The *in vivo* activity of the two *T. cruzi* peroxiredoxin enzymes has also been established by over-expression of each enzyme within the parasite. Such recombinant cell lines exhibited an increased resistance towards exogenous peroxide [9]. An alternative peroxide-scavenging system has also been reported in *T. cruzi*, based on the high levels of ascorbate and a dihydroascorbate reductase activity [15], but the reductase activity may be attributed to non-enzymic interactions with trypanothione [16]. The recent identification of thioredoxin within trypanosomatids suggests that these organisms contain additional redox systems [17]. Whether these play an important role in the parasites' oxidative defence pathways has yet to be established.

Here we demonstrate that *T. cruzi* also expresses a glutathione-dependent peroxidase. The enzyme can efficiently metabolize hydroperoxides of fatty acids and phospholipids, but not hydrogen peroxide.

## EXPERIMENTAL

### Reagents

GSH, *Saccharomyces cerevisiae* glutathione reductase,  $\beta$ -NADPH, Triton X-100 (peroxide free), t-butyl hydroperoxide, cumene hydroperoxide, hydrogen peroxide, linoleic acid, L- $\alpha$ -phosphatidylcholine, soya bean lipoxidase (type IV) and dithiothreitol were purchased from Sigma.

Linoleic acid hydroperoxide and L- $\alpha$ -phosphatidylcholine hydroperoxide were prepared using soya bean lipoxidase [18,19]. Hydroperoxide formation was monitored by following the

Abbreviations used: GPX, glutathione peroxidase; Ni-NTA, Ni<sup>2+</sup>-nitrilotriacetate; cGPX, cellular GPX; PHGPX, phospholipid hydroperoxide GPX; pGPX, plasma GPX; IPTG, isopropyl  $\beta$ -D-thiogalactoside.

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TcGPXI	-----MFRFGQLLRAAEMKSIYEFQVNAADG--KPYDLSQ	33
AtGPX	-----MAASS.P..L.D.T.KD.K.-NDV...I	27
NsGPX	-----MASQSSKPQ...D.T.KD.K.-NDV...I	28
HscGPX	-----MCAAR.A.AAQ.V.A.SARPLA.GEPVS.GS	32
HspGPX	--MARLLQASCLLSLLLAGFVSQSRGQEKSKMDCHGGISGT..EYGALTID.EEYIPFK.	58
HseGPX	--MTTQLRVVHLLPLLLACFVQTSPKQEKMKMDCHKD.KGT..DYEAI.LNKNEYVSFK.	58
HsPHGPX	-MSLRRLRLLKPALLCGALAAPLAGTMCASRDDWRCAR.MH..SAKDIDG-HMVN.DK	58
SmGPX	-----MSSSHKSWN-----T.KDIN.-VDVS.EK	28
Bpgp29	MSAQLLILSHMVLQLLIVAQLGPKIGKQFLKPKQCEITNQTV.D...QMLN.-AQKS.AE	59
	A	B
TcGPXI	HKGHPLLIY <b>NVASRCGYT</b> RGGYETATTLYNKYKGQFTVLAFCNQFAG <b>QEPG</b> CTALEVKE	93
AtGPX	Y..KV...V... <b>Q..L</b> .NSN.TELAQ..E...H..EI.....GN.....NE.IVQ	87
NsGPX	Y..KV.I.V... <b>Q..L</b> .NSN.TDL.EI.K...D..LEI.....G.....SIE.IQN	88
HscGPX	LR.KV...E... <b>LX.T</b> VRD.TQMNELQRRILGPR.LV..G.....GH... <b>NA</b> KNE.ILN	92
HspGPX	YA.KYV.FV... <b>YX.L</b> G-Q.IELNA.QEELAPF.LVI..G.....GR.....ENS.ILP	117
HseGPX	YV.KHI.FV... <b>TY.L</b> A-Q.PELNA.QEEL.PY.LV..G.....GK.....DNK.ILP	117
HsPHGPX	YR.FVCIVT... <b>QX.K</b> EVN.TQLVD.HAR.AEC.LRI.....GK.....SNE.I..	118
SmGPX	YR..VC...V... <b>CKX.A</b> .DKN.RQLQEMHTRLV.K.LRI.....G.....WAEAEI.K	88
Bpgp29	YRNKV...V... <b>TY.A</b> .M-Q.RDFNPILSNSNGTLNILG.....YL... <b>A</b> ENH.LLS	118
TcGPXI	FACTR-----FKADFPIMAKIDVNGSKAHPLYEFMKATIPGL-----	130
AtGPX	.....EY..FD.V...D..A.V.K.L.SSKG.....	124
NsGPX	MV.....EY..FD.V...DN.A...K.L.SSKGGF-----	125
HscGPX	SLKYVRPGGGFEP--N.MLFE.CE...AG...FA.LREAL.APSDDATALMTDPKLIW	150
HspGPX	TLKYVRPGGGFVP--N.QLFE.G...E.EQKF.T.L.NSC.PTS---ELLGTSDRLEW	171
HseGPX	GLKYVRPGGGFVP--S.QLFE.G...E.EQKVFS.L.HSC.HPS---EILGTFKSIW	171
HsPHGPX	..AG-----YNVK.DMFS..C...DD...WKW..IQPK.KG-----	155
SmGPX	.V.EK-----YGVQ.DMFS..K...D.DD..K.L.SRQHG-----	124
Bpgp29	GLKYVRPGHGWEPHKNMH.FG.LE...END...K.L.ERC.PTVP---VIGKRHQILIY	174
	C	
TcGPXI	--FGTKA <b>KWNE</b> TSFLIDRHGVPVERFSPG--ASVEDIEKLLPLLGGARI-----	177
AtGPX	--..DG...AK..V.KD.NV.D..A.T--T.PLS...DVKK...VTA-----	169
NsGPX	--..DS...SK..V.KE.NV.D.Y..T--TTPASM..DIKK...V-----	169
HscGPX	SPVCRNDVA <b>A</b> .EK..V.GPD..LR.Y.RR--FQTI...PDIEA..SQGFSCA---	201
HspGPX	EPMKVHD <b>R</b> ..EK..V.GPD..I..M.WHHR--T.T.SNVKMDI.SYMRQAALGVKRR	226
HseGPX	DPVKVHD <b>R</b> ..EK..V.GPD..I..M.W.HR--T.SSVKTDI..AY.KQFKTK---	221
HsPHGPX	--ILGN...K...K..CV.K.YG.M--EELV...D.PHYF-----	197
SmGPX	--TL.NN...SK..V.Q.Q..K.Y..T--TAPY..EGDIME..EKK-----	169
Bpgp29	DPI..NDV <b>I</b> ..EK..V.KK.R.RY..H.ENWVQGTAVKPYIDE.EREI-----	223

**Figure 1** Sequence alignment of *T. cruzi* GPX (TcGPXI) with other proteins from this family

AtGPX, *Arabidopsis thaliana* phospholipid hydroperoxide GPX-like protein, accession number BAA24226; NsGPX, *Nicotiana sylvestris* GPX, CAA42780; HscGPX, human cellular GPX, P07203; HspGPX, human plasma GPX, NP002075; HseGPX, human epididymis-specific GPX-like protein, O75715; HsPHGPX, human phospholipid hydroperoxide GPX, NP002076; SmGPX, *Schistosoma mansoni* GPX, Q00277; Bpgp82, *Brugia pahangi* surface glycoprotein, S23062. The residues that are in common with the TcGPXI sequence are represented by dots; dashes represent gaps in the sequence made to optimize the alignments. Differences between the sequences when compared with the TcGPXI are indicated. Boxes A, B and C represent the regions containing residues implicated in the redox activity of these enzymes [24]. The X in region A represents a selenocysteine residue.

change in absorbance at 234 nm and their concentrations calculated using  $\epsilon = 25000 \text{ M}^{-1} \text{ cm}^{-1}$ . The hydroperoxides were stored as pellets at  $-80^\circ\text{C}$  under argon and then dissolved in ethanol prior to use. Trypanothione disulphide (Bachem) was non-enzymically reduced to dihydrotrypanothione using dithiothreitol [20].

### Isolation of *T. cruzi* GPX

A DNA fragment encoding a *T. cruzi* GPX (designated *TcGPXI*) was amplified from *T. cruzi* CL Brener genomic DNA using the primers GPXI-1 (5'-GTTggatccATGTTTCGTTTCGGTCAA-TTG-3') and GPXI-2 (5'-GGGaagcttTCAAATCCCTAGCAC-CACCAA-3'). Restriction sites (shown here in lower case) were incorporated into the primers to facilitate cloning of the amplified product into pBluescript KS(-) (Stratagene). A derivative of *TcGPXI* was amplified from *T. cruzi* genomic DNA using the primers GPXI-5 (5'-GCGgatccGGGCAGCCGAGATGAA-AAGC-3') and GPXI-2. The amplified product was cloned into the *Bam*HI and *Hind*III sites of the *Escherichia coli* expression

vector pTrcHis-C (Invitrogen). The amplified products were sequenced using a dye terminator cycle sequencing kit (Applied Biosystems) and fractionated using an ABI Prism 377 DNA sequencer. A copy of *TcGPXI* was also isolated by screening a *T. cruzi* (CL Brener) cosmid library that had been plated as an array of 7200 clones [21].

### Heterologous expression and purification of His-tagged *TcGPXI*

*E. coli* XL-1Blue, transformed with the plasmid pTrcHis-GPX1, were grown in NZCYM broth (1 litre contains 10 g of enzymic casein digest, 5 g of yeast extract, 1 g of casamino acids, 5 g of NaCl and 0.98 g of  $\text{MgSO}_4$ ; Sigma) containing  $50 \mu\text{g ml}^{-1}$  ampicillin at  $37^\circ\text{C}$  with aeration. When the culture was in the mid-logarithmic phase of growth, isopropyl  $\beta$ -D-thiogalactoside (IPTG) was added to a concentration of 0.5 mM. Cultures were grown for a further 1 h at  $37^\circ\text{C}$  before harvesting the cells by centrifugation. Recombinant His-tagged *TcGPXI* was affinity purified on an  $\text{Ni}^{2+}$ -nitrilotriacetate (Ni-NTA) matrix column under native conditions as recommended by the manufacturer

(Qiagen; see Figure 3, below, for details). Fractions were analysed by SDS/PAGE. Protein concentrations were determined by the BCA protein assay system (Pierce). As a control, cultures of non-transformed *E. coli* XL-1Blue were treated as above in parallel experiments.

### Enzyme activity

Glutathione-dependent peroxidase activity was measured by monitoring NADPH oxidation [18,19]. A standard reaction mixture (1 ml) containing 100 mM Tris/HCl, pH 7.4, 5 mM EDTA, 0.2 mM  $\beta$ -NADPH, 1 mM  $\text{NaN}_3$ , 3 mM GSH, 0.1% (v/v) Triton X-100, 1.4 units of glutathione reductase and 40  $\mu\text{g}$  of *TcGPXI* was incubated at 30 °C for 5 min. The background rate of NADPH oxidation was determined and the reaction initiated by the addition of the peroxide substrate. The non-enzymic activity due to the auto-oxidation of GSH and the activity of any potentially co-purified *E. coli* proteins were also examined. The enzyme activity was calculated using an  $\epsilon$  value of  $6220 \text{ M}^{-1} \text{ cm}^{-1}$ . Data were analysed by fitting to a rectangular hyperbola (Kinenort program, supplied by Dr A. G. Clark, University of Wellington, Wellington, New Zealand).

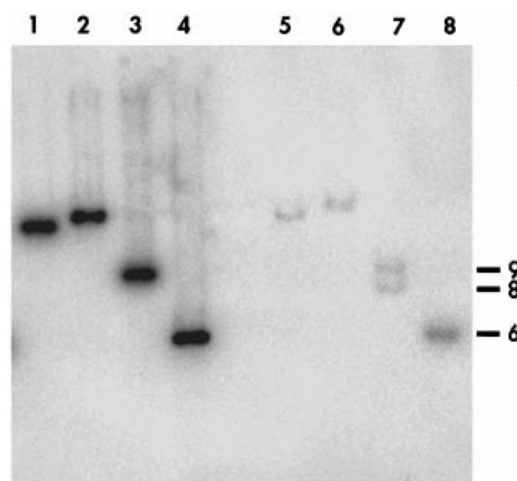
Trypanothione-dependent peroxidase activity was assayed in a similar fashion except that 1.5 mM dihydrotrypanothione and 0.22 units of trypanothione reductase were used in place of GSH and glutathione reductase. Positive controls for the activity of a trypanothione-dependent redox cycle were performed by comparing the peroxide metabolism of cell extracts from wild-type *T. cruzi* against *T. cruzi* cells overexpressing a cytoplasmic peroxidase [9].

## RESULTS

### Isolation of a *T. cruzi* GPX

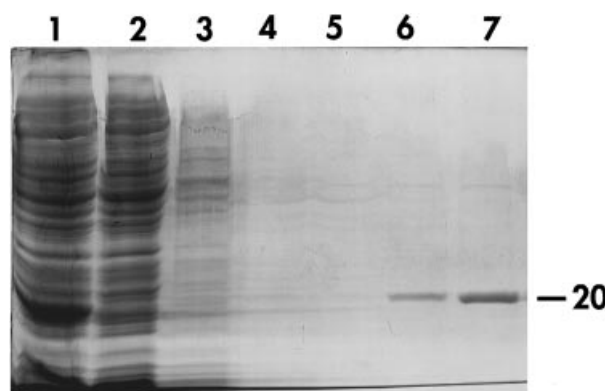
Two DNA sequences from the *T. cruzi* genome project (accession numbers AI069627 and AI035005) were identified as having similarity to GPXs in other organisms. However, *T. cruzi* has previously been shown to lack this activity. We designed DNA primers corresponding to the putative start and stop codons and amplified the complete open reading frame from *T. cruzi* genomic DNA. The resultant 550 bp fragment (designated *TcGPXI*) was cloned and the inferred amino acid sequence compared with other GPXs (Figure 1). Analysis of the *TcGPXI* protein sequence identified three regions characteristic of the GPX family of antioxidant enzymes (Figure 1, regions A, B and C). Each region contributes a residue that participates in the catalytic activity [22,23]. In many enzymes, such as the human cellular GPX (cGPX) and human phospholipid hydroperoxide GPX (PHGPX), region A (Figure 1) contains the amino acid selenocysteine. In other cases, such as the plant GSH peroxidases, this residue is substituted by cysteine. It has been demonstrated that the selenocysteine/cysteine at this position plays a critical role in the activity of these enzymes in determining their specificity for various substrates and their sensitivity towards peroxide-mediated inactivation [23,24]. The *T. cruzi* enzyme shows the cysteine substitution (Figure 1).

The genomic organization of *TcGPXI* was determined by Southern hybridization. This indicated that it was a single-copy gene per haploid genome. With certain restriction enzymes, such as *SalI*, two bands that correspond to the allelic forms of the gene were detected (Figure 2). This arrangement was confirmed after the isolation of a *T. cruzi* cosmid clone where one of the allelic forms was identified (the upper *SalI* band; Figure 2).



**Figure 2** Genomic organization of *TcGPXI*

Cosmid DNA digested with the enzymes *HindIII* (lane 1), *BamHI* (lane 2), *SalI* (lane 3) and *EcoRI* (lane 4), and *T. cruzi* CL Brener genomic DNA digested with the enzymes *HindIII* (lane 5), *BamHI* (lane 6), *SalI* (lane 7) and *EcoRI* (lane 8), were analysed by Southern hybridization using *TcGPXI* as a DNA probe. Sizes given are in kb.



**Figure 3** Purification of *TcGPXI* on an Ni-NTA column

Fractions obtained at various stages of the purification of *TcGPXI* were resolved by SDS/PAGE (10% gel) and visualized by Coomassie Brilliant Blue staining. A clarified fraction of an *E. coli* (pTrcHis-GPX1) cell lysate after 1 h of IPTG induction (lane 1) was loaded onto a Ni-NTA column and the flow-through collected (lane 2). The column was washed extensively with 20 mM imidazole (lanes 3 and 4). Progressively higher concentrations of imidazole were then used to elute bound protein (e.g. lanes 5 and 6, both 50 mM imidazole and lane 7, 100 mM imidazole). The band of 20 kDa (indicated) corresponds to recombinant *TcGPXI*.

### Heterologous expression and enzyme activity

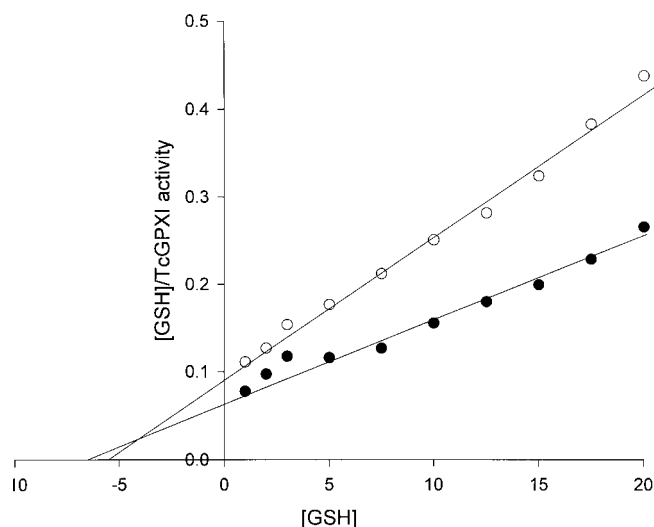
To investigate the activity of the putative peroxidase, *TcGPXI* was amplified and cloned into the vector pTrcHis-C. In this system the expressed protein is tagged at its N-terminal with a histidine-rich sequence and an epitope detectable with the anti-Xpress monoclonal antibody (Invitrogen). *E. coli* XL-1Blue cells were transformed with this plasmid (pTrcHis-GPX1). After induction with IPTG, a 20 kDa band was detected in the soluble fraction of lysates by Western-blot analysis (results not shown). The native protein could be purified readily by one round of affinity chromatography on an Ni-NTA column (Figure 3).

The activity of *TcGPXI* towards several hydroperoxides was determined in the presence of glutathione/glutathione reductase.

**Table 1 Activity of TcGPXI towards different substrates**

Glutathione-dependent peroxidase assays were performed in triplicate using between 9 and 11 different hydroperoxide concentrations with a fixed concentration of GSH (3 mM). The data were analysed statistically using the Kinenort program. The values for apparent  $K_m$  and  $V_{max}$  ( $\pm$  S.E.M.) are given. ND, no activity detected.

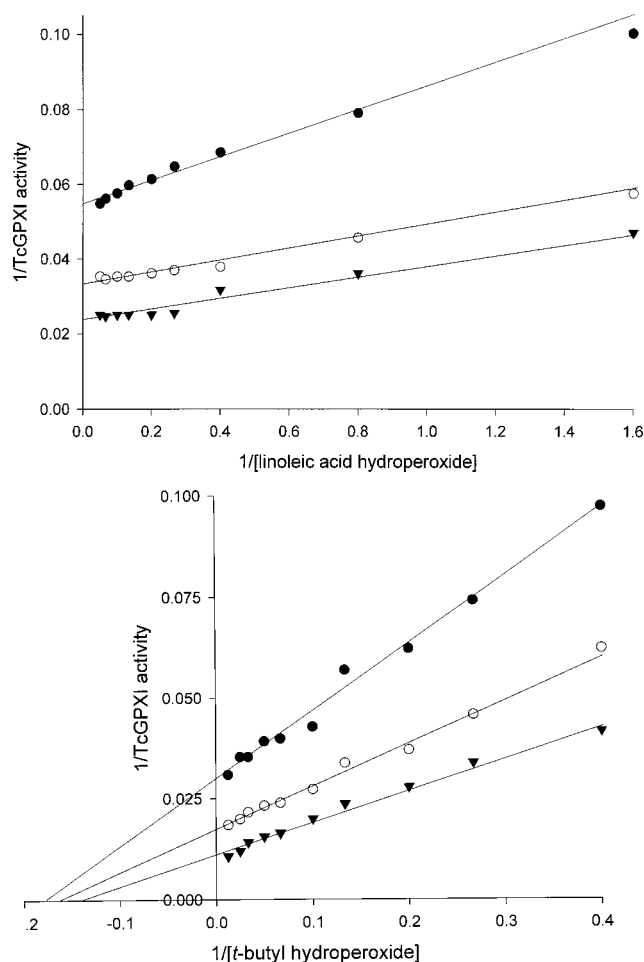
Substrate	Apparent $K_m$ ( $\mu$ M)	Apparent $V_{max}$ (nmol of NADPH oxidized $\text{min}^{-1}$ mg of protein $^{-1}$ )
t-Butyl hydroperoxide	$5.64 \pm 0.53$	$33.66 \pm 0.89$
Cumene hydroperoxide	$1.71 \pm 0.16$	$18.21 \pm 0.22$
Linoleic acid hydroperoxide	$0.58 \pm 0.05$	$18.28 \pm 0.2$
L- $\alpha$ -Phosphatidylcholine hydroperoxide	$0.74 \pm 0.09$	$32.24 \pm 0.89$
Hydrogen peroxide	ND	ND

**Figure 4 Saturation of TcGPXI activity by GSH**

TcGPXI activity was assayed by following NADPH oxidation [18,19] in the presence of various concentrations of GSH (1–20 mM), glutathione reductase and the protein sample. The reactions were initiated by the addition of a fixed concentration (20  $\mu$ M) of either linoleic acid hydroperoxide ( $\bullet$ ) or t-butyl hydroperoxide ( $\circ$ ). TcGPXI activity is expressed as nmol of NADPH oxidized  $\text{min}^{-1}$  mg of protein $^{-1}$  and [GSH] is expressed in mM.

This involved following NADPH oxidation by measuring changes in absorbance at 340 nm. Assays were carried out using either purified recombinant TcGPXI or extracts from *E. coli* XL-1Blue that had been affinity purified in parallel. Such control assays accounted for any non-enzymic background due to auto-oxidation of GSH and also any *E. coli* peroxidase activity that may have been co-purified with TcGPXI. Double reciprocal plots of  $1/\text{TcGPXI activity}$  against  $1/[\text{peroxide}]$  at a fixed concentration of GSH (3 mM) were linear in each case. The apparent  $K_m$  and  $V_{max}$  values for a variety of substrates under these conditions were calculated (Table 1). TcGPXI exhibited a higher affinity towards fatty acid and phospholipid hydroperoxides, as indicated by their lower (submicromolar)  $K_m$  values. There was no detectable activity with hydrogen peroxide.

To determine if TcGPXI activity could be saturated by GSH, assays were carried out using various concentrations of GSH with a fixed amount of either linoleic acid hydroperoxide or t-butyl hydroperoxide (20  $\mu$ M). These data were analysed by using a Hanes plot, which demonstrated a linear relationship between  $[\text{GSH}]/\text{TcGPXI activity}$  and  $[\text{GSH}]$  (Figure 4). From this we determined the  $K_m$  for GSH when using linoleic acid

**Figure 5 Analysis of TcGPXI activity with different concentrations of linoleic acid hydroperoxide (top panel) and t-butyl hydroperoxide (bottom panel)**

TcGPXI activity was assayed by following NADPH oxidation [18,19] in the presence of 3 ( $\bullet$ ), 5 ( $\circ$ ) or 10 ( $\blacktriangledown$ ) mM GSH. The reactions were initiated by the addition of peroxide (0.625–20  $\mu$ M for linoleic acid hydroperoxide or 2.5–80  $\mu$ M for t-butyl hydroperoxide). TcGPXI activity is expressed as nmol of NADPH oxidized  $\text{min}^{-1}$  mg of protein $^{-1}$  while [linoleic acid hydroperoxide] and [t-butyl hydroperoxide] are expressed in  $\mu$ M.

hydroperoxide to be  $5.85 \pm 0.58$  mM and the  $K_m$  for GSH when using t-butyl hydroperoxide to be  $7.86 \pm 1.16$  mM. This study was expanded to investigate whether TcGPXI displays the Ping Pong

kinetics exhibited by the mammalian selenium-dependent GPXs [25–27]. Here GPX assays were carried out using at three different GSH concentrations (3, 5 and 10 mM) over a wide range of peroxide concentrations. Double reciprocal plots of  $1/\text{TcGPXI}$  activity versus  $1/[\text{linoleic acid hydroperoxide}]$  were linear at all three glutathione concentrations (Figure 5, top panel). These converged and allowed a true  $K_m$  for linoleic acid hydroperoxide of  $0.99 \pm 0.24 \mu\text{M}$  to be determined. Similar experiments using *t*-butyl hydroperoxide as substrate also gave linear plots and again these converged, yielding a higher true  $K_m$  of  $4.64 \pm 0.24 \mu\text{M}$  for this substrate (Figure 5, bottom panel).

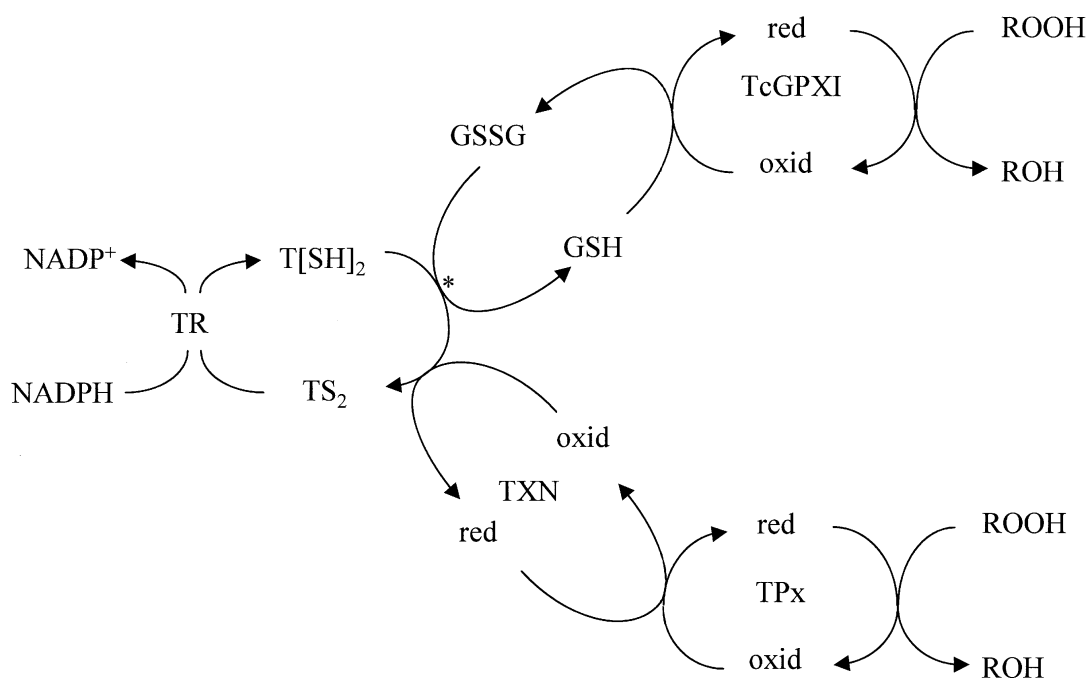
Within trypanosomatids the importance of trypanothione in peroxide metabolism has been well documented [5,7,9–11]. However, when we used trypanothione and the ancillary enzyme trypanothione reductase in place of their glutathione counterparts there was no detectable TcGPXI activity, indicating that trypanothione cannot directly interact with this peroxidase (results not shown).

## DISCUSSION

The only reported peroxidase activity detected within the trypanosomatids has been associated with enzymes belonging to the trypanothione-dependent peroxiredoxin family [9–11,14] (Scheme 1). Here we demonstrate that for the American trypanosome, *T. cruzi*, an additional pathway exists involving a glutathione-dependent peroxidase.

Based on amino acid sequence, subcellular localization and substrate specificity, GPXs can be divided into the three clades; PHGPX, cGPX and plasma GPX (pGPX) [22]. TcGPXI shows extensive similarity to the PHGPX group, which includes GPXs from plants and the mammalian PHGPXs (Figure 1). This clade

differs from other groups in that the proteins contain deletions in two regions thought to mediate dimerization/tetramerization (amino acids 98–105 and 135–152 in the human cGPX sequence; Figure 1). This is believed to account for the observed monomeric nature of mammalian PHGPX enzymes and may play a role in their substrate specificity; mammalian PHGPX and TcGPXI can metabolize phospholipid hydroperoxides whereas other GPXs cannot [22]. Additionally, members of the PHGPX clade do not contain all the residues found in the cGPXs that contribute to the binding of glutathione. Glutathione may not be the principal physiological reductant but no alternative cascade has yet been identified [22]. A significant difference between members of the PHGPX clade is seen in region A (Figure 1). In mammalian PHGPX this region contains a selenocysteine residue, whereas TcGPXI and plant GPXs contain a cysteine. This residue plays a critical role in the antioxidant function of these enzymes. It has been demonstrated that conversion of the selenocysteine into a cysteine in mammalian cGPX and PHGPX affects both the peroxidase activity and alters their sensitivity toward peroxide inactivation [23,24]. These structural features of PHGPXs coupled with their substrate specificity indicate that they may play a significant role in protecting membranes from oxidative damage [28,29]. Evidence to support this comes from subcellular localization studies, which suggest that mammalian PHGPX is found both within the cytosol and as a membrane-bound form [18], and from overexpression studies, which indicate that elevated levels of PHGPX activity protect cells from agents that mediate lipid peroxidation [30]. Recently it has also been shown that PHGPXs may be involved in modulating the activity of other enzymes responsible for the formation of prostaglandins [31–33]. As TcGPXI can metabolize phospholipid hydroperoxides, by analogy with other PHGPXs, its main role within



**Scheme 1** Proposed scheme for trypanothione-dependent peroxide metabolism in trypanosomatids

Trypanothione disulphide ( $\text{TS}_2$ ) is reduced to dihydrotrypanothione ( $\text{T[SH]}_2$ ) by the NADPH-dependent flavoprotein trypanothione reductase (TR). Peroxiredoxins (TPx) reduce hydroperoxide (ROOH) to the corresponding alcohol (ROH) at the expense of dihydrotrypanothione, but only in the presence of the thioredoxin-like molecule, trypanoxin (TXN) [11,12]. Dihydrotrypanothione can also interact (\*) with oxidized glutathione (GSSG) via non-enzymic [33] and enzymic [34,35] mechanisms to generate reduced glutathione (GSH). TcGPXI reduces hydroperoxides at the expense of GSH.

*T. cruzi* may be to minimize or prevent cellular damage due to lipid peroxidation. It will also be of interest to determine if TcGPXI can affect the activity of other enzymes.

The type of kinetics displayed by GPXs towards GSH varies. For the selenium-dependent cGPXs, first-order kinetics have been observed [22,25]. In contrast, the activity of other GPXs, such as pGPX and a *Brugia* GPX, can be saturated readily by this thiol [19,26]. For TcGPXI,  $K_m$  values for GSH were determined when using a saturating concentration (20  $\mu$ M) of either linoleic acid hydroperoxide or t-butyl hydroperoxide (Figure 4). This indicated that TcGPXI displays kinetics towards GSH similar to pGPX and the *Brugia* GPX [19,26]. To examine the effect of GSH in more detail, a series of assays was carried out where the TcGPXI activity was determined over a range of peroxide concentrations at different concentrations of GSH. With linoleic acid hydroperoxide, a group of narrowly converging lines was obtained (Figure 5, top panel) that differed somewhat from the parallel plots characteristic of the Ping Pong kinetics of the mammalian, selenium-dependent GSH peroxidases [25–27]. The low  $K_m$  ( $\approx 1 \mu$ M) renders a more accurate kinetic analysis of the mechanism difficult. The data for t-butyl hydroperoxide yielded double-reciprocal plots that converged to give a higher  $K_m$  (4.64  $\mu$ M; Figure 5, bottom panel). This suggests that the overall rate is limited predominantly by the glutathione-mediated reduction of the oxidized enzyme (as for the selenium-dependent GPX). In the case of linoleic acid hydroperoxide, the reaction with the reduced enzyme is rapid such that the overall rate is only significantly affected at very low hydroperoxide concentrations. For t-butyl hydroperoxide the rate of oxidation with the reduced enzyme is slower and this affects the overall rate of reaction over a wide range of hydroperoxide concentrations. It can therefore be inferred from the difference in the  $K_m$  values for linoleic acid hydroperoxide and t-butyl hydroperoxide that the structural similarity between TcGPXI and mammalian PHGPXs extends to substrate specificity. Thus it appears that the selenium-independent enzyme TcGPXI is most efficient at metabolizing hydrophobic molecules such as fatty acid and phospholipid hydroperoxides. Short-chain organic hydroperoxides such as t-butyl hydroperoxide may be more efficiently detoxified by other components of the *T. cruzi* oxidative defence system.

TcGPXI has been shown to mediate glutathione-dependent metabolism of a wide range of hydroperoxides (Table 1 and Figure 5). Attempts to use the trypanothione system as an electron donor gave no demonstrable activity. These findings now allow us to propose a new, expanded model for enzyme-mediated peroxide metabolism in *T. cruzi* (Scheme 1). Within trypanosomatids, glutathione reductase activity has not been detected and glutathione is maintained in its reduced form by interaction with trypanothione. This can occur non-enzymically [34] or via the activity of a thiol transferase [35,36]. Whichever mechanism occurs (possibly both), glutathione is reduced within the parasite at the expense of dihydrotrypanothione and this ultimately maintains TcGPXI in its reduced active form (Scheme 1).

Sequences with the potential to encode GPXs are also present in the *Trypanosoma brucei* database (accession numbers AQ647037 and AQ651227). Thus it appears that these enzymes, which were believed to be absent from trypanosomatids, probably occur across the group. The enzymic basis of peroxide metabolism in these organisms is therefore more complicated than previously thought. However, our results also indicate that the trypanothione system remains central to the process (Scheme 1). This strengthens further the case for trypanothione reductase and enzymes involved in trypanothione biosynthesis being considered as potential chemotherapeutic targets.

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