Distinct Mitochondrial and Cytosolic Enzymes Mediate Trypanothione-dependent Peroxide Metabolism in Trypanosoma cruzi

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The American trypanosome Trypanosoma cruzi is exposed to toxic oxygen metabolites that are generated by drug metabolism and immune responses in addition to those produced by endogenous processes. However, much remains to be resolved about the parasite oxidative defense system, including the mechanism(s) of peroxide reduction. Here we show that reduction of peroxides in T. cruzi is catalyzed by two distinct trypanothione-dependent enzymes. These were localized to the cytosol and mitochondrion. Both are members of the peroxiredoxin family of antioxidant proteins and are characterized by the presence of two conserved domains containing redox active cysteines. The role of these proteins in protecting T. cruzi from peroxide-mediated damage was demonstrated following overexpression of enzyme activity. The parasite-specific features of T. cruzi cytoplasmic peroxiredoxin and T. cruzi mitochondrial peroxiredoxin may be exploitable in terms of drug development.

In South America up to 20 million people are infected with the protozoan parasite Trypanosoma cruzi, the causative agent of Chagas’ disease (1). There is no immediate prospect of a vaccine, and the development of more effective chemotherapy is a priority (2). The drugs in current use, benznidazole and nifurtimox, often have toxic side effects and can fail to eradicate parasitemia. The precise mechanisms of action of these drugs are unknown, although both have been shown to undergo redox cycling within the parasite (3–5). It has been proposed that the trypanocidal activity of nifurtimox may result in part from the generation of nitro radicals mediated by various parasite reductases (5, 6). Under aerobic conditions, futile cycling of these nitro radicals gives rise to the formation of toxic oxygen metabolites (5, 7, 8). This, together with reports that T. cruzi has a limited ability to metabolize peroxides (9), has encouraged the view that components of the parasite oxidative defense system may have potential as chemotherapeutic targets (10).

T. cruzi is also exposed to toxic oxygen/nitrogen metabolites generated by immune processes. The major T. cruzi-killing mechanism in activated macrophages involves the production of nitric oxide (11). In combination with the superoxide anion (O2·−), this results in the formation of peroxynitrite, a toxic molecule that kills T. cruzi in a dose-dependent manner (12). In T. cruzi, O2·− is removed by iron superoxide dismutase, and genes encoding both cytosolic- and mitochondrial-localized enzymes have been isolated (13, 14). Trypanosomatid, including T. cruzi, are devoid of catalase and glutathione peroxidase (10), and it is presumed that the low molecular weight thiol trypanothione plays a central role in peroxide metabolism (15–17). Trypanothione is a glutathione:serendipine conjugate (N2,N8-bisglutathionylserendipine) and is maintained in its reduced form by the NADPH-dependent flavoprotein trypanothione reductase. In the cytosol of the insect trypanosomatid Crithidia fasciculata, trypanothione-dependent peroxide metabolism has been proposed to involve a unique cascade of enzymes (18, 19). One of these (tryparedoxin) has some similarity to thioredoxin proteins (20). A second, tryparedoxin peroxidase, is a member of the peroxiredoxin family of antioxidant proteins (21). In T. cruzi, the presence of the cytosolic peroxiredoxin system remains to be confirmed, and it has even been proposed that H2O2 metabolism is a nonenzymatic trypanothione-dependent process (9). An alternative H2O2-scavenging system has been suggested within T. cruzi based on the level of reduced ascorbate found in this organism (22). Ascorbate is maintained in this redox state by dihydroascorbate reductase, the activity of which has been detected within parasite extracts (22). However, this activity has also been attributed to a nonenzymatic interaction of dihydroascorbate with trypanothione (23). The mechanism of peroxide metabolism within the trypanosomatid mitochondrion is unknown.

Here we demonstrate that in T. cruzi distinct trypanothione-dependent members of the peroxiredoxin family are expressed in the mitochondrion and cytosol. Furthermore we show that overexpression of both enzymes results in parasites that are resistant toward exogenous peroxides.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—T. cruzi (MHOM/BR/78/Silvio-X106 or CL-Brener) epimastigotes were grown at 28 °C in RPMI 1640 medium (Sigma) containing the supplements previously described (24). Recombinant T. cruzi were maintained in the same medium containing 200 μg ml−1 of G418.

DNA and RNA Extractions—T. cruzi genomic DNA was isolated from exponentially growing cells using the proteinase K/SDS method (25). Intact T. cruzi chromosomes for contour clamped homogenous electric field (CHEFE) analysis were extracted using an agarose-embedding technique (26). T. cruzi total RNA was prepared using the guanidinium thiocyanate lysis method (25).

Cloning of TcCPX and TcMPX—DNA fragments containing the

1 The abbreviations used are: TcCPX, T. cruzi cytoplasmic peroxiredoxin; TcMPX, T. cruzi mitochondrial peroxiredoxin; TcTS, trypanothione reductase.
5'-coding sequence of TcMPX and TcCPX were amplified from parasite epimastigote RNA using a sense primer corresponding to the T. cruzi spliced leader sequence (TcSL, gcattcGAGTCTGCTGCTGATGTTAG) and a degenerate antisense primer (TP1, gcattcGAG/GCAIAAC/GAAICTAGCAAATGCTG, designed to the conserved amino acid sequence, DPTFVC) (Fig. 1) (27). Restriction sites (lower case, italics) were incorporated into the primers to facilitate DNA cloning. A full-length copy of the TcMPX gene was obtained by screening a T. cruzi (CL-Brener) genomic DNA cosmid library that had been plated as an array of 7200 clones (25). The remainder of the TcCPX coding sequence was amplified from T. cruzi genomic DNA using the primers TPx1 (gcattcGAGTCTGCTGCTGACGT) and TPx2 (gcattcGAGGCGGGGGTTGGTTAG).  

Construction of T. cruzi Transformation Vectors and Parasite Transformation—The vectors used in the localization of the peroxiredoxin enzymes were constructed as follows. The primers tagg (gcattcTGGTTTCGCGGTAAGG) and tagt (gcattcGCGTTTTTCTCAAAAAATTTCC) were used to amplify the TcMPX coding sequence from T. cruzi cDNA. The amplified DNA fragment was purified and ligated into the BamHI/EcoRV sites of the trypanosomal vector pTEX-9E10 (29). The ligation was performed such that a c-myc-derived epitope (9E10) was inserted in-frame to the 3' end of the peroxiredoxin gene to produce the construct pTEX-MPX-9E10. The same approach was used to construct the vector pTEX-CPX-9E10. Here the primers TPx4 (gcattcGAAACAATGTCCTGGCTAGGA) and TPx5 (gcattcGCGGGAAGATTCC) were used to amplify the TcCPX coding sequence. The amplified DNA fragment was purified and ligated into the EcoRI/EcoRV sites of pTEX-9E10.  

The two DNA vectors used in overexpression studies were constructed as follows. For TcMPX, the entire gene plus flanking sequences were excised from a cosmid as an 880-bp HindIII/Csal fragment. This was then cloned into the HindIII/EcoRV sites in the trypanosomal expression vector pTEX (30) to form pTEX-MPX. For TcCPX, the complete gene was amplified using the primers TPx4 and TPx6 (gcattcTCA-CAATGGAGACTGTGAAAC). The resulting 600-bp fragment was purified and cloned into the HindIII/EcoRI sites within pTEX to form pTEX-CPX. These plasmids were introduced into T. cruzi by electroporation and selected using the conditions previously described (31).  

Localization of TcMPX and TcCPX by Electron Microscopy—The experiments described were carried out by the electron microscopy unit at the London School of Hygiene and Tropical Medicine. Parasite samples (2 × 10⁶ cells ml⁻¹) were fixed in 2% paraformaldehyde in growth medium (minus fetal calf serum) and embedded in agar. The blocks were infiltrated with polyvinylpyrrolidone/sucrose and snap-frozen in liquid nitrogen. Ultrathin sections (90–100 nm) were made and collected on pilaform-coated nickel grids, and immunogold labeling was performed as described (32). The primary antibody used in this detection was as a mouse anti-c-myc (9E10) monoclonal antibody (Santa Cruz) (33). Controls were carried out on all samples where the primary antibody step was replaced with a phosphate-buffered saline incubation.  

Enzyme Assay—T. cruzi epimastigotes in the logarithmic phase of growth were pelleted and washed in argon-saturated ice-cold HE buffer (50 mM HEPES, 1 mM EOTA, pH 7.6). The cell pellet was resuspended (2 × 10⁹ cells ml⁻¹) in argon-saturated HE buffer containing 1 mM dithiothreitol plus a mixture of protease inhibitors, and the cells were lysed by repeated (3×) freeze-thawing. The lysate was clarified (13,000 × g, 10 min, 4 °C), and the supernatants were dialyzed against argon-saturated HE (3 × 100 vol) at 4 °C. The protein concentrations of the parasite extracts were determined using the BCA protein assay system ( Pierce). Peroxidase activity was measured by coupling it to NADPH oxidation and following the change in absorbance at 340 nm as described (19). To determine the relative level of overexpression of TcMPX and TcCPX, the enzymatic peroxidase activities were compared with the activity of the housekeeping enzyme alanine aminotransferase (34).

RESULTS

Isolation of T. cruzi Peroxiredoxin Gene Homologues—Comparison of peroxiredoxin proteins from various organisms has shown that this family of antioxidant enzymes contains two conserved domains (27). A degenerate oligonucleotide primer, TP1, was designed to one of these regions (Fig. 1, region I) and used in conjunction with the T. cruzi spliced leader primer in a reverse transcriptase-polymerase chain reaction reaction with T. cruzi epimastigote RNA. This generated two DNA fragments of 230 and 400 bp. Sequence analysis indicated that the two fragments were derived from distinct genes. The 230-bp fragment contained an open reading frame (ORF) of 157 bp with a putative 5’-untranslated region of 60 bp, whereas the 400-bp fragment contained an ORF of 246 bp and a 5′-untranslated region of 118 bp. BLAST searches revealed that the deduced amino acid sequence encoded by both ORFs had similarity to the amino termini of proteins in the peroxiredoxin family.  

Using the 400-bp fragment, a clone was isolated from a cosmid library (28, 35). The full-length gene (designated TcMPX) was localized to a 3.8-kb MinI fragment. DNA sequencing identified an ORF of 681 bp with the potential to encode a peptide of 25 kDa (Fig. 1). Southern hybridization indicated that TcMPX was a single-copy gene, which in the CL-Brener clone of T. cruzi, was localized on two chromosome homologues of 160 kb and 1000 kb (data not shown). A distinctive feature of the TcMPX sequence was the presence of an amino-terminal extension of 30 amino acids, characterized by the presence of hydrophobic and basic amino acids and a lack of acidic residues. Analysis using the PSORT program (36) predicted that this leader region contained a mitochondrial-targeting signal.  

To clone a full-length copy of the second gene (designated TcCPX), a DNA amplification approach was used. Southern analysis had demonstrated that multiple copies of this gene were present within the T. cruzi genome, organized predominantly as a 2.8-kb tandem repeat and localized to a single chromosome band of 800 kb (data not shown). DNA primers were designed to sequences within the 230-bp fragment and used to amplify across the tandem repeats. DNA sequencing of the product identified an ORF of 600 bp with the potential to encode a protein with a molecular mass of 20 kDa (Fig. 1).
Sequencing of several cloned tandem repeat fragments obtained from various DNA amplification reactions suggested that all copies of TcCPX within the repeat were identical. TcCPX and TcMPX both contain the two domains characteristic of the 2-cys subgroup of the peroxiredoxin family of antioxidant enzymes (Fig. 1; regions I and II). The domains have cysteine residues that in other peroxiredoxins have been implicated in mediating peroxidase activity (37, 38). Although both TcCPX and TcMPX are identical in region I (PTFVCPE), they diverge within region II. The sequence in this region for TcCPX is identical to that found in most other 2-cys peroxiredoxins (HGEVCPA), whereas for TcMPX the sequence is slightly different (NGDVIPC) (Fig. 1). Some other 2-cys peroxiredoxin proteins, including yeast thioredoxin peroxidase, also show differences within this region, but this does not appear to affect the antioxidant activity of these enzymes (39).

**Dimerization of TcCPX and TcMPX Is Mediated by Intermolecular Disulfide Bonds**—TcMPX and TcCPX were tagged at the carboxyl terminus with a 10-amino acid epitope from the human c-myc protein. This was carried out using the T. cruzi expression vector pTEX-9E10 (29). Each ORF was ligated upstream to and in-frame with the epitope to produce the constructs pTEX-MPX-9E10 or pTEX-CPX-9E10. These were introduced into T. cruzi by electroporation, and G418 resistant cells were selected. The recombinant cell lines were verified by hybridization (data not shown) and by Western blot analysis using a monoclonal antibody against the epitope (Fig. 2). The antibody did not cross-react with wild type T. cruzi cell extracts but did detect bands within cell extracts derived from the recombinant lines. For both proteins, a single band of approximately 25 kDa was observed in cell extracts treated with the reductant 2-mercaptoethanol, whereas in nonreduced samples, a predominant band of approximately 50 kDa was observed (Fig. 2). The 25-kDa band is approximately the size expected for the tagged peroxidases and corresponds to the reduced, monomeric form, whereas the 50-kDa band corresponds to the oxidized, homodimeric form. The capacity of peroxiredoxins to form dimers is mediated by the cysteines within the two conserved domains (37, 38). The data indicate that this process is not affected by the presence of the carboxyl tag. It can be inferred from the amino acid sequences (Fig. 1) that TcMPX is slightly larger than TcCPX. However, the proteins detected here are approximately the same size (Fig. 2). This suggests that TcMPX may undergo post-translational processing.

**Subcellular Localization of TcMPX and TcCPX**—The T. cruzi cell lines expressing the carboxyl-terminal-tagged proteins were used to determine the subcellular location of TcMPX and TcCPX using immunoelectron microscopy (Fig. 3). Little or no labeling was observed in T. cruzi wild type cells (Fig. 3A) or in controls where the primary antibody (mouse anti c-myc (9E10)) had been replaced with a phosphate-buffered saline incubation step (data not shown). Analysis of parasite cells expressing tagged TcMPX showed extensive labeling of the mitochondrion (Fig. 3, B and C). T. cruzi and other trypanosomatids have a single large mitochondrion that contains the kinetoplast, an electron dense region situated between the nucleus and flagellar pocket, within which the mitochondrial genome is located. In T. cruzi, the mitochondrial DNA constitutes approximately 20% of the cellular total. In Fig. 3, B and C, the labeling is found predominantly toward the periphery of the cell with an apparent concentration around the kinetoplast. Therefore, one role of TcMPX may be to protect mitochondrial DNA from peroxide-mediated damage. In contrast to the localization of TcMPX, cells expressing the recombinant TcCPX showed extensive labeling throughout the cytosol (Fig. 3, D and E). No labeling was detected within the mitochondrion. Thus T. cruzi expresses at least two distinct peroxiredoxin proteins with different subcellular localizations.

**Overexpression of TcCPX and TcMPX in T. cruzi Confers Resistance to Exogenous Peroxides**—To investigate the role of TcCPX and TcMPX, we overexpressed both proteins within the parasite. For TcMPX, this was achieved by cloning a cosm id DNA fragment containing the entire TcMPX ORF plus 5′- and 3′-flanking sequences into the polylinker of the trypanosomal expression vector pTEX (30). For TcCPX, the entire ORF was
amplified from cDNA and subcloned (“Experimental Procedures”). The resulting constructs, pTEX-MPX and pTEX-CPX, were then introduced into T. cruzi by electroporation, and G418 resistant cell lines were selected. The presence of multiple episomal copies of each plasmid within the parasite was verified by Southern hybridization (data not shown), and elevated expression was confirmed by RNA hybridization (Fig. 4).

The parasite cell lines which overexpress TcCPX or TcMPX were grown in the presence of either H_{2}O_{2} or the organic peroxide t-butyl hydroperoxide, and the concentrations that inhibit parasite growth by 50% (IC_{50}) were determined. When exposed to exogenous H_{2}O_{2}, both cell lines showed an approximate 2-fold increase in resistance compared with control cultures (Table I). A similar increase in resistance to t-butyl hydroperoxide was observed (Table I). Thus, both proteins, when expressed within the parasite, confer resistance toward peroxides even though they have different subcellular locations. The studies were extended to investigate the effect of the trypanocidal agents nifurtimox and benzimidazole on parasite growth. These drugs are believed to undergo redox cycling within T. cruzi, leading to the production of toxic oxygen metabolites. However, after treatment with these agents, the IC_{50} of both cell lines did not differ significantly from controls (Table I).

**TcCPX- and TcMPX-mediated Peroxide Metabolism Is Trypanothione-dependent—**We investigated the biochemical nature of the peroxide metabolism by assaying cell extracts obtained from wild type and pTEX-MPX- or pTEX-CPX-transformed T. cruzi for total peroxidase activity. To determine if the excess activity associated with TcMPX and TcCPX overexpressed from episomal copies of each plasmid within the parasite was verified, as described previously (51). The data are the mean from three experiments ± S.D. The differences observed for susceptibility toward peroxides in the control and transformed cell lines were statistically significant (P < 0.01), as assessed by Student’s t test.

### Table I

| Agent                  | Wild type cells | pTEX-MPX-transformed cells | pTEX-CPX-transformed cells |
|------------------------|-----------------|----------------------------|---------------------------|
|                        | IC_{50} (μM)    |                            |                           |
| H_{2}O_{2}             | 94.2 ± 15.3     | 199.2 ± 20.0               | 194.2 ± 6.6               |
| t-Butylhydroperoxide   | 10.9 ± 0.1      | 18.4 ± 0.8                 | 17.7 ± 0.2                |
| Nifurtimox             | 1.6 ± 0.1       | 1.6 ± 0.1                  | 1.5 ± 0.1                 |
| Benzimidazole          | 3.1 ± 0.1       | 3.3 ± 0.4                  | 3.3 ± 0.3                 |

**FIG. 5.** Trypanothione-dependent H_{2}O_{2} metabolism by T. cruzi cell extracts overexpressing TcMPX and TcCPX. Total peroxide reduction was assayed in a reaction mixture containing 100 mM HEPES, pH 7.6, 0.5 mM EDTA, 150 μM NADPH, and 1 mg of dialyzed cell extract to which 20 μM TS_{2} (Bachem) and 100 μM H_{2}O_{2} (Sigma) were added where indicated. A and E, H_{2}O_{2} metabolism in the absence of a cell extract but in the presence of trypanothione reductase (TR, 220 milliunits); B and F, H_{2}O_{2} metabolism by T. cruzi wild type cell extract; C and G, H_{2}O_{2} metabolism by T. cruzi cell extract overexpressing TcMPX; D and H, H_{2}O_{2} metabolism by T. cruzi cell extract overexpressing TcCPX. All assays were carried out at 27 °C. The peroxidase activity is expressed as nmol of NADPH oxidized min^{-1} mg^{-1} (traces B-D and F-H) or nmol NADPH oxidized min^{-1} ml^{-1} (traces A and E). The peroxidase activity value in traces B-D and F-H represent the enzymatic component of the total peroxidase activity, i.e., the nonenzymatic activity subtracted from total peroxidase activity. The activities are the mean from three experiments ± S.D. from the mean.

order in which TS_{2} and H_{2}O_{2} were added to the assay. In B–D, the observed increase in NADPH oxidation is due to the H_{2}O_{2} alone, whereas the increase in NADPH oxidation in F–H is due to the combined effect of TS_{2} and H_{2}O_{2}. These experiments...
clearly demonstrate that H$_2$O$_2$ does not affect the rate of NADPH oxidation unless trypanothione is present. Denaturation of the cell extract before the assay abolished this activity (data not shown). Previous studies have suggested that peroxidase metabolism in *T. cruzi* is nonenzymatic and occurs via the spontaneous interactions of the oxidant with trypanothione (9). To determine the capacity for nonenzymatic trypanothione-mediated peroxide metabolism, parallel assays were carried out where the cell extract was replaced with trypanothione reductase. In these experiments, a nonenzymatic activity was observed, but this was considerably lower than the total peroxidase activity detected when a cell extract was present (Fig. 5, A and E). The enzymatic peroxidase activities for each extract were calculated and standardized against the activity of an unrelated enzyme (alanine aminotransferase). This showed that cell extracts from the TcMPX-overexpressing cell line had a peroxidase activity 2-fold that of the wild type, whereas the extract prepared from cells overexpressing TcCPX showed a 5-fold increase in activity. These values underestimate TcCPX and TcMPX overexpression, since the level of activity in control extracts is derived from both enzymes plus any other NADPH-dependent peroxidase activities present in the cell. Experiments were carried out to determine if TcMPX and TcCPX exhibited peroxidase activity in the presence of glutathione/glutathione reductase or *Escherichia coli* thioredoxin/thioredoxin reductase. No differences in NADPH oxidation were observed between the dialyzed extracts when either of these redox systems were used as electron donor (data not shown). Thus, under the conditions used in these assays, both TcMPX and TcCPX display a peroxidase activity that is trypanothione-specific.

**DISCUSSION**

Since the observation was made that the cytosolic and mitochondrial fractions of *T. cruzi* extracts can both generate significant levels of H$_2$O$_2$ (40, 41) it has been assumed that an effective peroxide metabolizing system would be present in both compartments. However the mechanisms of peroxide detoxification in *T. cruzi* have remained unresolved, and it has even been proposed that the process is not enzyme-mediated (9). In this paper we now demonstrate the existence of distinct mitochondrial (TcMPX) and cytosolic (TcCPX) peroxidases in *T. cruzi* and show that both enzymes play important roles in peroxide metabolism.

TcMPX and TcCPX are members of the peroxiredoxin family of antioxidant proteins but differ from their mammalian counterparts in being trypanothione-dependent. In most eukaryotes, peroxiredoxins are part of a three-component pathway that also includes thioredoxin and thioredoxin reductase. These proteins act in concert to mediate electron transfer to the peroxiredoxin, with NADPH acting as the initial donor (39, 42, 43). Recent studies with the insect trypanosomatid *C. fasciculata* have led to the identification of a cytosolic peroxiredoxin, designated tryparedoxin peroxidase (18, 19). This was shown to undergo trypanothione-dependent redox cycling but only in the presence of tryparodoxin, a thioredoxin-like molecule. On the basis of amino acid sequence (Fig. 1), cytosolic location (Fig. 3), and trypanothione-dependent activity (Fig. 5), TcCPX appears to be a homologue of tryparedoxin peroxidase. The widespread distribution of TcCPX throughout the cytosol of *T. cruzi* (Fig. 3) may reflect a general peroxide-scavenging role.

TcMPX is also a trypanothione-dependent peroxidase (Fig. 5) but is characterized by a mitochondrial location (Fig. 3). By implication, either the mitochondrion contains all the redox machinery required to maintain TcMPX in its reduced form, or the redox pathway is partitioned between the cytosol and the mitochondrion. The subcellular distribution of trypanothione has not been reported, but biochemical evidence from the African trypanosome *Trypanosoma brucei* strongly suggests that the ancillary enzyme trypanothione reductase is restricted to the cytosol (44). If this is also the case in *T. cruzi*, our identification of a mitochondrial enzyme that is trypanothione-dependent suggests the presence of an unidentified thiol transporter and/or reduction system. Such a system would be of considerable interest, particularly as a target for chemotherapy. In other eukaryotic cells, the predominant low molecular weight thiol glutathione is found at high levels in the mitochondria. In the absence of a biosynthetic pathway within the organelle, glutathione is translocated from the cytosolic pool to the mitochondrial matrix. The exact transport mechanism(s) is unknown but may be multicomponent (45) and involve the dicarboxylate and 2-oxoglutarate carriers of the mitochondrial inner membrane (46). The presence of other glutathione transporter systems has not been excluded. In trypanosomatids, because of the unique nature of trypanothione, it is likely that any transporter/exchanger system will be mechanistically distinct from the corresponding glutathione system(s) in the mammalian host. It is also possible that TcMPX is part of a mitochondrial redox pathway, analogous to that present in the cytosol. Attempts to address this using heterologous gene expression systems have been limited by the observation that both TcMPX and a deletion derivative (minus the putative mitochondrial leader sequence) are insoluble when expressed in *E. coli*. Mitochondrial-localized thioredoxins have been identified in mammalian cells (42, 47) but not so far in trypanosomes.

The precise role of the TcMPX peroxidase activity within the mitochondrion of *T. cruzi* remains to be determined. H$_2$O$_2$ can react with a variety of biological macromolecules including DNA. However the major cellular damage is thought to result from the formation of highly active hydroxyl radicals (HO$^·$), which are generated via the Fe$^{2+}$-mediated Fenton reaction. HO$^·$ reacts at the site of formation and can cause DNA strand breakage and mutagenesis (48). There is no enzymatic defense against this short-lived radical (half-life 10$^{-9}$ seconds) except by preventing the build up of the reactive oxygen species that lead to its production. The concentration of TcMPX in the vicinity of the kinetoplast (Fig. 3) suggests that a major function of this enzyme may be to protect the mitochondrial genome from direct or indirect peroxide-mediated damage.

Previous experiments with *Leishmania* have shown that although trypanothione reductase appears to be essential to the parasite (49, 50), the level of enzyme activity is not a rate-limiting step in peroxide metabolism in situations where the enzyme is overexpressed (51). Here we have investigated the relative importance of TcCPX and TcMPX in this process. We found that cell extracts obtained from transformed *T. cruzi* that overexpressed either peroxidase exhibited enhanced trypanothione-dependent peroxide metabolism (Fig. 5). Furthermore, the recombinant cells were also found to have increased resistance to exogenous H$_2$O$_2$ and t-butylhydroperoxide (Table I). It can therefore be inferred that the level of each enzyme is a rate-limiting factor in peroxide metabolism within their respective subcellular compartments. Peroxides are uncharged molecules and can cross plasma or organelle membranes. Our observation that overexpression of compartmentalized peroxidases in *T. cruzi* protects the cell from the toxic effects of exogenous peroxides indicates that these oxidants readily gain entry into the cytosol and the mitochondrion and that the increased peroxide metabolism at both these sites can confer protection against cellular damage. Thus in their biological context, both TcMPX and TcCPX are capable of protecting the parasite from peroxides of both endogenous and exogenous significance.
origin. We also investigated the importance of TcCPX and TcMPX to benzimidazole- and nifurtimox-treated \textit{T. cruzi} parasites. The generation of toxic oxygen metabolites by nifurtimox is well documented (5, 7, 8), although the precise \textit{T. cruzi}-killing activity has yet to be established. Cell lines overexpressing either peroxidase were equally as susceptible to these agents as the wild type (Table I), suggesting that the predominant killing activity of these drugs is not by peroxide-mediated damage.

In summary, we have demonstrated an enzymatic basis for cytosolic and mitochondrial peroxide metabolism in \textit{T. cruzi}. The parasite-specific features of these enzymes suggest that they may have potential as chemotherapeutic targets.

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REFERENCES

1. World Health Organization (1990) \textit{Weekly Epidemiol. Rep.} \textbf{65}, 257–262
2. Twelfth Programme Report of the UNDP/World Bank/WHO Programme for Research and Training in Tropical Diseases (TDR) (1995) World Health Organization, Geneva
3. Giuli, C., Turrens, J. F., and Boveris, A. (1988) \textit{Biochem. Pharmacol.} \textbf{37}, 218–226
4. Peng, C., Bettache, N., Cenas, N., Krauth-Siegel, R. L., Chauviere, G., and Giulivi, C. (1998) \textit{Free Radicals in Tropical Diseases} (Aruoma, O. I., ed) Harwood Academic Publishers GmbH, Chur Switzerland
5. Moreno, S. N., Mason, R. P., and Docampo, R. (1984) \textit{Infect. Immun.} \textbf{5}, 127–136
6. Tovar, J., Wilkinson, S., Mottram, J. C., and Fairlamb, A. H. (1998) \textit{Mol. Biochem. Parasitol.} \textbf{96}, 111–123
