Substrate Specificity, Localization, and Essential Role of the Glutathione Peroxidase-type Tryparedoxin Peroxidases in Trypanosoma brucei

Received for publication, November 27, 2004, and in revised form, January 12, 2005
Published, JBC Papers in Press, January 21, 2005, DOI 10.1074/jbc.M413338200

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Trypanosoma brucei, the causative agent of African sleeping sickness, encodes three nearly identical cysteine homologues of the classical selenocysteine-containing glutathione peroxidases. Although one of the sequences, peroxidase III, carries both putative mitochondrial and glycososomal targeting signals, the proteins are detectable only in the cytosol and mitochondrion of mammalian bloodstream and insect procyclic T. brucei. The enzyme is a trypanothione/tryparedoxin peroxidase as are the 2 Cys-peroxiredoxins of the parasite. Hydrogen peroxide, thymine hydroperoxide, and linoleic acid hydroperoxide are reduced with second order rate constants of 8.7 × 10^4, 7.6 × 10^4, and 4 × 10^4 M^-1 s^-1, respectively, and represent probable physiological substrates. Phosphatidylcholine hydroperoxide is a very weak substrate and, in the absence of Triton X-100, even an inhibitor of the enzyme. The substrate preference clearly contrasts with that of the closely related T. cruzi enzyme, which reduces phosphatidylcholine hydroperoxides but not H_2O_2. RNA interference causes severe growth defects with second order rate constants of 8.7 × 10^4, 7.6 × 10^4, and 4 × 10^4 M^-1 s^-1, respectively, and represent probable physiological substrates. Phosphatidylcholine hydroperoxide is a very weak substrate and, in the absence of Triton X-100, even an inhibitor of the enzyme. The substrate preference clearly contrasts with that of the closely related T. cruzi enzyme, which reduces phosphatidylcholine hydroperoxides but not H_2O_2. RNA interference causes severe growth defects in bloodstream and procyclic cells in accordance with the peroxidases being essential in both developmental stages. Thus, the cellular functions of the glutathione peroxidase-type enzymes cannot be taken over by the 2 Cys-peroxiredoxins that also occur in the cytosol and mitochondrion of the parasite.

The causative agents of African sleeping sickness (Trypanosoma brucei gambiense and T. brucei rhodesiense), Nagana cattle disease (Trypanosoma congolense, T. brucei brucei), South-American Chagas' disease (Trypanosoma cruzi), and the different forms of leishmaniasis, differ from all other known eukaryotes and prokaryotes in their unique thiolo redox system. The thiol-polyamine conjugate trypanothione (N^1,N^8-bis(glutathionyl)spermidine) (1) and the flavoenzyme trypanothione reductase (TR)^1 replace the nearly ubiquitous glutathione/gluta-

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* This work was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 544 "Control of Tropical Infectious Diseases," project B3. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: TR, trypanothione reductase; ROOH, hydroperoxide; GPX, glutathione peroxidase; PhGPX, phospholipid hydroperoxide glutathione peroxidase; Px, glutathione peroxidase-type tryparedoxin peroxidase (Cys homologue of glutathione peroxidase); Pex, peroxin; BiP, endoplasmic reticulum chaperon (binding protein).
(GPX I), also known as classical or cellular glutathione peroxidase, the gastrointestinal Gl-GPX (GPX II), the extracellular pGPX (GPX III) found in human plasma, and the phospholipid hydroperoxide glutathione peroxidase (PhGPX) (GPX IV). The cytosolic glutathione peroxidase uses glutathione as reducing substrate, whereas the extracellular enzyme accepts thioredoxin and glutaredoxin and PhGPX can be reduced by a variety of protein thiols (18, 19). Selenocysteine-containing glutathione peroxidases have so far only been found in vertebrates and a GPX IV type enzyme in the helminth Schistosoma mansoni (20). In contrast, genes encoding cysteine homologues such as the T. cruzi and T. brucei peroxidases are widely distributed in nature. Only a few of these enzymes have been functionally characterized. All of them have much lower peroxidase activities than the mammalian seleno-enzymes (14, 21).

A cysteine homologue from the malarial parasite Plasmodium falciparum reduces H$_2$O$_2$ but not at all phosphatidylcholine hydroperoxide and proved to be much more efficient with thioredoxin than with glutathione as the electron source (21). An enzyme from the nematode Brugia pahangi reduces linoleic acid hydroperoxide and phosphatidylcholine hydroperoxide but not H$_2$O$_2$ with glutathione as the reducing substrate. It is a secreted glycoprotein, and the natural electron source is not known (22).

Here we will report on the substrate specificity and subcellular occurrence of the T. brucei glutathione peroxidase-type enzymes. African trypanosomes multiply as bloodstream parasites in the mammalian host and as procyclic parasites in the tsetse fly. Cell fractionation studies revealed the proteins in the cytosol and the mitochondrion of both cell types. No peroxidase was detectable in glycosomes, although one protein sequence contains a putative glycosomal/peroxisomal targeting signal. Hydrogen peroxide, thymine hydroperoxide, and linoleic acid hydroperoxide are probable physiological substrates of the T. brucei peroxidase. The results of RNA interference experiments demonstrated that the enzyme is also essential for procyclic in addition to bloodstream T. brucei.

**EXPERIMENTAL PROCEDURES**

**Materials**—The recombinant glutathione peroxidase-type T. brucei trypanothione reductase (24) were prepared as described. Polyclonal rabbit antibodies against the recombinant His-tagged T. brucei peroxidase III (14), T. brucei trypanothione reductase (25), and T. cruzi trypanothione reductase (24) were prepared as described. Polyclonal rabbit antibodies against the recombinant His-tagged T. brucei peroxidase III (14), T. brucei trypanothione reductase (25), and T. cruzi trypanothione reductase were produced by Bioscience, Göttingen, Germany and Eurogentec, Belgium, respectively. The TR antibodies were purified by affinity chromatography on recombinant T. cruzi TR.

**Peroxidase Standard Assay**—The trypanothione/tryparedoxin-dependent peroxidase activity of Px III was followed at 25 °C in a total volume of 150 μl of 0.1 mM Tris, 5 mM EDTA, pH 7.6, containing 240 μM NADPH, 100 μM trypanothione disulfide, 150 milliunits of TR, 10 μM trypanothione disulfide, and 0.05 μM peroxidase in the case of thymine hydroperoxide and 0.11 μM enzyme when H$_2$O$_2$ or linoleic acid hydroperoxide and 0.05 μM peroxidase when 1-butyl hydroperoxide or phosphatidylcholine hydroperoxide were studied as substrates. The reaction was started by adding 100 μM hydroperoxide substrate, and NADPH consumption was followed at 340 nm. The total concentration of hydroperoxide in the assay was determined by allowing the reaction to run to completion. Reduction of phosphatidylcholine hydroperoxide by rat PhGPX was measured as the control.

**Kinetic Analysis of Different Hydroperoxide Substrates**—The assays contained in a total volume of 150 μl of 0.1 mM Tris, 5 mM EDTA, pH 7.6, 240 μM NADPH, 100 μM trypanothione disulfide, 150 milliunits of TR, 1–10 μM trypanothione disulfide, and 0.4 or 1.5 μM peroxidase when thymine hydroperoxide and linoleic acid hydroperoxide were studied as the substrate, respectively. The reaction was started by adding 50 μM hydroperoxide. Concentration dependence of the initial velocities was obtained by analyzing the time progression curve of NADPH consumption in a Beckman DU65 spectrophotometer with data acquisition every 1.5 s. The data were analyzed using the integrated Dalziel equation for a two-substrate enzyme reaction (30, 31) (Equation 1).

\[
\text{Reaction rate} = \frac{[\text{ROOH}]}{[\text{ROH}]} = \frac{\phi_0 + \phi_1 [\text{ROOH}][\text{ROH}]}{\phi_0 + \phi_1 [\text{Thiol}]}(1)
\]

where [ROOH] is the initial hydroperoxide concentration, [ROH] is the concentration of the product at a time t, [Thiol] is the concentration of the substrate, [E], the total concentration of the peroxidase, and \(\phi_0\), \(\phi_1\), and \(\phi_2\) are the kinetic Dalziel coefficients (30, 31) as described in Hillebrand et al. (14).

**Cultivation of Bloodstream and Procyclic T. brucei**—For all experiments described here, culture-adapted bloodstream and procyclic T. brucei of cell line 449 were used. The cells are descendants of strain Lister 427 (32) that were stably transfected with pHD449 encoding the tetracycline repressor (33). Bloodstream T. brucei were grown at 37 °C in a humidified atmosphere with 5% CO$_2$ in HMI-9 medium supplemented with 1.5 mM cysteine, 0.0014% (v/v) β-mercaptoethanol, 1% heat-inactivated fetal calf serum (v/v), 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.2 μg/ml phytohemagglutinin to select for cells containing the tetr repressor. Procyclic T. brucei were grown in the absence of pHD449 encoding the tetracycline repressor in the presence of 10 μM thymine hydroperoxide and 50 μM NADPH. Concentration dependence of the initial velocities was obtained by analyzing the time progression curve of NADPH consumption in a Beckman DU65 spectrophotometer with data acquisition every 1.5 s. The data were analyzed using the integrated Dalziel equation for a two-substrate enzyme reaction (30, 31) (Equation 1).

\[
\text{Reaction rate} = \frac{[\text{ROOH}]}{[\text{ROH}]} = \frac{\phi_0 + \phi_1 [\text{ROOH}][\text{ROH}]}{\phi_0 + \phi_1 [\text{Thiol}]}(1)
\]

where [ROOH] is the initial hydroperoxide concentration, [ROH] is the concentration of the product at a time t, [Thiol] is the concentration of the substrate, [E], the total concentration of the peroxidase, and \(\phi_0\), \(\phi_1\), and \(\phi_2\) are the kinetic Dalziel coefficients (30, 31) as described in Hillebrand et al. (14).
same gel. After electrophoresis, the proteins were transferred onto a Hybond™-N* membrane (Amersham Biosciences) by wet electrophoretic blotting and probed with the polyclonal rabbit antiserum against the recombinant peroxidase (dilution 1:2000). Anti-rabbit IgG (dilution 1:20,000, Santa Cruz Biotechnology) served as secondary antibody. The immune complex was visualized by the SuperSignal® West Pico chemiluminescent substrate (Pierce). The intensity of the protein bands was quantified by the program Gel Pro Analyzer 3.1 as absolute integrated optical density.

Subcellular Localization of the Peroxidase in Procyclic and Bloodstream T. brucei—A sucrose gradient was prepared following a modified protocol of Opperoeds et al. (13). 1 × 10⁶ parasitoids were collected by centrifugation at 800 g for 30 min at 4 °C. The cell pellet was washed with TEDS buffer (25 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose, pH 7.8), resuspended in TEDS, 15% glycerol, and frozen at −80 °C. The cells were thawed at room temperature and washed in TEDS, 10% sucrose, 5% Museq and Incubator Mini. By centrifugation in 2–3 ml TEDS was added, and the cells were manually broken with silicon carbide (Merck). The silicon carbide was removed by centrifugation at 100 × g, and the supernatant was loaded on top of a 39-ml semi-continuous 30–60% sucrose gradient, with a 68% sucrose cushion and 30% sucrose as the top concentration. Separation of the different cell compartments was achieved by centrifugation at 170,000 g in a vertical rotor VT502 for 4 h (Beckman). 700-μl fractions were collected from the bottom of the centrifuge tube, and the protein concentration in each fraction was determined using the bichoncinic acid kit (Pierce). To 250 μl of each fraction trichloroacetic acid and deoxycholate were added at 20% (w/v) and 0.4% (w/v) concentrations, and the mixture was incubated overnight at 4 °C. After a 15-min centrifugation at 13,000 g, the supernatant containing 50% acetone, dissolved in 50 or 25 μl of 1× Laemmli buffer, and boiled for 2 min. For the bloodstream cells 8 μl of the 5 times-concentrated gradient fractions were applied on 10 or 15% SDS gels. In the case of the procyclic parasitoids, 20-μl aliquots of the 10 times-concentrated gradient fractions were analyzed. Western blots were performed as described above with the polyclonal rabbit antiserum against the recombinant peroxidase (dilution 1:2000). To distinguish the different cell compartments, the blots were developed with antibodies directed against the marker proteins Pex11 (1:1000), lipoamide dehydrogenase (1:20,000, BiP (1:2000), and TR (1:500). Anti-rabbit IgG (dilution 1: 20,000, Santa Cruz) served as secondary antibody.

RNA Interference Constructs and Transfection—A 452-bp fragment of the peroxidase III coding region was amplified twice from genomic DNA of procyclic and bloodstream strain T. brucei strain ArTAT 1.1. Pcs (5′-GAC GCT TTC-3′) and Pcs-as (5′-GAC CGT ATC ATG ATG ACT-3′) correspond to the transcription start sites of the T. brucei Peroxidase III has previously been shown to catalyze the trypanothione/tryparedoxin-dependent reduction of H₂O₂, β butyl hydroperoxide, and cumene hydroperoxide (14). The aim of this study was to reveal probable physiological substrates of the enzyme and to compare its substrate specificity with that of a homologous T. cruzi peroxidase (12, 13) as well as the mammalian PhGPX (37), which represents the structurally closest related host enzyme. Reduction of thymine hydroperoxide and linoleic acid hydroperoxide by peroxidase III was studied by single curve progression analysis according for Forstrom et al. (31) using the integrated Dalziel equation (Equation 1) as outlined in Hillbrand et al. (14). The hydroperoxide concentration was chosen such that the reaction rate was dependent on its concentration. The thiol concentration was kept constant over time by controlling the reaction to that of trypanothione reductase, which allowed the reactions to be followed by measuring NADPH consumption. As shown in Fig. 1A for thymine hydroperoxide, the integrated reciprocal initial velocities multiplied by the enzyme concentration were plotted against the integrated reciprocal concentrations of the hydroperoxide substrate. The slope of this primary plot is the Dalziel coefficient k₁, which corresponds to the reciprocal of rate constant k for the reaction of the peroxide (see the legend of Table 1). A re-plot of the ordinate intercepts, which represent the reciprocal apparent maximum velocities at infinite peroxide concentration against the reciprocal tryparedoxin concentration, yielded a straight line that cut the ordinate at kᵢ (Fig. 1B). This means that the enzyme does not show saturation at high concentrations of both substrates but that the limiting V_max and K_m values are infinite. The kinetic pattern can be due to two distinct catalytic phenomena. Either formation of enzyme-substrate complexes...
hydroperoxide substrates, standard assays were performed measuring initial ΔA/min at fixed concentrations of trypano-thione/tryparedoxin and hydroperoxide (Table II). As expected, thymine hydroperoxide and H$_2$O$_2$ are efficient substrates, linoleic acid hydroperoxide and t-butyl hydroperoxide are reduced at lower rates, and phosphatidylcholine hydroperoxide is almost not reduced. Moreover, Px III is inactivated by the latter compound. Preincubation of the enzyme with 30 μM phosphatidylcholine hydroperoxide for 30 min before starting the reaction by adding 80 μM H$_2$O$_2$ lowered the enzyme activity to ~5% of an untreated enzyme sample. When the parasite enzyme was treated with 80 μM phosphatidylcholine hydroperoxide in the presence of 0.5% Triton X-100 (8.45 mM) for 30 min, the activity with 100 mM H$_2$O$_2$ remained at 80% of the control. 

Preincubation of peroxidase III with H$_2$O$_2$ did not cause any inactivation excluding a general substrate inactivation. In the presence of 0.1% Triton X-100, phosphatidylcholine hydroperoxide is reduced by peroxidase III but is still the poorest substrate (Table II). Phosphatidylcholine hydroperoxide preparations may contain up to 2% deoxycholate,$^3$ which is equivalent to a final concentration of ≤2 μM in a standard assay with 100 μM phosphatidylcholine hydroperoxide. Because deoxycholate has been shown to strongly influence the activity of mammalian PhGPX (38) 4.5 μM Px III was preincubated with up to 33 mM deoxycholate for 30 min, and then the activity was measured with 100 μM H$_2$O$_2$ in a standard assay. With ≤21 μM deoxycholate in the assay, no effect on the enzyme activity was observed. Thus, inhibition of Px III was caused by phosphatidylcholine hydroperoxide and is not due to the possible presence of deoxycholate. Because Px III showed higher activity toward all hydroperoxides in the presence of the detergent, control assays contained 0.1% and 0.3% Triton X-100 but no deoxycholate. Because Px III showed higher activity toward all hydroperoxides in the presence of the detergent, control assays contained 0.1% and 0.3% Triton X-100 but no deoxycholate. No peroxidase activity was detectable excluding the presence of Triton X-100 hydroperoxides, which might have functioned as substrates (39). The reasons for the higher activity of peroxidase III in the presence of Triton X-100 are not known but may be an improved solubility or activation of the enzyme. In the case of human PhGPX, Triton X-100 specifically stimulated the reduction of phosphatidylcholine hydroperoxide, which was interpreted as a consequence of the transformation of the lipid bilayer into a solution of mixed micelles (40). Because the effect of Triton was not observed with H$_2$O$_2$, the parasite peroxidase with phosphatidylcholine hydroperoxide is very low when compared with the other hydroperoxides studied. Thus the T. brucei enzyme clearly differs in its substrate preference from T. cruzi glutathione peroxidase I and the mammalian PhGPX. With 0.1% Triton X-100 in the assay, the latter enzyme shows a significantly higher activity with phosphatidylcholine hydroperoxide than with H$_2$O$_2$ (39). T. cruzi glutathione peroxidase I reduces phosphatidylcholine hydroperoxide but does not accept H$_2$O$_2$ as substrate (12).

**Quantification and Subcellular Localization of the Peroxidase(s) in Bloodstream and Procyclic T. brucei**—On chromosome 7, T. brucei encodes three nearly identical genes for cysteine homologues of glutathione peroxidases. Western blot analysis with polyclonal antibodies against the recombinant His$_6$-peroxidase III revealed a single protein band in both bloodstream and procyclic T. brucei (14). Purification of the recombinant tag-free protein allowed us to estimate the cellular concentration of the enzyme. Western blots with 1 × 10$^6$ and 2 × 10$^6$ parasites in comparison with different amounts of

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$^3$ R. Brigelius-Flohé, personal communication.
The Dalziel coefficients $\Phi_1$ and $\Phi_2$ for lipoic acid hydroperoxide and thymine hydroperoxide are the means of two and three independent series, respectively. The $\Phi$ coefficients were determined in the case of lipoic acid hydroperoxide for four concentrations and with thymine hydroperoxide for five different concentrations of tryparedoxin. $\Phi_1$ and $\Phi_2$ correspond to the reciprocal of the apparent rate constants $k_1$ and $k_2$ for the two half reactions. $k_1$ is $k_0 - k_2$ and may be regarded as $k_1$ because the partial reaction should be irreversible. Peroxidase$_{red} + ROOH \rightarrow$ peroxidase$_{ox} + ROH + H_2O (k_0 = 1/ \Phi_1)$. $k_2$ is the overall rate constant for the two-step regeneration of reduced enzyme by tryparedoxin. Peroxidase$_{ox} +$ tryparedoxin$_{red} \rightarrow$ peroxidase$_{ox} +$ tryparedoxin$_{red} (k_0 = 1/ \Phi_2)$. The extrapolated $\Phi$ coefficients were zero within the experimental error. No limiting $K_m$ values, given as $\Phi_0/\Phi_1$ and $\Phi_0/\Phi_2$, were obtained for all hydroperoxide substrates studied.

The recombinant protein yielded 22 $\mu M$ peroxidase in bloodstream $T. brucei$ using a cell volume of 58 femtoliters (34, 41) for calculation. For procyclic cells, a cell volume of 100 femtoliters was assumed that resulted in a cellular enzyme concentration of about 10 $M$ (Fig. 2). This analysis does not allow us to distinguish between the probable expression of the individual peroxidase I-III that consist of 166, 169, and 169 (after cleavage of the presumed mitochondrial targeting sequence) amino acid residues, respectively.

In addition to the putative mitochondrial signal peptide, Px III has a C-terminal ARL motif that may represent a glycosomal membrane protein. The other two proteins lack known signal sequences (14). To determine the subcellular localization of the putative mitochondrial signal peptide, Px II and Px III were identified by Western blots with antibodies against Pex11, a glycosomal membrane protein, against lipoamide dehydrogenase, and against the endoplasmic reticulum chaperon BiP and the cytosolic trypanothione reductase. As shown in Fig. 3A, the peroxidases were detectable in fractions 36–50 of the bloodstream gradient in accordance with a cytosolic and mitochondrial localization with most of the protein in the cytosol. Also in procyclic $T. brucei$, the peroxidases co-eluted with trypanothione reductase and lipoamide dehydrogenase and, thus, occur in the cytosol and mitochondrion (Fig. 3B).

The Peroxidases Are Essential for $T. brucei$—For RNA interference experiments, two copies of a 452-bp fragment of the peroxidase gene were cloned in sense and antisense orientation on either side of a stuffer sequence in the vector pH678. Bloodstream and procyclic $T. brucei$ 449 cells were transfected

### Table I

**Apparent rate constants of $T. brucei$ peroxidase III for the reduction of different hydroperoxides**

<table>
<thead>
<tr>
<th>Hydroperoxide</th>
<th>$\Phi_0$</th>
<th>$\Phi_1$</th>
<th>$k_1$</th>
<th>$\Phi_2$</th>
<th>$k_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumene hydroperoxide</td>
<td>s</td>
<td>s</td>
<td>$1.3 \times 10^4$</td>
<td>s</td>
<td>$2.1 \times 10^3$</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>0</td>
<td>9.7 ± 2.1</td>
<td>10.3</td>
<td>4.75 ± 2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Thymine hydroperoxide</td>
<td>0</td>
<td>11.5 ± 1.8</td>
<td>8.7</td>
<td>5.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Linoleic acid hydroperoxide</td>
<td>0</td>
<td>13.2 ± 2.8</td>
<td>7.6</td>
<td>3.5 ± 0.5</td>
<td>2.8</td>
</tr>
<tr>
<td>t-Butyl hydroperoxide</td>
<td>0</td>
<td>25 ± 6</td>
<td>4</td>
<td>4 ± 0.2</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>81 ± 43</td>
<td>1.2</td>
<td>4.2 ± 2.1</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*From Hillebrand et al. (14).*

### Table II

**Specific activity of $T. brucei$ Px III for different hydroperoxide substrates in the absence and presence of 0.1% Triton X-100**

<table>
<thead>
<tr>
<th>Hydroperoxide</th>
<th>Triton X-100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mg</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>3.8</td>
</tr>
<tr>
<td>Thymine hydroperoxide</td>
<td>4.2</td>
</tr>
<tr>
<td>Linoleic acid hydroperoxide</td>
<td>1.4</td>
</tr>
<tr>
<td>t-Butyl hydroperoxide</td>
<td>1.1</td>
</tr>
<tr>
<td>Phosphatidylcholine hydroperoxide</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*From Hillebrand et al. (14).*

**Fig. 2. Quantification of the peroxidases in bloodstream and procyclic $T. brucei$**

A Western blot analysis of total cell lysates from cultured bloodstream and procyclic $T. brucei$ and 7, 15, 28, and 56 ng of tag-free recombinant peroxidase III. Polyclonal antibodies against the peroxidase together with the SuperSignal West Pico chemiluminescent substrate were used for visualization as described under “Experimental Procedures.” B, standard diagram based on four different amounts of the recombinant protein (●) using the program GelPro Analyzer 3.1. +, 2 × 10^6 procyclic parasites; *, 2 × 10^6 bloodstream parasites. IOD, integrated optical density.
Subcellular localization of the peroxidases in bloodstream and procyclic *T. brucei*. A. Western blot analysis of subcellular fractions of bloodstream cells with antibodies against Pex11, lipoamide dehydrogenase, and trypanothione reductase identified 28–32 as the main glycosomal fractions, fractions 36–38 as peak mitochondrial fractions, and the cytosol starting with fraction 48, respectively. Antibodies against the endoplasmic reticulum matrix protein BiP recognized the protein starting with fraction 30 nearly throughout the gradient. The peroxidases were detectable in the cytosolic and mitochondrial fractions. B. Western blots of subcellular fractions of procyclic cells showed the glycosomal Pex11 in fractions 23–31, the mitochondrial lipoamide dehydrogenase (LipDH) mainly in fractions 29–37 and the cytosolic TR from fraction 43 onward. Also in procyclic cells, the peroxidases are detectable in the cytosol and mitochondrion. The bands of Pex11 and lipoamide dehydrogenase in fraction 45 of the procyclic gradient are probably due to some broken organelles. The bands below the peroxidase are caused by cross-reactions with other cytosolic proteins. The protein concentration of the unconcentrated fractions are given in mg/ml. For each protein to be analyzed, two gels were run in parallel and developed together under identical conditions. A dotted line is drawn between the two gels.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fraction Protein concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pex11</td>
<td>24</td>
</tr>
<tr>
<td>LipDH</td>
<td>55</td>
</tr>
<tr>
<td>BiP</td>
<td>71</td>
</tr>
<tr>
<td>TR</td>
<td>50</td>
</tr>
<tr>
<td>Px</td>
<td>19</td>
</tr>
</tbody>
</table>

Table showing the distribution of proteins in different fractions of bloodstream and procyclic cells.

**DISCUSSION**

The glutathione peroxidase-type *T. brucei* tryparedoxin peroxidases studied here readily reduces $\text{H}_2\text{O}_2$, thymine hydroperoxide, and linoleic acid hydroperoxide, which probably repre-
cultivated bloodstream parasites. The cultures were diluted to 2 (1:1000). As a loading control, the blots were stripped and developed with the purified TR antibodies (1:500). against /H9252 hybridized with a digoxigenin-labeled probe of the detergent, even an inhibitor of the enzyme. The hydroperoxide is a very poor substrate and, in the absence of sent physiological substrates. In contrast, phosphatidylcholine hydroperoxide is a very poor substrate and, in the absence of detergent, even an inhibitor of the enzyme. The T. brucei enzyme is the first trypanosomatid peroxidase shown to reduce thymine hydroperoxide. This model compound for DNA oxidative damage is an excellent substrate of human phospholipid hydroperoxide glutathione peroxidase, and a role of the enzyme in the repair of oxidatively damaged DNA has been suggested (44). Thymine hydroperoxide is not reduced by the cytosolic mammalian GPX. Of the four mammalian selenoglutathione peroxidases, PhGPX is the most similar of the T. brucei enzyme. The two enzymes show an overall sequence identity of 39% and are monomeric proteins. In addition, both peroxidases occur in the cytosol and mitochondria (39, 45). PhGPX accepts a broad spectrum of hydroperoxides, particularly fatty acid and phospholipid hydroperoxides (19, 37, 46). This lack of substrate specificity has been attributed to the fact that PhGPX acts as a monomer, whereas the other mammalian glutathione peroxidases are tetrameric proteins (47). Because the T. brucei peroxidase as well as a cysteine-containing glutathione peroxidase from P. falciparum (21) are also monomeric but do not reduce phosphatidylcholine hydroperoxide, it is unlikely that the broad specificity of the PhGPX is mainly due to a better accessibility of its redox center.

The closest relative of the T. brucei peroxidase is a T. cruzi enzyme first described as glutathione peroxidase I (12) and later shown also to prefer the trypanothione/tryparedoxin system as electron source (13). T. cruzi glutathione peroxidase I has been reported to be encoded by a single copy gene (12). This is not the case. In T. cruzi, as is the case in T. brucei and L. major, a genomic locus encodes a cluster of three, at least 95% identical genes. Despite this conserved genomic organization and an amino acid sequence identity of 72%, the T. brucei and T. cruzi peroxidases have remarkably different substrate specificities. In contrast to the T. brucei peroxidase studied here, the T. cruzi enzyme does not accept H_{2}O_{2} (12, 13) but prefers fatty acid and phospholipid hydroperoxides. The assumption that the T. brucei peroxidase behaves like the T. cruzi glutathione peroxidase I and is mainly engaged in the removal of oxidatively damaged lipids (17) is, therefore, unlikely. There is some evidence for a general function of the T. cruzi peroxidase in oxidative defense since overexpression of the enzyme conferred a slight increase in resistance toward exogenous H_{2}O_{2} and t-butyl hydroperoxide (13), but a direct role in membrane protection has yet to be shown. The subcellular distribution of these peroxidases in African and American trypanosomes is also remarkably different. T. cruzi glutathione peroxidase I has been reported to occur in glycosomes and the cytosol (13). In contrast, the T. brucei glutathione peroxidase-type enzymes described here are detectable in the cytosol and
mitochondria but not the glycosomes of bloodstream and procyclic parasites. This subcellular localization coincides with that of the parasite peroxiredoxins (11).

Besides the N-terminal mitochondrial signal sequence, *T. brucei* peroxidase III has a putative C-terminal peroxisomal targeting signal. The sole mitochondrial and cytosolic localization of the enzyme in bloodstream and procyclic cells indicates that the peroxisomal targeting signal must have been repressed in some way. Studies on mammalian proteins revealed that in many cases the mitochondrial signal sequence is functionally dominant (48). One explanation is that the mitochondrial signal, which is translated first, causes the protein to be immediately recognized and translocated into the mitochondrion. Another possibility has been reported in the case of the rat liver pyruvate/alanine glyoxylate aminotransferase. Here the presence of the mitochondrial-targeting signal causes the protein to become unfolded, which represses the peroxisomal targeting signal (49). The putative mitochondrial signal peptide of *T. brucei* peroxidase III is only seven residues long. Such exceptionally short targeting sequences have been observed in other trypanosomatid proteins (50). In the Western blots of the mitochondrial and cytosolic fractions of the cell gradient the size of the protein bands was indistinguishable, suggesting cleavage of the leader sequence in Px III.

Depletion of the glutathione peroxidase-type enzyme(s) causes severe growth defects in bloodstream and procyclic *T. brucei*. The same is true for the cytosolic 2 Cys-peroxiredoxin in bloodstream cells (17). Obviously the two types of peroxiredoxins cannot functionally substitute for each other. In the insect trypanosomatid *Crithidia fasciculata*, cytosolic peroxiredoxin represents about 6% of the total soluble protein and, thus, is an extremely abundant protein (10). If this is also the case in *T. brucei*, the cellular concentration of the peroxiredoxin would be at least an order of magnitude higher than that of the glutathione peroxidase-type enzymes. The fact that the latter enzymes are nevertheless essential strongly argues for physiological roles different from a general defense against oxidative stress. This assumption is in agreement with data from Wilkinson *et al.* (17), where the H$_2$O$_2$ sensitivity of bloodstream cells depleted of the mitochondrial and cytosolic peroxiredoxins as well as the glutathione peroxidase-type enzyme was studied. Only depletion of the cytosolic 2 Cys-peroxiredoxin increased the sensitivity of the parasites toward exogenous H$_2$O$_2$. In mammalian cells, peroxiredoxins are both peroxide-destroying enzymes and peroxide targets being over-oxidized (51). The latter property is presumably not valid for the kinetoplastid peroxiredoxins (52), but the cytosolic peroxiredoxin obviously plays the major role in the defense against exogenous oxidative stress. The fact that the intracellular concentration of the glutathione peroxidase-type enzymes is probably more than an order of magnitude lower than that of the peroxiredoxins and that in general the cysteine homologues of the classical glutathione peroxiredoxins have much lower activities than the mammalian seleno-enzymes support the assumption that these

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**Fig. 5.** Tetracycline (TET)-induced RNA-interference response in procyclic *T. brucei brucei*. Wild-type (WT) procyclic PC449 cells (●, -TET) and control PC449 cells with pH678, (○, -TET; □, +TET) were compared with three tetracycline-induced and non-induced clones of PC449 containing pH678px, clone 2 (gray box, -TET; open square, +TET), clone 3 (▲, -TET; Δ, +TET), clone 4 (light gray inverted triangle, -TET; open inverted triangle, +TET). A, Northern blot analysis. A culture with $4 \times 10^5$ cells/ml was induced with 1 µg/ml tetracycline, and the cells were harvested after 24 h (control, clone 4) or 48 h (clone 3). 12–15 µg of total RNA from 5 × 10$^6$ cells was applied per lane. B, Western blot analysis. Total cell lysate from 2 × 10$^6$ cells was applied per lane. 4 × 10$^5$ cells/ml were treated with 1 µg/ml tetracycline, and the cells were harvested after 24 h. C, growth curves of continuously cultivated parasites. A cell culture with 4 × 10$^5$ cells/ml was induced with 1 µg/ml tetracycline, and the cells were cultivated for 72 h. D, growth curves of cells cultivated in 24-h cycles. 1 × 10$^6$ cells/ml were treated daily with 1 µg/ml tetracycline for a 6-day period. For details see the legend of Fig. 4 and “Experimental Procedures.”
enzymes have specialized functions. Although the RNA interference approach could not discriminate between the cytosolic and mitochondrial enzymes, one may speculate that the mitochondrial glutathione peroxidase-type enzyme plays an important role because the mitochondrial peroxiredoxin proved to be not essential (17). The source of reducing equivalents for the mitochondrial peroxidases remains unclear because trypanothione reductase has been found only in the cytosol of T. brucei (53) (Figs. 4 and 5). There may be some mitochondrial trypareodoxin. Immunofluorescence showed only a cytosolic localization. In C. fasciculata tryparedoxin activates the binding of a universal minicircle sequence-binding protein to the kinetoplast DNA, and it was postulated that tryparedoxin-dependent peroxidases could regulate the redox state of tryparedoxin and, thus, influence this replication initiation process (6). Another possible reason for the requirement of both types of tryparedoxin peroxidases is that mammalian PhGPX (44). Mitochondrial DNA is much more sensitive to oxidative damage than nuclear DNA (54). The peroxidase could also be involved in redox regulation processes. In T. brucei Peroxidase

REFERENCES

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doi: 10.1074/jbc.M413338200 originally published online January 21, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413338200

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