Macrophage activation is one of the hallmarks observed in trypanosomiasis, and the parasites must cope with the resulting oxidative burden, which includes the production of peroxynitrite, an unusual peroxy-acid that acts as a strong oxidant and trypanocidal molecule. Cytosolic tryparedoxin peroxidase (cTXNPx) has been recently identified as essential for oxidative defense in trypanosomatids. This peroxiredoxin decomposes peroxides using tryparedoxin (TXN) as electron donor, which in turn is reduced by dihydrotrypanothione. In this work, we studied the kinetics of the reaction of peroxynitrite with the different thiol-containing components of the cytotoxic tryparedoxin peroxidase system in Trypanosoma brucei (Tb) and Trypanosoma cruzi (Tc), namely trypanothione, TXN, and cTXNPx. We found that whereas peroxynitrite reacted with dihydrotrypanothione and TbTXN at moderate rates (7200 and 3500 M⁻¹ s⁻¹, respectively, at pH 7.4 and 37 °C) and within the range of typical thiols, the second order rate constants for the reaction of peroxynitrite with reduced TbTXNPx and TcTXNPx were 9 × 10⁵ and 7.2 × 10⁴ M⁻¹ s⁻¹ at pH 7.4 and 37 °C, respectively. This reactivity was dependent on a highly reactive cTXNPx thiol group identified as cysteine 52. Competition experiments showed that TbTXNPx inhibited other fast peroxynitrite-mediated processes, such as the oxidation of Mn³⁺-porphyrins. Moreover, steady-state kinetic studies indicate that peroxynitrite-dependent TbcTXNPx and TecTXNPx oxidation is readily reverted by TXN, supporting that these peroxideroxins would be not only a preferential target for peroxynitrite reactivity but also be able to act catalytically in peroxynitrite decomposition in vivo.

Trypanosoma brucei and Trypanosoma cruzi are the causative agents of African trypanosomiasis and Chagas disease, respectively, major public health problems affecting millions of people in Africa and Latin America. Both diseases are characterized by an increase in the number of macrophages and the presence of macrophage activation markers (1, 2). However, T. brucei is an extracellular parasite, whereas T. cruzi proliferates inside the macrophages and in the cytoplasm of other nucleated cells. Macrophages from T. brucei- and T. cruzi-infected mice produce high levels of nitric oxide (NO), which has antiparasitic effects in vitro and in vivo (3–5). In addition, reactive oxygen intermediates such as superoxide radical (O₂⁻) and hydrogen peroxide (H₂O₂) are synthesized as a result of the oxidative burst by inflammatory cells from T. brucei- and T. cruzi-infected animals (6–8). Peroxynitrite can also be formed by the parasite itself (e.g. during the generation of the iron-tyrosyl radical center in the small subunit of ribonucleotide reductase (9), by mitochondrial respiration (10), or by redox cycling of antichagasic drugs (11)). The diffusion-controlled reaction between NO and O₂⁻ leads to the formation of peroxynitrite anion (12), a strong oxidizing and cytotoxic effector molecule against T. cruzi (13, 14). Moreover, inflammatory lesions in the central nervous system of mice chronically infected with T. brucei brucei and in the myocardium of acute chagasic rats express type II nitric-oxide synthase and show protein 3-nitrotyrosine immunoreactivity, which has been ascribed to peroxynitrite¹ and/or nitrogen dioxide (‘NO₂) formation (15–17). Peroxynitrous acid is an unusual peroxy-acid, since it has a low pKₐ value (6.8 versus 11.6 of the first proton dissociation in hydrogen peroxide, H₂O₂) and a weak O–O bond (bond strength of 90 kJ mol⁻¹ versus 170 kJ mol⁻¹ H₂O₂) (18) that makes it an unstable species that decomposes by homolysis (k = 0.9 s⁻¹ in phosphate buffer, pH 7.4 and 37 °C) to yield hydroxyl radical (‘OH) and NO₂, which either recombine to form nitrate or react with substrates. The short lifetime of peroxynitrous acid and its fast reaction with carbon dioxide (CO₂) frequently present in buffers and in biological systems (12) makes biochemical studies more difficult to perform than with H₂O₂ and organic hydroperoxides. Preferential targets for peroxynitrite in vivo are thiols that can be oxidized both by direct bimolecular reaction and by the reactions with peroxynitrite-derived radicals (12). The direct peroxynitrite-mediated thiol oxidation is a two-electron oxidation process that leads to the formation of nitrate and the thiol-derived sulfenic acid, which, in the presence of an

¹ The term peroxynitrite is used to refer to both peroxynitrite anion (ONOO⁻) and peroxynitrous acid (ONOOH). IUPAC-recommended names are oxoperoxynitrate (1⁻) and hydrogen oxoperoxynitrate, respectively.
accessible thiol group, forms a disulfide, resulting in an overall stoichiometry of two thiols oxidized per peroxirexin (19). The reaction involves peroxynitrous acid and the deprotonated form of the thiol (thiolate, RS⁻). The second order rate constants for the reactions between peroxynitrous acid and low molecular weight thiols at pH 7.4 (10⁻⁵ to 10⁻⁸ M⁻¹ s⁻¹) are inversely related to the thiol pKₛₐ (20). However, there is an increasing number of highly reactive protein thiols that react with peroxynitrite at rates of 10⁻⁹ to 10⁻¹ M⁻¹ s⁻¹ at pH 7.4 and 37 °C (21, 22). Among them, the bacterial peroxiredoxin alkylhydroperoxide reductase subunit C serves to catalytically detoxify peroxirexin (23). It has been postulated that highly reactive cysteines in proteins are located close to positively charged amino acids (24) or at the positive edges of aromatic rings, which promote dissociation of the thiol (pKₛₐ as low as ~5 (23)) (i.e. the local electrostatic environment of cysteine is an important, although not necessarily the only, determinant of its reactivity (25)).

Hydroperoxide detoxification in trypanosomatids is achieved by a series of linked redox pathways that depend on the parasite-specific dithiol dihydrotrypanothione (26) (N(1)V(1)N(1)bis(glutathionyl)peroxirexin) for the supply of reducing equivalents. These pathways differ in subcellular location and contain different reducing components of the tryparedoxin peroxidase antioxidant system against peroxynitrite (27), which promote dissociation of the thiol (pKₛₐ as low as ~5 (23)) (i.e. the local electrostatic environment of cysteine is an important, although not necessarily the only, determinant of its reactivity (25)).

Trypanothione refers to the pool of reduced and oxidized forms of T. brucei trypanothione, respectively. Moreover, isolated T. brucei trypanothione disulfide indicates the oxidized (TS2) form of the compound; dihydrotrypanothione indicates the reduced (T(SH)2) form of trypanothione, respectively.

The abbreviations used are: cTXNPs, cystolic trypanothione peroxidase; TbTXNPs, T. brucei cTXNPs; TcTXNPs, T. cruzi cTXNPs; NEM, N-ethylmaleimide; DTPA, diethylenetriaminopentaacetic acid; DTNB, 5,5’-dithio-bisnitrobenzoic acid; Mn²⁺-TM-4-PyP, manganese (III)-meso-tetraakis(4-methyl)pyridinium-4-yl)porphyrin; DHR, dihydrorhodamine. However, the biochemical mechanism of dihydrotrypanothione oxidation and peroxynitrite detoxification at the cellular level remains unknown. Indeed, since trypanothione reductase, dihydrotrypanothione, trypanothione, and tryparedoxin peroxidase are all thiol-containing molecules, they could all in theory react with peroxynitrite. However, the relative importance of these biotargets as reactants of peroxynitrite in vivo would be, in good part, dictated by rate constants and reactant concentrations.

Herein, we present kinetic studies on the reactivity of different components of the trypanothione peroxidase antioxidant system in trypanosomes, namely trypanothione, TbTXN, TbTXNPs, and TcTXNPs with peroxynitrite, with the aim to rationalize the biochemical mechanisms of peroxynitrite detoxification in T. brucei and T. cruzi.

MATERIALS AND METHODS

Chemicals—Trypanothione was obtained from Bachem. Sodium borohydride, dithiothreitol, N-ethylmaleimide (NEM), diethyleneetriaminopentaacetic acid (DTPA), 5,5’-dithio-bisnitrobenzoic acid (DTNB), manganese (III) dithiocarboxylic acid (Mn²⁺-TM-4-PyP), sodium dithiothreitol, tryparedoxin, and tryparedoxin peroxidase (TXP) were from Sigma. Dihydrotironohemoglobin was prepared by co-oxidation of high-purity human (Eugene, OR) R-B-Formylmethylaminodinitroamine hydrochloride (SIN-1) was from Casella AG. Argon (~99.5% pure) was purchased from AGA Gas Company (Montevideo, Uruguay). Manganese (III)-meso-tetraakis(4-methyl)pyridinium-4-yl)porphyrin (Mn³⁺-TM-4-PyP) was kindly supplied by Ines Batinic-Haberle (Duke University). All other chemicals were reagent grade. Solutions were prepared with highly pure water (Barnstead D18528, resistance >18 megaohm cm⁻¹) to minimize trace metal contamination.

Expression and Purification of T. brucei Trypanothione and T. brucei Cytosolic Trypanothione Peroxidase—Proteins were obtained by heterologous expression of the respective genes in Escherichia coli. The gene of TXN of T. brucei brucei, as identified by LuDEMANN et al. (40), was expressed as an N-terminally His-tagged protein that was purified as described previously (32). Molecular variants of TcTXN were obtained according to Ref. 32. T. brucei cytosolic TXNPs were purified as in Ref. 41 with the following modifications. The amplified T. cruzi TXNPs gene was cloned into the pQE-30 vector (Qiagen) between SacI and HindIII. The purification was performed in a 5-ml HiTrap affinity column (Amersham Biosciences) and equilibrated with binding buffer (50 mM sodium phosphate, pH 7.6, containing 300 mM NaCl). The purified recombinant Trypanothione Peroxidase—Peroxynitrite was synthesized in a quenched flow reactor from sodium nitrite and H₂O₂ under acidic conditions and quantitated as described previously (19). Treatment of a stock solution of peroxynitrite with granular manganese dioxide eliminated H₂O₂ remaining from the synthesis. Nitrite (NO₂⁻) present in samples of peroxynitrite decomposed at acidic pH was typically 0.6. The purification was performed in a 5-ml HiTrap affinity column (Amersham Biosciences) charged with Ni²⁺ and equilibrated with binding buffer (50 mM sodium phosphate, pH 7.6, containing 10 mM imidazole, 500 mM NaCl) at a flow rate of 3 ml/min. The His-tagged TXNPs was eluted in 50 mM sodium phosphate, pH 7.6, eluting 300 mM imidazole, 500 mM NaCl.

Peroxynitrite Synthesis—Peroxynitrite was synthesized in a quenched flow reactor from sodium nitrite and H₂O₂ under acidic conditions and quantitated as described previously (19). Treatment of a stock solution of peroxynitrite with granular manganese dioxide eliminated H₂O₂ remaining from the synthesis. Nitrite (NO₂⁻) present in samples of peroxynitrite decomposed at acidic pH was typically <30% of peroxynitrite concentration.

Trypanothione Reduction—Stock solutions of trypanothione disulfide (2.6 mM) were treated with excess sodium borohydride during 30 min and adjusted to pH 2 in order to eliminate excess borohydride. Then samples were adjusted to pH 7.4 and extensively bubbled with argon and stored in ice in the dark. The amount of reduced trypanothione was measured by quantitation of reduced thiols, using the DTNB assay (42).

Protein Thiol Reduction and Alkylation—TbTXN, TbTXNPs, and TcTXNPs were reduced overnight by the addition of a 10-fold excess of dithiothreitol. Excess dithiothreitol was removed immediately before use by passing proteins through a high pressure liquid chromatography-connected Hitrap column (Amersham Biosciences) with a UV-visible detector. Injections were performed manually in 1:10 volume fractions of elution buffer, which was extensively degassed before use. Once collected, samples were bubbled for 5 min with argon at 4 °C. Protein concentration was measured by the Bradford method, as well as by their absorb-
ance at 280 nm as previously described (31). Alkylation of cTXNPx thiol groups by NEM was performed by incubation of the enzyme with a 10–20-fold excess of NEM for 5 min.

**Thiol Measurements**—Low molecular weight thiols as well as protein thiols were quantitated using the DTNB assay (42).

**Dihydrorhodamine Oxidation**—Stock solutions of dihydrorhodamine (DHR) (28.9 mM) in dimethyl sulfoxide were purged with argon and stored at −20 °C. Dihydrorhodamine oxidation to rhodamine was followed spectrophotometrically at 500 nm (ε_{500} = 78.8 mM⁻¹ cm⁻¹) (43).

**Direct Kinetic Studies**—The kinetics of peroxynitrite decomposition were studied in a stopped-flow spectrophotometer (SF17MV, Applied Photophysics) with a mixing time of <2 ms. Although peroxynitrite decomposition is usually measured at 302 nm (ε_{302} = 1670 mM⁻¹ cm⁻¹) (44), we monitored it at 310 nm (ε_{310} = 1600 mM⁻¹ cm⁻¹) in order to avoid interferences by background protein absorption at 302 nm. When the initial rate approach was used (45), the peroxynitrite decomposition at 2–10 ms was fitted to a linear plot. To calculate initial rates of peroxynitrite decomposition, the slopes were divided by the molar extinction coefficient of peroxynitrite at 310 nm and multiplied by 1.2 (since the absorption at 310 nm derives from peroxynitrite anion, which represents 80% total peroxynitrite concentration at pH 7.4). In pseudo-first-order analysis, apparent rate constants for peroxynitrite decomposition, k_{obs} (s⁻¹) values were determined by fitting stopped-flow data to single exponential decays with floating end point. Reported values are the average of at least seven separate determinations. Temperature was maintained at 37 ± 0.1 °C, and the pH was measured at the outlet.

**Computer-assisted Simulations**—Mn³⁺ porphyrins are rapidly oxidized by peroxynitrite (k = 10^6 to 10^7 M⁻¹ s⁻¹ at pH 7.4 and 37 °C) to the O•–Mn⁴⁺ derivative, in a process that can be conveniently monitored at the Soret band as the decay of absorbance at 462 nm (46). In the case of Mn³⁺-TM-4-PyP, the second order rate constant for its reaction with peroxynitrite was determined previously as 3.7 × 10^6 M⁻¹ s⁻¹ at pH 7.4 and 37 °C (46). The effect of increasing concentrations of TbcTXNPX on peroxynitrite-mediated Mn³⁺-TM-4-PyP oxidation was determined by stopped flow (47). Experiments were performed at 37 °C, and the pH was maintained at the outlet. Kinetics of peroxynitrite-dependent TbcTXNPX oxidation in the presence of Mn³⁺-TM-4-PyP was estimated by computer-assisted simulation, varying the apparent second order rate constant for the reaction of the enzyme and peroxynitrite at pH 7.4 and 37 °C so as to get the best fit to experimental data. In this system, peroxynitrite can (a) decompose to nitrate after proton-catalyzed isomerization (k = 0.9 s⁻¹) (12) (Reaction 1), (b) react with the reduced enzyme (Reaction 2), or (c) react with Mn³⁺-TM-4-PyP (Reaction 3).

**Statistics**—All experiments reported here were repeated and reproduced at different days. Results are expressed as mean values with the corresponding S.D. values. Graphics and data analysis were performed using Sigma Plot.

**RESULTS**

**The Reaction of Peroxynitrite with Trypanothione**—The addition of increasing excess dihydrotrypanothione concentrations to peroxynitrite led to an increase of the exponential decay of peroxynitrite at pH 7.4 and 37 °C. Dihydrotrypanothione reacted with peroxynitrite with an apparent (pH-dependent) second order rate constant of 7200 M⁻¹ s⁻¹ at pH 7.4 and 37 °C (Fig. 1). In contrast, similar concentrations of trypanothione disulfide did not lead to any...
Tryparedoxin Peroxidase Reaction with Peroxynitrite

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_2a$</th>
<th>$s^{-1} \cdot s^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrotropanethione</td>
<td>7.2 ± 0.5 × 10³</td>
<td></td>
</tr>
<tr>
<td>Trypanothione disulfide</td>
<td>No reaction</td>
<td></td>
</tr>
<tr>
<td>TcTXN</td>
<td>$-3.5 \times 10^3$</td>
<td></td>
</tr>
<tr>
<td>TbcTXNPx (wild type)</td>
<td>$9 \pm 1 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>TbcTXNPx (C52S)</td>
<td>$\sim 1 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>TbcTXNPx (C173S)</td>
<td>$3.5 \pm 0.5 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td>TcTXNPx</td>
<td>$7.2 \pm 0.6 \times 10^5$</td>
<td></td>
</tr>
</tbody>
</table>

a At pH 7.4 and 37 °C.

increase on the rate of peroxynitrite decomposition, indicating that the thiol groups of the molecule are the responsible for the reactivity (Table I).

Up to 150 μM reduced T. brucei tryparedoxin caused only a modest effect on peroxynitrite (24 μM) decomposition rate, from 0.89 ± 0.04 to 1.4 ± 0.05 s⁻¹, indicating that the second order rate constant between peroxynitrite and the reduced protein is $7.5 \times 10^{-5} \cdot s^{-1}$ at pH 7.4 and 37 °C (Table I).

Kinetics of the Reaction of T. brucei Cytosolic Tryparedoxin Peroxidase with Peroxynitrite—Reduced TbcTXNPx increased the rate of peroxynitrite decomposition (Fig. 2). Using an initial rate approach, an apparent second order rate constant of 9 ± $1 \times 10^4 \cdot m^{-1} \cdot s^{-1}$ at pH 7.4 and 37 °C was determined. A kinetic study in the presence of peroxynitrite decomposition was abolished by pretreatment of reduced TbcTXNPx with excess NEM, thus pointing to thiol groups as responsible for the fast reactivity of peroxynitrite with the enzyme (data not shown). However, there are six cysteine residues in the TbcTXNPx primary sequence and, at least three of them can be easily titrated with DTNB (see Fig. 5, y axis intercept). In order to unambiguously identify the cysteine residue responsible for the fast reaction between TbcTXNPx and peroxynitrite, we performed kinetic studies using site-directed TbcTXNPx modified proteins in which Cys-52 or Cys-173 was replaced by serine (C52S or C173S, respectively). C173S TbcTXNPx continued to react fast with peroxynitrite, with an apparent second order rate constant of 3.3 ± 0.4 × 10⁶ M⁻¹ s⁻¹, whereas C52S TbcTXNPx had only a marginal effect on the initial rate of peroxynitrite decomposition (Fig. 2), and pseudo-first-order analysis yielded a second order rate constant of 1 × 10⁵ M⁻¹ s⁻¹ at pH 7.4 and 37 °C (Table I).

TbcTXNPx Catalytically Detoxifies Peroxynitrite—In order to test whether peroxynitrite oxidizes TbcTXNPx to a form that can be re-reduced by its natural reductant TbTXN, we performed stopped-flow experiments following peroxynitrite decomposition in the presence of low TbcTXNPx concentrations in the absence or in the presence of reduced TbTXN. Steady-state studies have indicated that tert-butyl hydroperoxide (t-BuOOH)-oxidized TbcTXNPx is reduced by reduced TbTXN with a net forward second order rate constant of 1.1 × 10⁵ M⁻¹ s⁻¹ at pH 7.6 and 25 °C (32). If peroxynitrite oxidizes the enzyme to the same intermediate as t-BuOOH does, it can be expected that the same second order rate constant will apply for the re-reduction of peroxynitrite-oxidized TbcTXNPx by TbTXN. Then, by computer-assisted simulation studies, we calculated a concentration of TbTXN (≥70 μM) that would allow us to maintain reduced TbcTXNPx (1.5–6 μM) concentrations relatively unchanged (~30% change) in the presence of peroxynitrite (18 μM) (see Supplemental Material). As has already been mentioned, peroxynitrite does not react very rapidly with reduced TbTXN. TbTXN alone caused only a slight increase in peroxynitrite decomposition rate, whereas the addition of peroxynitrite (18 μM) to reduced TbcTXNPx (18 μM) produced a rapid initial decrease on peroxynitrite concentration, followed by a slower second phase of peroxynitrite decomposition, that reflected consumption of the enzyme. However, in the presence of both reduced TbcTXNPx (1.5–6 μM) and reduced TbTXN (70 μM), peroxynitrite had basically an exponential decay that was faster at higher TbcTXNPx concentrations tested (Fig. 3). From the slope of the plot of the apparent first-order rate constants of peroxynitrite decay at each TbcTXNPx concentration (in the presence of TbTXN) versus TbcTXNPx concentration, an apparent second order rate constant for the reaction between peroxynitrite and reduced TbcTXNPx of $7 \pm 1 \times 10^4 \cdot M^{-1} \cdot s^{-1}$ at pH 7.4 and 37 °C was obtained (Fig. 3, inset). This value is very similar to the value obtained by the initial rate approach (9 ± 1 × 10⁵ M⁻¹ s⁻¹; Fig. 2) for the same reaction. In contrast, both C40S TbTXN and C48S TbTXN were unable to sustain a catalytic TbcTXNPx-mediated peroxynitrite decomposition (data not shown).

Kinetics of the Reaction of T. cruzi Cytosolic Tryparedoxin Peroxidase with Peroxynitrite—The peroxynitrite (18 μM) decomposition rate was accelerated by reduced TcTXNPx (35–70 μM) but not by the NEM-pretreated enzyme. An initial rate approach indicated a second order rate constant of 7.2 ± 0.3 × 10⁵ M⁻¹ s⁻¹ for the reaction between peroxynitrite and reduced TcTXNPx at pH 7.4 and 37 °C (Table I).

Peroxynitrite decomposition in the presence of a heterologous system (41) formed by reduced TcTXNPx (0–9 μM) and reduced TbTXN (70 μM) had an exponential behavior, indicating that peroxynitrite-oxidized TcTXNPx could be re-reduced by the heterologous TbTXN. The second order rate constant for the reaction between peroxynitrite and TcTXNPx obtained with the enzyme in turnover was 8.5 ± 1 × 10⁷ M⁻¹ s⁻¹, at pH 7.4 and 37 °C (Fig. 4), consistent with the value obtained by the initial rate approach.

Peroxynitrite-dependent Oxidation of TbcTXNPx Thiol Groups—The addition of increasing concentrations of peroxynitrite to reduced TbcTXNPx (130 μM) led to an oxidation of the thiol groups. The slope of the curve was ~2, indicating...
that each peroxynitrite molecule led to the oxidation of two thiol groups of the enzyme (Fig. 5), in agreement with the stoichiometry of the reaction established previously for direct peroxynitrite-mediated oxidation of thiols (19). The addition of predecomposed peroxynitrite (60 μM) did not lead to any thiol oxidation in the enzyme (Fig. 5). Even in the presence of physiological concentrations of CO₂ (1.3 mM), there was still significant enzyme thiol oxidation, although it was less than in the absence of CO₂ (Fig. 5).

**FIG. 3.** Peroxynitrite decay in the presence of reduced TbcTXNPx is catalytic. Peroxynitrite (18 μM) decomposition in the presence of TbcTXN (70 μM) and increasing TbcTXNPx concentrations (a = 0; b = 1.5 μM; c = 3 μM; d = 4.5 μM; e = 6 μM) in 50 mM potassium phosphate buffer, pH 7.4 and 37 °C, was followed at 310 nm. The inset shows a plot of the k_{obs} of peroxynitrite decomposition versus TbcTXNPx concentration.

**FIG. 4.** TcTXNPx catalytically decomposes peroxynitrite in the presence of TcTXN. Peroxynitrite decomposition in the presence of increasing concentrations of TcTXNPx (0–9.2 μM) and 70 μM reduced TcTXN in 50 mM potassium phosphate buffer pH 7.4 and 37 °C plus 0.1 mM DTPA was followed at 310 nm. Primary curves were fitted to exponential decays and apparent first-order rate constants (k_{obs}) were plotted versus TcTXNPx concentrations.

**FIG. 5.** TbcTXNPx thiol oxidation by peroxynitrite. Increasing concentrations of peroxynitrite (filled symbols) or predecomposed peroxynitrite (open symbol) were added to reduced TbcTXNPx (130 μM) in 100 mM potassium phosphate buffer, pH 7.4 and 37 °C, in the absence (circles) or presence (triangles) of 25 mM sodium bicarbonate. The remaining thiols were measured by the DTNB assay.

Inhibition of Peroxynitrite-mediated Mn²⁺-TM-4-PyP Oxidation by TbcTXNPx—The fast reaction between peroxynitrite and reduced TbcTXNPx indicates that the enzyme should inhibit other direct oxidations performed by peroxynitrite. However, peroxynitrite-dependent oxidation of thiols leads to their sulfenic acid derivatives, which are not inert products but typically unstable and reactive, and could promote further oxidations. Therefore, we performed competition experiments in order to determine whether TbcTXNPx could protect other targets from peroxynitrite-dependent oxidation. We chose
Mn$^{3+}$-TM-4-PyP as a target, since it reacts directly and rapidly with peroxynitrite and can be conveniently followed spectrophotometrically. Thus, the effect of reduced TbcTXNPx on peroxynitrite-mediated Mn$^{3+}$-TM-4-PyP oxidation was evaluated. At increasing concentrations of TbcTXNPx, the maximum of Mn$^{3+}$-TM-4-PyP oxidation (at 100–200 ms) decreased, and the apparent first-order rate of peroxynitrite reduction increased (Fig. 6, a–d). Computer-assisted simulations allowed an estimation of the second order rate constant between peroxynitrite and the enzyme as $2 \times 10^6$ M$^{-1}$ s$^{-1}$. Percentage of Mn$^{3+}$-TM-4-PyP oxidation by peroxynitrite was calculated as ($\Delta$ absorbance in the presence of the enzyme/$\Delta$ absorbance in the absence of the enzyme) $\times$ 100. Absorbance change in the absence of the enzyme, which corresponds to 100% Mn$^{3+}$-TM-4-PyP oxidation, was 0.16 absorbance units. The inset shows the observed rate constants for peroxynitrite reduction versus TbcTXNPx concentration.

**FIG. 6.** Effect of TbcTXNPx on peroxynitrite-mediated Mn$^{3+}$-TM-4-PyP oxidation. Mn$^{3+}$-TM-4-PyP (10 $\mu$M) oxidation by peroxynitrite (2 $\mu$M) in 100 mM potassium phosphate buffer, pH 7.4 and 37 °C, was followed at 462 nm by the stopped-flow technique in the presence of increasing concentrations of TbcTXNPx ($a = 0$; $b = 2.5 \mu$M; $c = 5.0 \mu$M; $d = 6.2 \mu$M) and best fit to the experimental data using computer-assisted simulations using a second order rate constant for the reaction between peroxynitrite and the enzyme as $2 \times 10^6$ M$^{-1}$ s$^{-1}$. Percentage of Mn$^{3+}$-TM-4-PyP oxidation by peroxynitrite was calculated as ($\Delta$ absorbance in the presence of the enzyme/$\Delta$ absorbance in the absence of the enzyme) $\times$ 100. Absorbance change in the absence of the enzyme, which corresponds to 100% Mn$^{3+}$-TM-4-PyP oxidation, was 0.16 absorbance units. The inset shows the observed rate constants for peroxynitrite reduction versus TbcTXNPx concentration.

Mn$^{3+}$-TM-4-PyP oxidation by peroxynitrite (Reaction 4) is the sum of two contributions (48), one arising from the reaction of peroxynitrite with Mn$^{3+}$-TM-4-PyP, which is the $y$ intercept in Fig. 6, inset, and the acceleration in rate arising from the reaction of peroxynitrite with TbcTXNPx (slope in Fig. 6, inset).

$$k_{obs} = k(Mn^{3+} \cdot TM-4-PyP + ONOO^-) \cdot (Mn^{3+} \cdot TM-4-PyP)$$

$$+ k(TbcTXNPx + ONOO^-) \cdot (TbcTXNPx)$$

**REACTION 4**

Linear regression (Fig. 6, inset) yielded a second order rate constant for the reaction between peroxynitrite and Mn$^{3+}$-TM-4-PyP of $3.2 \times 10^6$ M$^{-1}$ s$^{-1}$ at pH 7.4 and 37 °C, in close agreement with the literature (46), and yielded a second order rate constant for the reaction of peroxynitrite with TbcTXNPx of $2.5 \pm 0.8 \times 10^6$ M$^{-1}$ s$^{-1}$ at pH 7.4 and 37 °C, which is not so
The addition of peroxynitrite to reduced TbCTXNPx lead to the oxidation of its thiol groups (Fig. 5). Under conditions of excess enzyme, the stoichiometry of the reaction was two thiols oxidized by each peroxynitrite added, consistent with the reported stoichiometry for direct thiol oxidation, which involves a two-electron oxidation of the thiol to its sulfenic derivative, an intermediate detected in the case of peroxynitrite-mediated oxidation of the single thiol group of human serum albumin (54). Peroxynitrite-derived radicals CO$_3^-$ and NO$_2^-$ oxidize thiols to their corresponding thyl radicals, which could lead to inactive forms of the enzyme either through the reaction with oxygen and thyl peroxyl radical formation (RSEO$^-$) and a sulfenic acid-containing end product or, in the presence of an accessible thiol group, to a mixed disulfide through the formation of a disulfide radical anion (RSSR$^-$). However, under the conditions of our experiments, kinetic analysis indicates that, at least initially, one-third of peroxynitrite would be reacting with CO$_2$ and the other 70% with TbCTXNPx$^4$, and therefore the thiol consumption observed in Fig. 5, even in the presence of physiological CO$_2$ concentrations, is mainly dependent on the direct reactions between the enzyme and peroxynitrite. Tryparedoxin peroxidases are highly abundant enzymes in trypanosomatids, constituting up to 5% of total soluble protein (26, 36) and representing about 0.5–1.0 mM active site thiol, which makes this an even more physiological relevant target for peroxynitrite reactivity inside the parasite.

If peroxynitrite oxidizes Cys-52 in the enzyme to the same sulfenic acid derivative implicated in hydroperoxide detoxification, it is very likely that the rest of the catalytic process (i.e. the formation of the postulated intersubunit disulfide with Cys-173 and the reduction of the oxidized enzyme by TbcTXNPx) functions in a way similar to that described for other hydroperoxides. This was indeed the case, since although TbcTXNPx did not react very rapidly with peroxynitrite (Table 1) and, at the concentration tested in Fig. 3, had only a marginal effect on peroxynitrite decomposition rate, the presence of both TbcTXNPx and TbcTXN led to an increase in the rate of exponential peroxynitrite decay, indicating that TbcTXNPx concentrations were maintained relatively constant during the time course of the experiment when TbcTXN was also present (Fig. 3 and Supplemental Matte-
Tryparedoxin Peroxidase Reaction with Peroxynitrite

T. cruzi macrophage-derived O2− and ‘NO generate peroxynitrite inside the phagosome, minimizing diffusional restrictions for enacting target molecule reactions inside the parasite during the infection process. In the case of T. brucei, which is an extracellular parasite, macrophage-derived peroxynitrite is expected to be consumed to some extent in the extracellular space. However, O2− is also formed inside T. brucei (e.g. by the tyrosyl radical formed during ribonucleotide reductase turnover), in particular in the proliferative stages of the parasite. Thus, macrophage-derived ‘NO, which is a long-lived free radical, could reach T. brucei, react with endogenous O2− and form peroxynitrite intracellularly. Therefore, the tryparedoxin:peroxynitrite oxidoreductase activity of T. brucei and T. cruzi cTXNPx reported herein supports its role as an important factor for the survival and proliferation of trypanosomatids in the presence of activated macrophages.

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