Trypanosoma brucei and Trypanosoma cruzi Tryparedoxin Peroxidases Catalytically Detoxify Peroxynitrite via Oxidation of Fast Reacting Thiols

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 peroxo-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 cytosolic tryparedoxin peroxidase system in T. brucei (Tb) and T. 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 \(9 \times 10^5\) and \(2.2 \times 10^8\) 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 Mn\(^{3+}\)-porphyrins. Moreover, steady-state kinetic studies indicate that peroxynitrite-dependent TbTXNPx and TcTXNPx oxidation is readily reverted by TXN, supporting that these peroxiredoxins would be not only a preferential target for peroxynitrite reactivity but also 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 (O\(_2^-\)) and hydrogen peroxide (H\(_2\)O\(_2\)) are synthesized as a result of the oxidative burst by inflammatory cells from T. brucei- and T. cruzi-infected animals (6–8). Superoxide can be also 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 O\(_2^-\) 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 peroxynitrite and/or nitrogen dioxide (NO\(_2\)) formation (15–17).

Peroxynitrous acid is an unusual peroxo-acid, since it has a low \(pK_a\) value (6.8 versus 11.6 of the first proton dissociation in hydrogen peroxide, H\(_2\)O\(_2\)) and a weak O–O bond (bond strength of 90 kcal mol\(^{-1}\) versus 170 kcal mol\(^{-1}\) H\(_2\)O\(_2\)) (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 NO\(_2\), which either recombine to form nitrate or react with substrates. The short lifetime of peroxynitrous acid and its fast reaction with carbon dioxide (CO\(_2\)) frequently present in buffers and in biological systems (12) makes biochemical studies more difficult to perform than with H\(_2\)O\(_2\) 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 nitrite and the thiol-derived sulfenic acid, which, in the presence of an
accessible thiol group, forms a disulfide, resulting in an overall stoichiometry of two thiols oxidized per peroxirexin (19). The reaction involves peroxynitrioxide acid and the deprotonated form of the thiol (thiolate, RS⁻). The second order rate constants for the reactions between peroxynitrioxide acid and low molecular weight thiols at pH 7.4 (~10² to 10⁶ M⁻¹ s⁻¹) are inversely related to the thiol pKₐ (20). However, there is an increasing number of highly reactive protein thiols that react with peroxirexin at rates of 10¹⁰ to 10¹³ M⁻¹ s⁻¹ at pH 7.4 and 37 °C (21, 22). Among them, the bacterial peroxirexin 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 (pKₐ 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) (N₁(V₁),N₈(bis(glutathionyl)permide)) for the supply of reducing equivalents. These pathways differ in subcellular location and substrate specificity (26–32). Cytosolic tryparedoxin peroxidase (cTXNPx), which is a two-cysteine peroxiredoxin, is essential for hydroperoxide detoxification in T. brucei (33) and for Leishmania chagasi survival from reactive oxygen and nitrogen species formed by infected macrophages (34). Moreover, cTXNPx is induced during H₂O₂ challenge to T. cruzi (35) and for Leishmania chagasi cells. Thus, reducing thiols oxidized per peroxynitrite (25). However, this general mechanism of peroxirexin detoxification has been challenged recently in the case of T. cruzi (36), thioredoxin peroxidases of yeast (37), bacterial alkyldihydroperoxide reductase (38), and mammalian peroxiredoxins (39), involves an initial Cys-52 oxidation to a sulfenic acid derivative that then reacts with Cys-173 of an inversely oriented identical subunit to form an interubunsh disulfide (32). The sequence of reactions has been described for bacterial alkyldihydroperoxide reductase decomposition of peroxirexin (23). However, this general mechanism of oxidant detoxification has been challenged recently in the case of L. chagasi cTXNPx (34). In this case, it has been postulated that mycobacterial reactive oxygen species react with Cys-52, peroxirexin does so with Cys-173.

Cytosolic TXNPx uses tryparedoxin (a thiol-disulfide oxidoreductase classified in the same family as the functional homologue thioredoxin (TXN) (26)) as an electron donor and reduces substrates including hydrogen peroxide and small chain organic hydroperoxides (32). Tryparedoxin is reduced by dihydrotrypanothione, which in turn is regenerated by trypanothenol reductase, a homodimeric enzyme containing FAD and a reducible active site disulfide per subunit, at the expense of NADPH. In T. cruzi, exogenous or endogenous peroxirexin leads to dihydrotrypanothione oxidation to the corresponding disulfide, and it has been postulated that the trypanothenole-dependent antioxidant system against peroxirexin may facilitate the survival of trypanosomes within the oxidative environment of activated macrophages (14). However, the biochemical mechanism of dihydrotrypanothione oxidation and peroxirexin detoxification at the cellular level remains unknown. Indeed, since trypanotheno reductase, dihydrotrypanothione, and tryparedoxin peroxidase are all thiol-containing molecules, they could all in theory react with peroxirexin. However, the relative importance of these biotargets as reactants of peroxirexin in vivo would be, in good part, dictated by rate constants and reactive concentrations.

Herein, we present kinetic studies on the reactivity of different components of the tryparedoxin peroxidase antioxidant system in trypanosomes, namely tryparanothione, TbvTXN, TbcTXNPx, and TcTrTXNPx with peroxirexin, with the aim to rationalize the biochemical mechanisms of peroxirexin detoxification in T. brucei and T. cruzi.

MATERIALS AND METHODS

Chemicals—Trypanothione was obtained from Bachem. Sodium borohydride, dithiothreitol, N-ethylmaleimide (NEM), diethylenetriaminepentaacetic acid (DTPA), 5,5'-dithio-2-nitrobenzoic acid (DTNB), manganese dioxide, sodium nitrite, sodium bicarbonate, hydrogen peroxide, and sodium hydroxide were from Sigma. Dihydrotachridamine 123 was purchased from Molecular Probes, Inc. (Eugene, OR). 3-Morpholinosydnonimine hydrochloride (SIN-1) was from Casella AG. Argon (99.5% pure) was purchased from AGA Gas Company (Montevideo, Uruguay). Manganes-(III)-meso-tetrakis[(N-methyl)pyridinium-4-yl]porphyrin (M₃⁺·TM-4-PyP) was kindly supplied by Ines Batinic-Haberle (Duke University). All other chemicals were reagent grade. Solutions were prepared with highly pure, oxygenated water (Barnstead NANOpure resist ance ~18 megaohm cm⁻¹) to minimize trace metal contamination.

Expression and Purification of T. brucei Tryparedoxin and T. brucei Cytosolic Tryparedoxin Peroxidases—Proteins were obtained by heterologous expression of the respective genes in Escherichia coli. The gene of TXN of T. brucei brucei, as identified by Luedemann et al. (40), was expressed as an N-terminally His-tagged protein that was purified as described previously (32). Molecular mutants of TbvTXN were obtained according to Ref. 32. T. brucei cytosolic TXNPx and mutated forms of the enzyme were also prepared as N-terminally His-tagged proteins as described previously (32). T. cruzi cytosolic TXNPx was purified as in Ref. 41 with the following modifications. The amplified T. cruzi TXNPx gene was cloned into the pQE-30 vector (Qiagen) between SacI and HindIII. pQE30-TcH6TXNPx in E. coli M15 cells was grown at 30 °C with vigorous aeration in LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. Expression of recombinant TcH6TXNPx was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside when the culture reached A₆₅₀ = 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 TXNPx was eluted in 50 mM sodium phosphate, pH 7.6, containing 300 mM imidazole, 500 mM NaCl.

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

Trypanothenone 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 on ice in the dark. The amount of reduced trypanothenone was measured by quantification of reduced thiol groups, using the DTNB assay (42).

Protein Thiol Reduction and Alkylation—TbvTXN, TbcTXNPx, and TcTrTXNPx 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 manually in 10 mM sodium phosphate, pH 7.4. The elution buffer was 100 mM potassium phosphate, pH 7, plus 100 µM DTPA, 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...
Fig. 1. Peroxynitrite reacts with reduced but not with oxidized trypanothione. Peroxynitrite (25 μM) decomposition rates in 100 mM potassium phosphate buffer, pH 7.4, and 37 °C and 0.1 mM DTPA were followed in the presence of increasing concentrations of dihydrotrypanothione (TS$_2$, closed circles) or trypanothione disulfide (TS$_2$, open circles). The graph shows the effect of trypanothione concentration on the pseudo-first-order decomposition rates ($k_{obs}$) of peroxynitrite. The inset shows a primary plot of peroxynitrite decay either with no further addition (a), in the presence of 270 μM dihydrotrypanothione (b), or in the presence of 260 μM oxidized trypanothione (c).

**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 M$^{-1}$ cm$^{-1}$) (44), we monitored it at 310 nm (ε$_{310}$ = 1600 M$^{-1}$ cm$^{-1}$) in order to avoid interferences by background protein absorption at 302 nm. When the initial rate approach was used (45), 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 are first-order 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**—Computer-assisted simulations were performed using the Gepasi program (49). 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 SigmaPlot.

**RESULTS**

The Reaction of Peroxynitrite with Trypanothione and T. brucei Tryparedoxin—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
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 ~3500 M⁻¹ 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 × 10³ M⁻¹ s⁻¹ at pH 7.4 and 37 °C was determined. The increase in peroxynitrite decomposition rates 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 mutated 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 × 10⁵ 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 × 10⁴ 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 redundant 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 net 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 essentially 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 ± 1 × 10⁴ M⁻¹ 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 × 10³ M⁻¹ s⁻¹; Fig. 2) for the same reaction. In contrast, both C40S TbTXN and C43S 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 × 10⁵ 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 enzyme thiol groups. The slope of the curve was ~2, indicating

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**Table 1.**

| Compound | \( k^2 \) |
|---------|---------|
| Dihydrotrypanothione | 7.2 ± 0.5 × 10³ |
| Trypanothione disulfide | No reaction |
| TbTXN | –3.5 × 10³ |
| TbcTXNPx (wild type) | 9 ± 1 × 10³ |
| TbcTXNPx (C52S) | ~1 × 10⁴ |
| TbcTXNPx (C173S) | 3.5 ± 0.5 × 10⁵ |
| TcTXNPx | 7.2 ± 0.6 × 10² |

\( ^* \) At pH 7.4 and 37 °C.
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 ±M) 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 TbcTXNPx (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 Mn³⁺-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 sulfinic 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$^{-1}$ (Fig. 6). The O$_7$Mn$_2$-TM-4-PyP is an unstable species that is readily reduced back to Mn$^{3+}$ state, the rate of reduction increasing with increasing TbcTXNPx concentration, and significant decay of the O$_7$Mn$_2$ complex took place over the time scale of its formation (Fig. 6) (i.e. see differences between experimental and simulated data at time $> 100$ ms). The observed first-order rate constant $k_{obs}$ for the reduction of 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 TTXNPx (slope in Fig. 6, inset).

$$k_{obs} = k(Mn^{3+}-TM-4-PyP + ONOO')([Mn^{3+}-TM-4-PyP] + k(TbcTXNPx + ONOO')(TbcTXNPx)$$

**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
far from the more reliable value determined directly for the same reaction ($9 \times 10^{-5} \text{ M}^{-1} \text{s}^{-1}$).

**Tryparedoxin Peroxidase Reaction with Peroxynitrite**

\[ \text{SIN-1} \rightarrow \text{ONOO}^- + \text{H}^+ \rightarrow \text{ONOOH} \]
\[ k_2 = 9 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \]

**Scheme 1. Mechanism of catalytic detoxification of peroxynitrite by cTXNPx.**

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$_2$ 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 (RSOO$^*$) 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*, 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.

**Discussion**

Peroxynitrite reacts directly with all of the thiol-containing components of the *T. brucei* cytosolic tryparedoxin peroxidase system tested (i.e. dihydrotrypanothione, TXN, and cTXNPx) (Figs. 1 and 2, Table 1). However, the second order rate constant for the reaction of peroxynitrite and reduced *TbcTXNPx* is ~100 times faster than with dihydrotrypanothione or reduced *TbTXN* at physiological pH. Cys-52 was identified as the initial peroxynitrite target in the enzyme (Fig. 2), as it is for the reaction with H$_2$O$_2$ and other hydroperoxides (32). The reason for its fast reactivity has been ascribed to its activation by a catalytic triad composed of Cys-52 that is hydrogen-bonded to the hydroxyl group of Thr-49 and electrostatically activated by Arg-128 (24, 36), which means that Cys-52 in tryparedoxin peroxidases is most probably deprotonated at physiological pH. Our observations contrast with a recent report on *L. chagasi* cTXNPx I, which proposed that in this enzyme the Cys-52 residue is essential for detoxifying H$_2$O$_2$, whereas the Cys-173 residue is essential for peroxynitrite reduction (34). Given the homology between *L. chagasi* cTXNPx I and *TbcTXNPx* (53) and the unique redox properties of Cys-52, this apparent discrepancy is intriguing; therefore, the reaction of peroxynitrite with peroxiredoxins of the *Leishmania* species deserves further investigation.

$^4$ The percentage of peroxynitrite that would react with each target is calculated dividing the pseudo-first-order rate constant of peroxynitrite decay in the presence of the target ($k_{TbTXNPx} + k_{CO_2}$) (i.e. $k_{CO_2} = k_C (\text{ONOO}^- + \text{CO}_2)^{12} \times [\text{CO}_2] = 4.3 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \times 1.3 \times 10^{-3} \text{ M} = 56 \text{ s}^{-1}$; $k_{TbTXNPx} = k_t (\text{ONOO}^- + \text{TbcTXNPx}) \times [\text{TbcTXNPx}] = 8 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \times 150 \times 10^{-8} \text{ M} = 120 \text{ s}^{-1}$.)
Tryparedoxin Peroxidase Reaction with Peroxynitrite

Peroxynitrite is expected to be consumed to some extent in the parasite, which is an extracellular parasite, macrophage-derived peroxynitrite inside the phagosome, minimizing degradation by a SIN-1-derived flux of peroxynitrite, where the lag phase of DHR oxidation was much longer in the presence of both TbcTXNpx and TbTXN than in the presence of TbcTXNpx alone (Fig. 7) confirms the catalytic activity of TbcTXNpx under steady-state conditions. The results obtained using TccTXNpx revealed that this enzyme also decomposes peroxynitrite in a catalytic manner (Fig. 4), and the second order rate constant for the reaction between the enzyme and peroxynitrite was similarly fast (Table I). Moreover, TbcTXNpx had an inhibitory effect on direct (Fig. 6) and radical-dependent (Fig. 7) peroxynitrite-mediated oxidations.

In the case of T. cruzi, macrophage-derived O2− and NO generate peroxynitrite inside the phagosome, minimizing diffusion 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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REFERENCES
1. Vincendeau, P., Jauberteau-Marchan, M. O., Daulouede, S., and Ayed, Z. (1999) in Progress in Human African Trypanosomiasis (Dumas, M., and Buguet, A., eds) pp. 157–156, Springer-Verlag, Berlin.
2. Celentano, A. M., and Gonzalez Cappa, S. M. (1992) Exp. Parasitol. 11, 211–215.
3. Celentano, A. M., and Gonzalez Cappa, S. M. (1992) Exp. Parasitol. 11, 211–215.