Kinetics, subcellular localization, and contribution to parasite virulence of a *Trypanosoma cruzi* hybrid type A heme peroxidase (TcAPx-CcP)

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The *Trypanosoma cruzi* ascorbate peroxidase is, by sequence analysis, a hybrid type A member of class I heme peroxidases (TcAPx-cytochrome c peroxidase [CcP]), suggesting both ascorbate (Asc) and cytochrome c (Cc) peroxidase activity. Here, we show that the enzyme reacts fast with H$_2$O$_2$ ($k = 2.9 \times 10^6$ M$^{-1}$s$^{-1}$) and catalytically decomposes H$_2$O$_2$ using CC as the reducing substrate with higher efficiency than Asc ($k_{cat}/K_m = 2.1 \times 10^4$ versus $3.5 \times 10^4$ M$^{-1}$s$^{-1}$, respectively). Visible-absorption spectra of purified recombinant TcAPx-CcP after H$_2$O$_2$ reaction denote the formation of a compound I-like product, characteristic of the generation of a tryptophan radical-cation (Trp$^{2+\bullet}$). Mutation of Trp$^{2+\bullet}$ to phenylalanine (W233F) completely abolishes the Cc-dependent peroxidase activity. In addition to Trp$^{2+\bullet}$, a Cys$^{222}$-derived radical was identified by electron paramagnetic resonance spin trapping, immunosuppressive trapping, and MS analysis after equimolar H$_2$O$_2$ addition, supporting an alternative electron transfer (ET) pathway from the heme. Molecular dynamics studies revealed that ET between Trp$^{2+\bullet}$ and Cys$^{222}$ is possible and likely to participate in the catalytic cycle. Recognizing the ability of TcAPx-CcP to use alternative reducing substrates, we searched for its subcellular localization in the infective parasite stages (intracellular amastigotes and extracellular trypomastigotes). TcAPx-CcP was found closely associated with mitochondrial membranes and, most interestingly, with the outer leaflet of the plasma membrane, suggesting a role at the host–parasite interface. TcAPx-CcP overexpressers were significantly more infective to macrophages and cardiomycocytes, as well as in the mouse model of Chagas disease, supporting the involvement of TcAPx-CcP in pathogen virulence as part of the parasite antioxidant armamentarium.

*Trypanosoma cruzi* | heme peroxidase | oxidants | virulence | kinetics

The protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas disease (CD; also known as American trypanosomiasis). Up to 10 million people across Latin America are infected with this protozoan parasite, a distribution range that is expanding driven by migration of infected insects and hosts, with CD now emerging as a public health problem at nonendemic sites (1, 2). *T. cruzi* strains are heterogeneous, exhibiting a high degree of biochemical and genetic variability. Such differences are believed, at least in part, to be responsible for disease outcome, which ranges from being asymptomatic during the course of infection to fatal severe cardiac and digestive complications (3). It has been shown that the parasite antioxidant systems are essential for parasite survival and establishment of the infection in the vertebrate host (4–7). In contrast to most eukaryotes, *T. cruzi* lacks catalase and selenium-dependent glutathione peroxidases, which are enzymes capable of rapidly metabolizing high levels of H$_2$O$_2$ (8, 9). Instead, it expresses an array of complex enzyme-mediated mechanisms in which the trypanosomatid-specific thiol trypanothione ([T(SH)$_2$], N$^\delta$N$^\delta$-bisglutathionylspermidine) plays a central role in the funneling of reducing equivalents to the different peroxidase antioxidant systems (10). Two typical 2-Cys peroxidoxins are located in the cytosol (CPX) and in the mitochondrial matrix (MPX), respectively, and efficiently scavenge H$_2$O$_2$, peroxynitrite, and small-chain organic hydroperoxides (11–13). Two glutathione-dependent peroxidases are located at the endoplasmic reticulum (ER) and in the cytosol, and seem to be important in the metabolism of lipid-derived hydroperoxides (14). Finally, a plant-like related heme peroxidase located at the ER displays ascorbate (Asc)-dependent peroxidase activity (APx) (15).

Because the *T. cruzi* antioxidant defense systems are distinct from its mammalian host, the trypanosomal activities are suitable targets for specific rationale pharmacological inhibition. During its life cycle, *T. cruzi* undergoes various morphological and biochemical changes. One of the most complex transformations occurs during

**Significance**

*Trypanosoma cruzi*, the causative agent of Chagas disease, affects 8–10 million people in Latin America. Parasite antioxidant systems are essential for parasite survival and infectivity in the vertebrate host. Herein, we characterized the enzymic properties, subcellular localization, and contribution to parasite virulence of a *T. cruzi* hybrid type A member of class I heme peroxidases. The enzyme reacts fast with hydrogen peroxide and utilizes both ferrocytochrome c and ascorbate as reducing substrates [*T. cruzi* ascorbate peroxidase (TcAPx)-cytochrome c peroxidase (CcP)]. A unique subcellular distribution of TcAPx-CcP in the infective stages suggests a role during parasite–host interactions. Infection of macrophages and cardiomycocytes, as well as in mice, confirmed the involvement of TcAPx-CcP in pathogen virulence as part of the parasite antioxidant armamentarium.


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metacyclogenesis, the process where a noninfective and replicative insect-derived epimastigote cell transforms to a highly infectious, nonreplicative, metacyclic trypomastigote. During this process, it has been shown that T. cruzi up-regulates several antioxidant enzymes to preadapt to the hostile environment of the vertebrate host (5, 16, 17). Enzymes known to be up-regulated during this differentiation process include peroxiredoxins, [T(3H)] synthase (5), Fe-containing superoxide dismutases, and APx (16).

*T. cruzi* ascorbate peroxidase (TcAPx) was first reported to reduce \( \text{H}_2\text{O}_2 \) catalytically in the presence of Asc as the reducing substrate (APx activity), but with a catalytic efficiency several fold lower than plant APx (18, 19). Its overexpression confers parasite resistance toward exogenously added \( \text{H}_2\text{O}_2 \) toxicity (13, 15, 20).

Recent studies have shown that *T. cruzi* lacking TcAPx is still capable of infecting cultured mammalian cells, albeit at a reduced level, and can still establish an infection in the mouse model of CD (20). Although the enzyme does not seem to be essential for parasite infectivity, its enhanced expression may represent an additional skill for the parasite to deal with host-derived oxidant toxicity both in the acute and chronic stages of the disease (6, 21).

TcAPx shares significant homology (62% identity and 86% similarity) with its *Leishmania* major peroxidase (Lmp) counterpart (18). Both parasite enzymes are related to the class I heme-peroxidase group of antioxidant enzymes that includes catalase-peroxidase (KatG), APx, and peroxiredoxin (4, 5). Lmp is encoded by the genome of *Lm*. Phylogenetic studies have classified the *T. cruzi* and *Leishmania* enzymes as members of the hybrid type A, subfamily A1, heme peroxidases (23), with other sequences in this group displaying both Asc- and cytochrome c (Cc)-dependent peroxidase activities (22, 24). Based on this fact, we have renamed TcAPx as TcAPx-CcP.

These hybrid type A peroxidases represent a real turning point in the evolution of ancient bifunctional catalase-peroxidase toward monofunctional specialization within this family, and their biochemical characterization may help to unravel the evolution of structure-function relationships of these ancient heme peroxidases (23).

*Lmp* can use both Asc (as monofunctional APx) and Cc (as monofunctional CcP) as electron donors (25). \( \text{H}_2\text{O}_2 \) reacts with the heme generating the oxoferryl (Fe(V=O)) and the porphyrin-centered radical (P•) forming the classical compound I species. The presence of tryptophan located near the heme (Trp•) in *Lmp* rapidly reduces the P•, generating an amino acid-derived cation radical (Trp0•, compound I-like). Finally, using either Asc or Cc as the reducing substrate, the enzyme recovers the resting state (Fe(II)-P). Classical compound I formation in these hybrid type A peroxidases was established by direct electron paramagnetic resonance (EPR) analysis (25) and/or by spectroscopic studies using a W208F mutant (25, 26). To date, *Lmp* represents the only trypanosomatid hybrid type A peroxidase characterized as using Asc and Cc as electron donors, with its activity implicated in parasite differentiation and oxidative stress protection (27). Biochemical characterization of other members of this family of peroxidases may help to unravel the catalytic mechanism of these ancient enzymes as well as to define their role in pathogen survival, proliferation, and virulence.

In the present work, we characterized the Cc-dependent peroxidase activity of TcAPx-CcP. After a fast reaction with \( \text{H}_2\text{O}_2 \), an intramolecular electron transfer (ET) pathway at the heme microenvironment necessary for the oxidation of Cc was revealed; indeed, in addition to Trp, the role of a Cys residue typically located in the active site of these hybrid type A peroxidases was disentangled. Also, in light of its CcP activity and the presence of a secretory motif, which predicts membrane localization in all other hybrid type A members studied (15, 22, 23), we explored the subcellular localization of the enzyme in the infective *T. cruzi* parasite stages. Finally, the role of TcAPx-CcP overexpression in parasite virulence was assessed through in vitro and in vivo infection in the cellular and acute murine model of CD.

**Results**

**Visible Spectral Studies of TcAPx-CcP Reaction with \( \text{H}_2\text{O}_2 \).** Recombinant *T. cruzi* wild-type (wt) and W233F APx-CcP were purified to homogeneity (>99% pure) by affinity chromatography as assayed by SDS/PAGE (Fig. 1A and Fig. S1A). For all purifications ca. 70% of protein had Fe incorporated (as determined by the Soret peak absorbance in relation to the total protein content). To investigate the reaction of TcAPx-CcP with \( \text{H}_2\text{O}_2 \), the optical spectra of TcAPx-CcP (from 350 to 650 nm) were recorded before and after the addition of equimolar concentrations of \( \text{H}_2\text{O}_2 \) to assay the spectral shifts associated with the formation of a compound I-like product (25, 26). The resting enzyme and after \( \text{H}_2\text{O}_2 \) reaction. Spectral shifts of TcAPx-CcP are indicative of compound I-like formation. The Soret peak (5 μM) (D) or W233F (E) was mixed with (dashed line) or without (solid line) \( \text{H}_2\text{O}_2 \) at 10 °C. Changes in the absorbance at the Soret peak [409 and 414 nm for TcAPx-CcP (D) and W233F (E), respectively] were followed. The decrease in the absorbance at the Soret peak is indicative of compound I generation.

**Fig. 1.** Spectroscopic analysis of TcAPx-CcP compound I and compound I-like intermediates. (A) SDS/PAGE of purified, recombinant TcAPx-CcP (1) and its W233F mutant (2) visualized following Coomassie blue staining. Absorption spectra of TcAPx-CcP (2 μM) (B) and the W233F mutant (C) before (solid line) and after (dashed line) equimolar \( \text{H}_2\text{O}_2 \) addition (2 μM). (Inset) Spectra from 500 to 650 nm. Arrows indicate the Soret peak of the resting enzyme and after \( \text{H}_2\text{O}_2 \) reaction. Spectral shifts of TcAPx-CcP are indicative of compound I-like formation. TcAPx-CcP (5 μM) (D) or W233F (E) was mixed with (dashed line) or without (solid line) \( \text{H}_2\text{O}_2 \) at 10 °C. Changes in the absorbance at the Soret peak [409 and 414 nm for TcAPx-CcP (D) and W233F (E), respectively] were followed. The decrease in the absorbance at the Soret peak is indicative of compound I generation.
in the W233F mutant, the data support that other amino acids, in addition to Trp$^{233}$, can participate in the reduction of the Fe($^{IV}$ = O)P$^{•+}$ radical in TcAPx-CcP.

**Kinetic Analysis of the Cc-Dependent Peroxidase Activity.** Both the formation of a compound I-like product (this work) and the phylogenetic analysis of TcAPx-CcP indicate that the T. cruzi enzyme might function using Asc and Cc as electron donors. The TcAPx-CcP activity with Cc as the reducing substrate was monitored spectrophotometrically at 550 nm following ferrocyanochrome c (Cc$^{2+}$) oxidation after H$_2$O$_2$ addition. As shown in Fig. 2A, Cc$^{2+}$ oxidation was observed in the presence of variable concentrations of wt enzyme (0.05–0.1 μM) after H$_2$O$_2$ addition (50 μM). When the W233F enzyme was used, even at high concentrations (0.1–0.5 μM), no Cc$^{2+}$ oxidation was observed, indicating the central role of Trp$^{233}$ for the Cc-dependent enzyme activity (Fig. 2A). In this mutant, a 10-fold decrease in Asc-dependent activity was also measured. To characterize the Cc-dependent peroxidase activity further, steady-state kinetic analysis was performed in the presence of wt TcAPx-CcP (0.05 μM), and variable amounts of Cc$^{2+}$ (0–60 μM) and H$_2$O$_2$ (0–50 μM) as shown in Fig. 2B. The activity of the wt enzyme obeys Michaelis–Menten kinetics as assessed by the dependency of the initial velocity ($V_{\text{max}}$) as a function of H$_2$O$_2$ concentration under different Cc$^{2+}$ concentrations (Fig. 2B). A secondary plot of $1/V_{\text{max}}$ vs. $1/K_{\text{cat}}$ generated linear parallel slopes, indicating that TcAPx-CcP displays a typical bisubstrate ping-pong kinetic mechanism (30, 31) (Fig. 2C). From the y-axis intercept, apparent $V_{\text{max}}$ ($V'_{\text{max}}$) values for the different Cc$^{2+}$ concentrations used were calculated. From the y- and x-axes intercepts of the secondary plot ($1/V'_{\text{max}}$ vs. $1/K_{\text{cat}}$; Fig. 2D), $1/V'_{\text{max}}$ and $1/K_{\text{cat}}$ values for Cc$^{2+}$ were determined, respectively: $K_{m}$ (Cc$^{2+}$) = 23.5 μM, $k_{\text{cat}}$ = 5.0 s$^{-1}$, and $k_{\text{cat}}$/$K_{m}$ (Cc$^{2+}$) = 2.1 × 10$^5$ M$^{-1}$s$^{-1}$. To determine the rate constant of the enzyme with H$_2$O$_2$, the absorbance at 390 nm ($\lambda$ value at which the resting and oxidized enzyme have significant absorbance differences; Fig. 1B) was followed by rapid stopped-flow techniques. The reaction of H$_2$O$_2$ (8 μM) with TcAPx-CcP (2 μM) led to a fast time-dependent decrease in absorbance (Fig. 2E). The time course fitted to a double exponential decay, with observed rate constants for both phases dependent on H$_2$O$_2$ concentration (Fig. 2F). The slopes of the plot (Fig. 2F) yielded second-order rate constants for the H$_2$O$_2$-dependent oxidation of TcAPx-CcP of (2.9 ± 0.5) × 10$^{5}$ M$^{-1}$s$^{-1}$ and (5.4 ± 0.2) × 10$^{5}$ M$^{-1}$s$^{-1}$ for the fast and slow processes, respectively. The k value on the order of 10$^{7}$ M$^{-1}$s$^{-1}$ for the initial reaction of H$_2$O$_2$ with the resting ferri state of the TcAPx-CcP is in good agreement with previous reports for other heme peroxidases [26, 32]. It was previously shown that APx enzymes (in the absence of reducing substrates) are inactivated by H$_2$O$_2$ (33). Thus, the slower process is likely to represent (because no reducing substrate was present) the reaction of a second molecule of H$_2$O$_2$ with the oxidized TcAPx-CcP species, leading to an inactive form of the enzyme. Indeed, under these experimental conditions, we also observed a time-dependent inactivation of the enzyme by H$_2$O$_2$ (SI Materials and Methods and Fig. S1). In Table 1, the parameters of the steady-state kinetic analysis at pH 7.4 of TcAPx-CcP when using Cc (Fig. 2) or Asc (Fig. 2) as the reducing substrates are shown. Overall, the efficiency of TcAPx-CcP when using Cc is one order of magnitude higher than with Asc.

**Detection of TcAPx-CcP Trp$^{233}$ Radical and Role of Cys$^{222}$.** To determine the amino acid-derived radicals participating in the TcAPx-CcP catalytic cycle after reaction with H$_2$O$_2$, EPR spin trapping studies were carried out. Upon addition of equimolar concentrations of H$_2$O$_2$ to the wt enzyme (60 μM) in the presence of the spin trap 3,5-dibromo-4-nitroso benzene sulfonate (DBNBS) (10 mM), an immobilized carbon-centered radical adduct signal that has a nitrogen hyperfine splitting constant ($a_N$) of 32 G (or 2a$N = 64$ G); this signal was absent in the W233F mutant (Fig. 3). Overall, these results strongly support the participation of a Trp$^{233}$-derived radical in the generation of the compound I-like product. Detailed inspection of the heme microenvironment in hybrid type A and CcP peroxidases revealed the presence of a Cys residue (Cys$^{222}$ for T. cruzi enzyme) located near Trp$^{233}$ (25, 34); this Cys residue is absent in the APx family (23). It has been proposed that the sulfur atom of this Cys residue favors stabilization of the LmP compound I-like product. Indeed, mutation of Cys$^{222}$ to Thr in LmP decreased more than 100-fold the Cc$^{2+}$-dependent peroxidase activity without affecting the Asc-dependent function (25, 26). We thus explored the involvement of Cys$^{222}$ in TcAPx-CcP peroxidase activity by means of alkylation of enzyme thiols with N-ethyl-maleimide. In the W233F mutant, the data support that other amino acids, in addition to Trp$^{233}$, can participate in the reduction of the Fe($^{IV}$ = O)P$^{•+}$ radical in TcAPx-CcP.
(NEM). Of the six Cys residues present in TcAPx-CcP, two are buried in the protein structure and four (including Cys\(_{222}\)) are solvent-accessible. As shown in Fig. 4, NEM-treated wt enzyme reacts with H\(_2\)O\(_2\), while the generation of a compound I-like product (Fig. 4A). Although the NEM-treated enzyme reacted with H\(_2\)O\(_2\), CcP\(_{222}\)-dependent peroxidase activity was decreased by more than 70\% compared with wt enzyme, although the Asc-dependent peroxidase activity was not affected (25, 26) (Fig. 4D). Interestingly, when the NEM-treated W233F mutant was exposed to H\(_2\)O\(_2\), no changes in the Soret peak at 414 nm of the resting Fe-III was observed (Fig. 4C). In this condition, a significant drop in Asc-dependent peroxidase activity was also evident for the W233F-NEM enzyme (Fig. 4D). Overall, the above results indicate that Cys\(_{222}\) (located near the heme and Trp\(_{233}\)) is necessary for compound I-like generation/stability.

To determine the generation of a Cys\(_{222}\) radical accurately after reaction of TcAPx-CcP with H\(_2\)O\(_2\), immunospin trapping assays using 5,5-dimethyl-1-pyreline-N-oxide (DMPO) were performed. Untreated or NEM-treated wt and W233F enzymes (10 \(\mu\)M) were exposed to H\(_2\)O\(_2\) (0–30 \(\mu\)M) in the presence of DMPO (100 mM). DMPO-protein adducts were evaluated by Western blot using the anti–DMPO-nitron antibody as previously reported (35). In the wt enzyme, DMPO-nitron adducts were observed with a signal intensity dependent on H\(_2\)O\(_2\) concentration (Fig. 5A). These immunoreactive signals were completely abrogated in the NEM-treated enzyme, strongly suggesting the participation of Cys in the detected protein adduct (Fig. 5A). Moreover, the signal was greatly enhanced in the W233F mutant, suggesting that in the absence of Trp\(_{233}\), Cys\(_{222}\) could be involved in the recovery of compound I, leading to an alternative ET pathway, as suggested from data in Fig. 1. These results were corroborated by EPR spin trapping analysis (Fig. 5B).

Reaction of the wt enzyme (60 \(\mu\)M) with equimolar concentrations of H\(_2\)O\(_2\) in the presence of DMPO (100 mM) yielded a detectable paramagnetic signal; notably, the EPR signal was significantly enhanced in the W233F mutant (Fig. 5B, line B). One hyperfine splitting constant of the beta hydrogen (\(a(H) = 16\) G) is fully consistent with the trapping of an APx-CcP-cysteinyln radical, whereas another (\(a(H) = 9.3\) G) suggests that a Tyr radical may be also trapped (36, 37). Furthermore, the formation of the DMPO radical adduct was almost fully inhibited in the NEM-treated wt and W233F enzymes (Fig. 5B, lines C). The results indicate the generation of a protein-derived radical located at the active site Cys\(_{222}\) (alternative compound I-like) that is important for the TcAPx-CcP Cc-dependent peroxidase activity (Fig. 4B). To obtain full confirmation of the formation of Cys\(_{222}\) radical upon H\(_2\)O\(_2\) reaction, peptide mapping-MS analysis of TcAPx-CcP-W233F was performed. A manual search for the peptide of interest revealed both L\(^{202}\)-K\(^{237}\) and DMPO-adduct peptides coeluting at 60.2 min. Most abundant ions were those ions tetra-charged for both peptides although triple-charged ions were also found. Enhanced resolution analysis was performed for best accuracy and mass-to-charge ratio (m/z) were obtained for each peptide: 1,258.9 ([M + 3H]\(^{3+}\)); 944.4 ([M + 4H]\(^{4+}\)); 1,294.9 ([M + DMPO + 3H]\(^{3+}\)); 971.4 ([M + DMPO + 4H]\(^{4+}\)). The fragmentation pattern of the DMPO-peptide adduct was obtained by an enhanced product ion of m/z 971.4 (Fig. 5C). Several b and y ions were found, ensuring identity of the peptide sequence. Interestingly, the y-series (\(y_{9}\)) suggests that Tyr\(^{229}\) is unmodified after treatment with H\(_2\)O\(_2\) in the presence of DMPO. The assignment of the DMPO-modified amino acid was finally obtained with the data of the b\(_{17}\) fragment (unmodified peptide) and the b\(_{22}\) fragment (modified peptide) in full support of Cys\(^{222}\) as the preferential site of adduct formation by DMPO in the W233F enzyme (Fig. 5C). Overall, EPR spin trapping, immunospin trapping, and peptide mapping-MS data support the critical role of Cys\(^{222}\) in the generation of the alternative compound I-like in TcAPx-CcP.

**Molecular Dynamics of ET Pathways at the Heme Microenvironment Involving Trp\(_{233}\) and Cys\(_{222}\)** Using the previously reported crystallographic structure of LmP [Protein Data Bank (PDB) ID code 3R1V], we performed homology models for the TcAPx-CcP (25) (Fig. S3), from which 100-ns-long molecular dynamics (MD) simulations were conducted. The proximal site of the heme in TcAPx-CcP contains the conserved residues Trp\(_{233}\), Cys\(^{222}\), and His\(^{237}\) present in all hybrid type A peroxidases (25). The critical Trp\(_{233}\) side chain is maintained nearby the His\(^{237}\), which coordinates the heme iron via \(\pi\)-stacking interactions (Fig. 6). This structural property determines the capability of Trp\(_{233}\) of being able to reduce the heme after its reaction with H\(_2\)O\(_2\). Particularly for TcAPx-CcP, Trp\(_{233}\) is located close to Cys\(^{222}\). MD simulations indicate that the interactions between the Trp\(_{233}\) and Cys\(^{222}\) side chains are influenced by the thiol or thiolate character of Cys\(^{222}\) ([i.e., Cys\(^{222}\) pK\(_a\)]. The thiol form shows a
Subcellular Localization of TcAPx-CcP in the Infective Parasite Stages.

Here, the kinetic data shown above indicate that the TcAPx-CcP–preferred source of reducing equivalents is Cc. In addition to the previously reported ER localization in the noninfective epimastigote stage (15), we searched for the presence of TcAPx-CcP in the mitochondrial compartment (where Cc is present) in the different parasite stages (noninfective epimastigotes, infective intracellular amastigotes, and extracellular trypomastigotes) using specific antibodies (Fig. S3). First, and using TcAPx-CcP overexpressers (epimastigote), we conducted controlled-digitonin permeabilization studies to evaluate the coelution of the enzyme with mitochondrial markers (Fig. S6A). TcAPx-CcP was found to coelute with Cc and Fe-superoxide dismutase A (SODA) (mitochondrial markers), and it was also present in the membrane fraction (Fig. S6A). This result indicates that at least a fraction of the enzyme is associated with mitochondria. Immunofluorescence microscopy of T. cruzi epimastigotes evidences a nonhomogenous distribution of the enzyme, compatible with ER localization (15). The enzyme is also present in close proximity to mitochondrial DNA and Fe-SODA (Fig. S6B). Finally, to localize proteins at the ultrastructural level precisely, providing simultaneous visualization of antigen epitopes together with the ultrastructure of membrane compartments, we conducted immunoelectron microscopy (immuno-EM) (38) of TcAPx-CcP in the infective T. cruzi stages. TcAPx-CcP enzyme was found to be located in mitochondria, particularly associated with membranes of the cristae (Fig. 8B and Figs. S7 and S8). The localization of TcAPx-CcP in mitochondria was different from the localization observed for MPX located at the mitochondrial matrix (11) (Fig. 8 C–E). Intriguingly, the enzyme was found to be associated with the plasma membrane of the intracellular amastigote and extracellular trypomastigote stages, potentially forming a “shield” at the host–parasite interface (Fig. S4 and Figs. S7 and S8). These observations were confirmed in T. cruzi pTEX-APx-9E10

direct interaction with Trp^{233}, but also with the heme porphyrin aromatic system mainly in the form of thiolate (Fig. 6 and Fig. S4A).

To understand the redox interplay at the heme microenvironment involving the heme, Trp, and Cys better, we performed ET probability calculations by using the “pathways” formalism. By means of this method, the possible paths for ET from Trp^{233} and Cys^{222} to the oxoferryl heme were computed throughout the MD trajectories. The best possible structural path (i.e., the higher electronic coupling matrix) connecting the electron donor and acceptor was obtained, allowing us to analyze and compare key structural features governing the ET process. In Fig. S4B, the average values are shown. The data support that the preferred ET pathway involved the heme-dependent oxidation of Trp^{233} (Fig. 7, route A) to the Tc^{233} intermediate; this intermediate may lose a proton with the generation of the neutral indolyl radical (NTrp^{233}) to be reduced by Cys^{222} to yield the corresponding cysteiny1 radical and establishing a redox equilibrium between the two amino acid-derived radical species. On the other hand, in the W233F mutant, the oxidized heme will promote the direct one-electron oxidation of the thiolate form of Cys^{222} (Fig. 7, route B), thus maintaining the Asc peroxidase activity of the enzyme in the absence of Trp^{233} (Fig. 4D).
overexpressers using the monoclonal anti-c-Myc antibody in both the trypanastigote and amastigote stages (Fig. S7). The presence of TcAPx-CcP at the host–parasite interface strongly suggests its participation in the parasite defense mechanisms toward host-derived toxicity. To date, the physiological reducing substrate (i.e., Asc, Cc, or both) for this ancient enzyme in T. cruzi is still elusive (Discussion).

Enhanced Virulence of TcAPx-CcP Overexpressers in Cellular and Mice Infections. T. cruzi interaction with macrophages leads to the assembly of the membrane-bound NADPH oxidase with the generation of sustained (90 min) and large amounts of superoxide radical, and thus hydrogen peroxide, directed toward the internalized parasite (7). Once internalized, T. cruzi has to deal with the oxidative environment of the phagosome to survive and escape to the host cytosol, where it proliferates as amastigotes. Thus, we first searched for parasite survival in naive macrophage infections. Macrophages were infected with wt or TcAPx-CcP–overexpressing trypomastigotes, and infection yields were evaluated after 24 h by intracellular amastigote counting. Trypomastigotes overexpressing TcAPx-CcP were more resistant to macrophage toxicity compared with wt parasites, indicating the participation of TcAPx-CcP in the H₂O₂ detoxification generated during parasite internalization (Fig. 9A). Finally, the infectivity of TcAPx-CcP overexpressers was evaluated in cardiomycocytes, cells where amastigotes can reside during chronic infection. It has been shown that T. cruzi cardiomycocyte infection leads to the establishment of host–mitochondrial dysfunction, with an increase in H₂O₂ and inflammatory cytokine (IL-1β and TNF-α) production (39). We evaluated the infectivity of TcAPx-CcP overexpressers following 96 h of infection, an incubation time sufficient to cause mitochondrial dysfunction in cardiomycocytes and to achieve amastigote replication in the host cell cytoplasm. Rat-derived ventricular cardiomycocytes (H9c2) were infected as above and incubated for 96 h. As was observed for macrophages, TcAPx-CcP overexpressors were more infective than wt parasites, supporting the participation of the enzyme in enhancing virulence (Fig. 9A).

These results correlate well with in vitro studies where TcAPx-CcP–null parasites infect myoblasts at a reduced level (20). Finally, and to validate the enhanced virulence of the TcAPx-CcP overexpressers in vivo, we conducted mouse (BALB/c susceptible and C57BL/6 resistance lineages; Fig. 9B) infections with culture-derived trypomastigotes from wt (CL-Brener) and TcAPx-CcP overexpressers. As shown in Fig. 9B, TcAPx-CcP overexpressers produced higher blood parasitemias in the acute infection model of CD, indicating the role of this enzyme in parasite virulence and consistent with its function as part of the pathogen defense against host-derived cytotoxic oxidants.

Discussion

By phylogenetic (23) and biochemical studies (this work), TcAPx-CcP is a hybrid type A peroxidase sharing both Asc- and Cc-dependent peroxidase activity. Although initially described to use Asc as a reducing substrate (15), the catalytic efficiency with Cc is higher, suggesting that, in vivo, the enzyme may also function as a CcP, so long as a component of TcAPx-CcP is present where this “alternative” reducing equivalent is found, such as the mitochondrion. Spectral analysis during the reaction of the enzyme with H₂O₂ showed the formation of a compound-I-like (Fig. 1B) characteristic of the generation of the Trp²⁺ in...
reaction (Fig. 1) is established by Lm Cys + E and Fig. 7), establishing the following equilibrium:

\[ \text{NTrp}^{2+} + \text{Cys}^{2-} - \text{SH} \rightleftharpoons \text{HNTrp}^{+} + \text{Cys}^{2-} - \text{S}^{-}. \]

It is important to note that the estimated one-electron redox potentials of the iron/oxo complex and the tryptophan and cysteiny radicals are approximately 1.156, 1.025, and 0.970 mV, respectively, at pH 7.4 (40, 41), all of which are thermodynamically consistent with preferential route “A” of the ET shown in Fig. 7. In light of these data, we propose that the equilibrium involving the presence of Cys^{2-} (Eq. 1) allows the stabilization of the protein-derived radicals, permitting a more selective reactivity of \( \text{Trp}^{2+} \) toward Cc^{2+} after binding of the reducing substrate to the active site. Overall, these observations indicate distinctive characteristics of the LmP enzyme compared with the \( T. cruzi \) enzyme (e.g., formation of compound-like I in \( Tc-\text{APx-CcP} \)) and provide a detailed atomistic picture of the redox processes occurring at the heme microenvironment during catalysis in this family of peroxidases. Future studies involving crystallization of \( Tc-\text{APx-CcP} \) and comparative analysis with \( LmP \) should help to rationalize the subtle differences observed herein in the reaction mechanism.

The hybrid type APx-CcP peroxidases have been proposed to represent an evolutionary link between catalase-peroxidase and Cc-peroxidases, although their physiological roles are still under discussion (23). \( LmP \), located at the parasite mitochondria, is involved in \( H_2O_2 \) detoxification during parasite differentiation and infectivity (27, 42). In light of its CcP activity, we searched for \( Tc-\text{APx-CcP} \) localization in the different \( T. cruzi \) parasitc stages. In addition to the early report of ER localization in the noninfective epimastigote stage, immuno-EM experiments revealed that the enzyme was also associated with the mitochondria cristae in all parasite stages (Fig. 8 and Fig. S7), suggesting that the mitochondrial-associated fraction of the enzyme may truly work as a CcP. In the intracellular amastigotes and extracellular trypomastigotes, \( Tc-\text{APx-CcP} \) was also found to be noticeably located at the plasma membrane of the parasite in close interaction with the host cytosol, creating an “antioxidant shield” at the host–parasite interface (Fig. 8). This membrane localization was not evident in the \( T. cruzi \) epimastigote stage (Fig. S7). In this subcellular localization, \( Tc-\text{APx-CcP} \) may principally use host cell-derived Asc as a reductant to protect the parasite from \( H_2O_2 \). Overall, we speculate that the infective stage of the parasite uses the reducing substrates alternatively depending on their availability. Although micromolar Asc is found in both the host cytoplasm (43) and \( T. cruzi \) trypomastigotes (17), micromolar Ce is also present in the mitochondrial intermembrane space (44), supporting that \( Tc-\text{APx-CcP} \) primarily uses Asc in its plasma membrane localization and Ce in the mitochondria (Table 1).

The cytochrome-protease cruzipain is an example of enzyme subcellular relocalization along the \( T. cruzi \) life cycle, where restriction to vesicles of the endosomal/lysosomal system in the epimastigote stage is relocalized to the parasite surface in the intracellular amastigote stage (45). The C-terminal extension in cruzipain was suggested to participate in this relocalization (46). \( Tc-\text{APx-CcP} \) contains a positively charged 17-aa sequence of unknown function close to the carboxyl terminus (15) that could be involved in such enzyme relocalization.

To evaluate the significance of \( Tc-\text{APx-CcP} \) in infectivity, studies using cultured cells and animals were performed. Two cellular models (macrophages and cardiomyocytes) were used for the in vitro infection studies. First, \( T. cruzi \) infection of naive macrophages leads to activation of the parasite membrane NADPH oxidase, yielding large amounts of superoxide radical, and subsequently \( H_2O_2 \) (due to superoxide dismutase-catalyzed dismutation), toward the internalized parasite (7, 47). Second, \( T. cruzi \) cardiomyocyte infection leads to the establishment of host–mitochondrial dysfunction with increased \( H_2O_2 \) production (39) that can diffuse and reach the intracellular amastigote. In

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**Fig. 9.** \( Tc-\text{APx-CcP} \) overexpression enhances parasite virulence. (A) Culture-derived \( T. cruzi \) trypomastigotes (CL-Brener and \( Tc-\text{APx-CcP} \) overexpressers) were used to infect macrophages at a ratio of five trypanosomes per cell. Following 24 h of infection, cells were stained with DAPI and the number of intracellular amastigotes was counted. Cardiomyocyte infection was performed as above, except that the time of infection was increased to 96 h. Results are expressed as the number of intracellular amastigotes per 100 cells. Data represent the mean ± SEM of at least three independent experiments. (B) BALB/c or C57BL/6 mice (five per group) were infected with 1.5 \( \times 10^7 \) culture-derived \( T. cruzi \) trypomastigotes (CL-Brener and \( Tc-\text{APx-CcP} \) overexpressers). Blood taken from each mouse at daily intervals from day 3 postinfection onward was microscopically examined for the presence of the bloodstream form of trypomastigotes. Data represent the mean ± SEM for each day. *\( P < 0.005 \) as assayed by the Student’s t test.

**Table 1.**

<table>
<thead>
<tr>
<th>Amastigotes/100 cells</th>
<th>CL-Brener</th>
<th>( Tc-\text{APx-CcP} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>1.2 ± 0.3</td>
<td>3.4 ± 0.7*</td>
</tr>
<tr>
<td>Cardiomyocytes</td>
<td>1.8 ± 0.4</td>
<td>4.2 ± 0.8*</td>
</tr>
</tbody>
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both cellular models, parasites overexpressing TcAPx-CcP were more infective than controls, thus supporting the hypothesis that this peroxidase plays a role in protecting T. cruzi from the cytotoxic effects of host cell-derived H$_2$O$_2$ (Fig. 9A). Recent proteomic data indicate that abundant T. cruzi metabolic enzymes are high-level expres-
sors of TcAPx-CcP, with this expression being further elevated in the infective stage (16, 48). However, it has now been shown that although TcAPx-CcP-null parasites are more susceptible to oxidant killing in vitro, the peroxidase activity is not a determinant for establishment of an infection in the animal model of CD, and is thus not essential for parasite survival within the mammalian host (20). As an ancient parasite, T. cruzi is well adapted to infect and persist in the mammalian host, developing redundant activi-
ties that have allowed it to succeed in hostile oxidative environments. Trypanosomes null for TcAPx-CcP contain other oxidant-
metabolizing systems, including CPX, that are able to detoxify H$_2$O$_2$ efficiently and make parasite infection possible. For comparative purposes, the reaction rate values of H$_2$O$_2$ with TcAPx-
CcP are 2.9 x 10$^{-4}$ M$^{-1}$ s$^{-1}$ (this work) and 3 x 10$^{-7}$ M$^{-1}$ s$^{-1}$ for CPX (49), supporting that both systems are, in principle, readily capable of eliminating cytotoxic levels of host cell-derived H$_2$O$_2$. Moreover, two other putative heme peroxidases are present in the parasite genome (TcCLB.507011.130 and TcCLB.511143.30), and the rele-
ance of their expression under different biological conditions remains to be defined. Finally, and to assay the infectivity of TcAPx-CcP-overexpressing parasites, infection trials in two differ-
te mouse models of acute CD for the TcAPx-CcP were conducted, and significantly different parasitemias were observed in both mouse models of acute CD for the TcAPx-CcP overexpressers with respect to wt parasites, confirming its role in parasite virulence (Fig. 9B).

Overall, the data presented herein provide conclusive bio-
chemical evidence of the hybrid nature of the type A heme peroxidase in T. cruzi (TcAPx-CcP); unravel mechanistic aspects of its redox enzymology with an atomic level of detail; and contribute to define its biological function, particularly as its virulence factor in CD.

Materials and Methods

Expression, Purification, and Site-Directed Mutagenesis of Recombinant TcAPx-
CcP. The plasmid for heterologous expression of TcAPx-CcP (pTrcHis-APX) was kindly provided by Shane Wilkinson, Queen Mary University, London (15). Purification of recombinant enzymes was performed as done previously with minor modifications, as noted in SI Materials and Methods (15). Purity of TcAPx-CcP preparations was evaluated by 15% SDS-PAGE (Fig. 5A), and the Soret peak at 414 nm was used to quantitate the heme protein content (c = 101 mm$^{-1}$ cm$^{-1}$) (18). Site-directed mutagenesis of TcAPx-CcP (W233F) was performed using a site-directed mutagenesis kit with the following primers: forward, 5’-GGGCTACCGTGTTCCGTGTTCAGGCAGGACGAC-3’ and reverse, 5’-CTTGGTCTGTCGGAAGGCCACCCAGTAGGCC-3’. Sequence fidelity was confirmed by DNA sequencing (Institut Pasteur, Montevideo, Uruguay). The TcAPx-CcP W233F mutant was purified as described above for the wt enzyme, and the Soret peak at 414 nm was used to quantitate the heme protein content as described above. Protein concentration was measured by the Bradford protein assay.

Thiol Alkylation. Alkylation of wt and W233F TcAPx-CcP was carried out in-
cubation of the enzyme (60 μM) with NEM (10 mM) for 2 h in phosphate buffer (100 mM, pH 7.4) at 4 °C. Excess NEM was immediately removed using HiTrap desalting columns (Amersham Biosciences) in PBS (100 mM, pH 7.4). Thiol al-
kylation was confirmed by the 5,5-dithiobis-2-nitrobenzoic acid assay (50).

Spectroscopic Analysis. UV-visible spectra were recorded at 25 °C from Asc-
free wt and W233F enzymes (2 μM) in the presence or absence of an equi-
molar H$_2$O$_2$ concentration in PBS (100 mM, pH 7.4).

Stopped-Flow Analysis. Rapid acquisition spectra and absorbance time courses were obtained following Asc-free wt and W233F enzymes before and after H$_2$O$_2$ addition by mixing equal amounts (4 μM each) in sodium phosphate buffer (100 mM, pH 7.4) containing diethylenetriaminepentaacetic acid (DTPA) (0.1 mM) at 10 °C, reaching a final concentration of 2 μM reagents. Absorbance was recorded with an Applied Photophysics SX-20 stopped-flow spectrophotometer (mixing time of ≤2 ms) equipped with a rapid-scanning diode array. Formation of compound 1 species was followed at a Soret peak of 409 nm for wt and 414 nm for W233F enzyme.

EPR-Spin Trapping and Immunospin Trapping Studies of wt and W233F TcAPx-
CcP. EPR spectra of Asc-free TcAPx-CcP, W233F mutant, and NEM-treated enzymes were recorded at room temperature (25 °C) on a Miniscope MS5400 (Magnetech Instruments). For EPR experiments, the reaction mixture con-
tained: wt or W233F (60 μM), DBNBS (10 mM) or DMPO (100 mM), H$_2$O$_2$ (60 μM), and DTPA (0.1 mM) in phosphate buffer (100 mM, pH 7.4). Imme-
diately after oxidation, samples were transferred to a 0.1-μL flat cell and the spectra recorded within 1 min (one-spectrum acquisition). In-
strumental conditions were as follows: microwave power, 20 mW; modula-
tion amplitude, 2.5 G; time constant, 0.2 s; and scan rate, 1.67 G/s. For
immunospin trapping, wt, W233F (10 μM), and NEM-treated enzymes were exposed to H$_2$O$_2$ (0–30 mM) in the presence of DMPO (100 mM). After treatment, proteins were subjected to SDS/PAGE, transferred to nitrocellu-
lose membranes, and blocked in PBS (50 mM, pH 7.4) containing dry milk (5% wt/vol) for 1 h. Protein-DMPO adducts were detected using a chicken-
derived polyclonal anti-DMPO-nitrotrone primary antibody (kindly provided by Ronald Mason, National Institutes of Environmental Health Sciences, Re-
search Triangle Park, NC) and rabbit polyclonal anti-TcAPx-CcP (1:2,000 di-
lution in PBS) containing Tween-20 (0.1% vol/vol) and BSA (4% vol/vol) as
previously described (5). Membranes were washed and then probed for 3 h with anti-chicken IgG (IR Dye-800; Licor Bioscience) and anti-rabbit IgG (IR Dye-680; Licor Bioscience) in PBS containing Tween 20 (0.1% vol/vol). Im-
munoreactive proteins were visualized using an infrared fluorescence de-
tection system (Odyssey; Licor Bioscience).

MD Simulations. MD simulations for wt and W233F mutant TcAPx-CcP were performed. Both initial structures were generated by homology modeling using the Swiss-Model package (51), using the structure of Lmp solved at 1.76 Å resolution as a template, complexed with a heme prostatic group and two cations: calcium and potassium (PDB ID code 3JMV). Both cations were kept in the systems, and the heme group was considered in the oxoferryl state. A P$\Delta$K$_{on}$ prediction of ionizable protein residues was made with PROPKA (52). Considering the PROPKA-computed values and examining the structures, protonation states were assigned for ionizable residues, promoting hydrogen...
bind. Because thioldithiole equilibrium may result critical in ET processes involving Cys residues, we also performed independent MD simul-
ations for both protonation states of Cys residues. The systems were placed into a truncated octahedral box of three point transferable intermolecular potential.

Immuno-EM Studies. The specificity of the antibodies used for the immuno-
localization studies was evaluated by Western blot of protein extracts (10 μg) from rat-derived cardiomyocytes (H9c2; American Type Culture Collection) and cardiomyocytes (H9c2) cultured in DMEM (Sigma) supplemented with γ-glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 μg/mL), and heat-inactivated FBS (10% vol/vol) at 37 °C in a 5% CO₂ atmosphere. Macrophages and/or cardiomyocytes were seeded in Lab-Tek chamber slides and infected with culture-derived trypomastigotes from wt and TcAPx-CCP overexpressers (parasite/cell ratio of 5:1). The infected cells were fixed in 4% (vol/vol) fresh FBS solution in PBS for 10 min at room temperature, washed with PBS containing glycine (100 mM), and permeabilized for 5 min with 0.1% (vol/vol) Triton X-100 in PBS. The number of parasites per 100 macrophages and/or cardiomyocytes was determined by DAPI staining (5 μg/mL). Preparations were analyzed using a microscope (Nikon Eclipse TE-200) at a magnification of 1,000×, and digital photographs of infected cells were recorded. At least 2,500 cells from three independent experiments were counted. Results are expressed as the number of amastigotes per 100 cells and represent the mean of three independent experiments.

Cellular Infections. Parasites were differentiated to the infective metacyclic stage, and metacyclic forms were purified by overnight incubation with fresh human serum as previously described (3). Metacyclic trypomastigotes were used to infect confluent Vero cells (American Type Culture Collection) at 37 °C in 5% CO₂ atmosphere. Culture-derived trypomastigotes were used to infect macrophages (U774A.1, ATCC-TIB-67; American Type Culture Collection) and cardiomyocytes (H9c2) cultured in DMEM (Sigma) supplemented with γ-glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 μg/mL), and heat-inactivated FBS (10% vol/vol) at 37 °C in a 5% CO₂ atmosphere. Macrophages and/or cardiomyocytes were seeded in Lab-Tek chamber slides and infected with culture-derived trypomastigotes from wt and TcAPx-CCP overexpressers (parasite/cell ratio of 5:1). Nonengulfed parasites were removed by washing twice in Dulbecco’s PBS at pH 7.4 (Sigma), and cells were further incubated for 24–96 h in DMEM at 37 °C. Infected cells were fixed in a 4% (vol/vol) fresh FBS solution in PBS for 10 min at room temperature, washed with PBS containing glycine (100 mM), and permeabilized for 5 min with 0.1% (vol/vol) Triton X-100 in PBS. The number of parasites per 100 macrophages and/or cardiomyocytes was determined by DAPI staining (5 μg/mL). Preparations were analyzed using a microscope (Nikon Eclipse TE-200) at a magnification of 1,000×, and digital photographs of infected cells were recorded. At least 2,500 cells from three independent experiments were counted. Results are expressed as the number of amastigotes per 100 cells and represent the mean of three independent experiments.

Animal Infections. Male BALB/c or female C57BL/6 mice (7–10 wk old, re-
spectively) were inoculated i.p. (five mice per group) with culture-derived trypomastigotes (1.5 × 10⁷ trypomastigotes) from wt (CL-Brener strain) or TcAPx-CCP overexpressers. The different susceptibility of these murine models to acute T. cruzi infection was previously characterized (67). Blood trypomastigote count (parasitemia) was assayed on blood (5 μL) drawn from the tail tips of mice, and the number of trypomastigotes per 32 fields was recorded from fresh blood in Neubauer chambers under a microscope (400× magnification).

Infection studies were approved by the Animal Ethics Committee (registration no. 070153-000119-5), registered with the Ethics Commission for the Use of Animals (CEUA), Vet-

erinary School, Uruguay), and mice were handled according to their guidelines.

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The specificity of the antibodies used for the immuno-


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