Structural and Molecular Basis of the Peroxynitrite-mediated Nitrination and Inactivation of Trypanosoma cruzi Iron-Superoxide Dismutases (Fe-SODs) A and B

DISPARATE SUSCEPTIBILITIES DUE TO THE REPAIR OF TYR\textsuperscript{35} RADICAL BY CYS\textsuperscript{83} IN Fe-SOD THROUGH INTRAMOLECULAR ELECTRON TRANSFER

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**Background:** Superoxide dismutases are inactivated by peroxynitrite.

**Results:** \textit{T. cruzi} cytosolic Fe-SOD is highly resistant toward peroxynitrite-mediated tyrosine nitrination and inactivation as compared with mitochondrial Fe-SODA.

**Conclusion:** Intramolecular electron transfer in Fe-SOD from Cys\textsuperscript{83} to critical Tyr\textsuperscript{35} prevents enzyme nitrination and inactivation.

**Significance:** Disparate susceptibilities of Fe-SODs to peroxynitrite can influence parasite virulence during \textit{T. cruzi} infection of mammalian cells.

\textbf{Trypanosoma cruzi}, the causative agent of Chagas disease, contains exclusively iron-dependent superoxide dismutases (Fe-SODs) located in different subcellular compartments. Peroxynitrite, a key cytotoxic and oxidizing effector biomolecule, reacted with \textit{T. cruzi} mitochondrial (Fe-SODA) and cytosolic (Fe-SODB) SODs with second order rate constants of 4.6 ± 0.2 × 10\textsuperscript{4} M\textsuperscript{−1} s\textsuperscript{−1} and 4.3 ± 0.4 × 10\textsuperscript{4} M\textsuperscript{−1} s\textsuperscript{−1} at pH 7.4 and 37 °C, respectively. Both isoforms are dose-dependently nitrated and inactivated by peroxynitrite. Susceptibility of \textit{T. cruzi} Fe-SODA toward peroxynitrite was similar to that reported previously for \textit{Escherichia coli} Mn- and Fe-SODs and mammalian Mn-SOD, whereas Fe-SODB was exceptionally resistant to oxidant-mediated inactivation. We report mass spectrometry analysis indicating that peroxynitrite-mediated inactivation of \textit{T. cruzi} Fe-SODs is due to the site-specific nitrination of the critical and universally conserved Tyr\textsuperscript{35}. Searching for structural differences, the crystal structure of Fe-SODA was solved at 2.2 Å resolution. Structural analysis comparing both Fe-SOD isoforms reveals differences in key cysteines and tryptophan residues. Thiol alkylation of Fe-SODB cysteines made the enzyme more susceptible to peroxynitrite. In particular, Cys\textsuperscript{83} mutation (C83S, absent in Fe-SODA) increased the Fe-SODB sensitivity toward peroxynitrite. Molecular dynamics, electron paramagnetic resonance, and immunospin trapping analysis revealed that Cys\textsuperscript{83} present in Fe-SODB acts as an electron donor that repairs Tyr\textsuperscript{35} radical via intramolecular electron transfer, preventing peroxynitrite-dependent nitrination and consequent inactivation of Fe-SODB. Parasites exposed to exogenous or endogenous sources of peroxynitrite resulted in nitrination and inactivation of Fe-SODB but not Fe-SODB, suggesting that these enzymes play distinctive biological roles during parasite infection of mammalian cells.

Superoxide dismutases (SODs\textsuperscript{5}; EC 1.15.1.1) are metalloenzymes essential for all living aerobic organisms that catalyze the detoxification of superoxide radicals (O\textsuperscript{2−}) to oxygen (O\textsubscript{2}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). They are subdivided into three structurally distinct families, depending on the metal of the active site:

\begin{itemize}
  \item SOD1: copper–zinc superoxide dismutase (Cu/Zn-SOD; EC 1.15.1.1)
  \item SOD2: manganese superoxide dismutase (Mn-SOD; EC 1.15.1.1)
  \item SOD3: iron superoxide dismutase (Fe-SOD; EC 1.15.1.1)
\end{itemize}

\begin{itemize}
  \item SOD1 is localized in the cytoplasm and is the most abundant enzyme in the cell.
  \item SOD2 is localized in the mitochondrial matrix and is the most abundant enzyme in the mitochondria.
  \item SOD3 is localized in the cytosol and is the most abundant enzyme in the cytoplasm.
\end{itemize}

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\*\*\* The abbreviations used are: SOD, superoxide dismutase; NEM, N-ethylmaleimide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PBN, α-phenyl N-tertiary butyl nitrite; NOC-12, 1-hydroxy-2-oxo-3-((N-ethyl-2-aminoethyl)-3-ethyl-1-triazene; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; IPG, immobilized pH gradient; AA, antimycin A; IET, intramolecular electron transfer.
Peroxynitrite-mediated Inactivation of T. cruzi Fe-SODs

The efficient removal of $O_2^\bullet$ by Fe-SODs can attenuate peroxynitrite formation in different T. cruzi subcellular compartments. However, the $NO$ and $O_2^\bullet$ reaction occurs in biological systems despite the presence of SODs because it can outcompete the enzyme-catalyzed $O_2^\bullet$ dismutation (13). Moreover, in the case of mammalian Mn-SOD, it is well established that it can be efficiently inactivated by peroxynitrite (14), which increases $O_2^\bullet$ levels, and subsequently peroxynitrite, creating a hazardous positive feedback loop that can impair mitochondrial energy metabolism (15) and the signaling of programmed cell death (16). In this scenario, it is reasonable to hypothesize that the contents of parasite Fe-SODs and the fluxes of $O_2^\bullet$, NO, and peroxynitrite may be important determinants in parasite survival or death during the infection process.

The mechanism of the peroxynitrite-dependent inactivation of mammalian Mn-SOD has been extensively studied and involves the preferential nitration of Tyr144, located 5 Å from the manganese ion of the active site (14, 17–20). This site-specific nitration has been ascribed to a kinetically favored reaction of peroxynitrite with the manganese ion, leading to the formation of oxidizing and nitrating species at the active site (18, 21). Due to the high degree of structural homology that Mn- and Fe-SODs share (2), a similar mechanism for peroxynitrite-dependent T. cruzi Fe-SOD enzyme inactivation may be expected. In this work, we have studied the reaction of peroxynitrite with the purified cytosolic and mitochondrial isoforms of T. cruzi Fe-SODs. The results obtained revealed outstanding differences between both T. cruzi SODs isoforms in terms of their susceptibility to peroxynitrite-mediated nitration and inactivation, with the cytosolic Fe-SOD being extremely resistant to oxidant treatment. The biochemical basis for the observed disparate oxidant susceptibility between both T. cruzi Fe-SODs was analyzed at the structural and molecular levels. Moreover, the occurrence of these biochemical events under cellular nitroxidative stress conditions was unambiguously established in T. cruzi Fe-SOD overexpressers, confirming the preferential nitration and inactivation of Fe-SOD in living parasites.

EXPERIMENTAL PROCEDURES

Chemicals

N-Ethylmaleimide (NEM), diethylenetriaminepentacetic acid, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), manganese dioxide, Dulbecco’s modified Eagle’s medium (DMEM), lipopolysaccharide (LPS), Geneticin (G418), l-cysteine-methyl ester, 4-(2-pyridylazo)-resorcinol, and α-phenyl N-tertiary-butyl nitrone (PBN) were from Sigma. The nitric oxide donor 1-hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC-12) was from Dojindo. Murine recombinant IFN-γ was from Calbiochem. Lab-Tek tissue culture chamber slides were from Nunc. All other chemicals were of reagent grade. Peroxynitrite was synthesized in a quenched flow reactor from sodium nitrite and hydrogen peroxide ($H_2O_2$) under acidic conditions and quantitated as described previously; excess $H_2O_2$ was removed by treatment with $MnO_2$, and $NO_2$ contamination was always <20% (22). Peroxynitrite concentration was determined spectrophotometrically at 302 nm ($\epsilon_{302\text{ nm}} = 1670 \text{ M}^{-1}$)
Expression, Purification, and Site-directed Mutagenesis of *T. cruzi* Cytosolic Fe-SOD and Mitochondrial Fe-SOD

Parasite SOD genes were amplified from *T. cruzi* genomic DNA (CL-Brener strain) using the following primers: Fe-SODA, 5′-GGATCCGCCCAGCGATGTGCAAA-3′ (forward) and 5′-GGAAGCTTTATTTTATGCTGGCATG-3′ (reverse); Fe-SODB, 5′-GGGGATCCTGCTTTCAGCATTCCTC-3′ (forward) and 5′-GGAAGCTTCTGCTGGTCAAAATGGTGCG-3′ (reverse) (restriction sites for BamHI and HindIII, respectively, are underlined). The purified PCR product (BIORON gel extraction kit) was ligated into the pGEM-T Easy vector (Promega) and transformed into electrocompetent *Escherichia coli* XL1 blue cells. The amplified *T. cruzi* Fe-SODA (without mitochondrial signal peptide) and Fe-SODB genes were cloned into the pQE-30 vector (Qiagen) between BamHI and HindIII. pQE30-Fe-SODs in *E. coli* M15 (pREP4) cells were grown at 37 °C in LB broth containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml). Expression of recombinant Fe-SODs was induced with isopropyl-β-D-thiogalactopyranoside (0.8 mM) when the culture reached A₆₀₀ = 0.6, and the temperature was lowered to 22 °C for overnight protein expression. 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 imidazole (10 mM) and NaCl (500 mM) at a flow rate of 3 ml/min. Fe-SODs were eluted with a linear imidazole (10–500 mM) gradient in sodium phosphate (50 mM, pH 7.6) containing NaCl (500 mM). Imidazole was removed by buffer exchange with sodium phosphate buffer (50 mM, pH 7.4) using HiTrap desalting columns (Amersham Biosciences). Purity of *T. cruzi* Fe-SOD preparations was evaluated by SDS-PAGE, and protein concentration was measured by the Bradford method (24). Molecular weight of the purified enzymes was evaluated by gel filtration chromatography in a Superdex™-200 column (GE Healthcare) calibrated with the following protein standards: 13,700, 29,000, 43,000, and 75,000 Da (GE Healthcare). Protein thiols were reduced by incubation with DTT (1 mM) for 30 min at room temperature. Alkylation of protein thiols was performed by incubation with iodoacetamide (100 µg/ml) and iodoacetate (50 µg/ml) for 30 min at room temperature. Activity of Fe-SODs was measured by the decrease in the rate of superoxide-dependent cytochrome *c* reduction at 550 nm using xanthine/xanthine oxidase as a superoxide source (26). The rate constants of *T. cruzi* Fe-SODA and Fe-SODB were determined by the kinetic competition method described previously. Xanthine-oxidase (6 millunits) and xanthine (50 µM) were used for the generation of O₂⁻ fluxes. The reduction of cytochrome *c* (20 µM) was monitored at 550 nm at 37 °C in phosphate buffer 50 mM, pH 7.8, using a 1-ml cuvette in the absence and presence of Fe-SODA or Fe-SODB (0–40 nm). The velocity of cytochrome *c* reduction in the absence (V₀) and presence of Fe-SOD (V₅₀) was employed to calculate an inhibition fraction (IF = 1 − (V₀ − V₅₀)/V₀). Utilizing the reported bimolecular rate constant for the reaction of O₂⁻ with cytochrome *c* at pH 7.8 (2.6 ± 0.1 × 10⁶ M⁻¹ s⁻¹), the rate constants were calculated as k₅₀ = k₅₀ ([cyt]/[Fe-SOD]₅₀). [Fe-SOD]₅₀ (the concentration of SOD that caused an inhibition fraction of 0.5) was obtained from the fitting of experimental data to a rectangular hyperbolic curve (27).

Peroxynitrite Treatment of *T. cruzi* Fe-SODs

Peroxynitrite (0–2000 µM) was added as a single dose under vortex to the purified enzymes (8 µM) in sodium phosphate buffer (200 mM, pH 7.4), and activity was measured as described.

Determination of *T. cruzi* Fe-SOD Rate Constants with O₂⁻

The O₂⁻ dismutase activity of the purified *T. cruzi* recombinant Fe-SOD and Fe-SODB was measured by the decrease in the rate of superoxide-dependent cytochrome *c* reduction at 550 nm using xanthine/xanthine oxidase as a superoxide source (26). The rate constants of *T. cruzi* Fe-SODA and Fe-SODB were determined by the kinetic competition method described previously. Xanthine-oxidase (6 millunits) and xanthine (50 µM) were used for the generation of O₂⁻ fluxes. The reduction of cytochrome *c* (20 µM) was monitored at 550 nm at 37 °C in phosphate buffer 50 mM, pH 7.8, using a 1-ml cuvette in the absence and presence of Fe-SODA or Fe-SODB (0–40 nm). The velocity of cytochrome *c* reduction in the absence (V₀) and presence of Fe-SOD (V₅₀) was employed to calculate an inhibition fraction (IF = 1 − (V₀ − V₅₀)/V₀). Utilizing the reported bimolecular rate constant for the reaction of O₂⁻ with cytochrome *c* at pH 7.8 (2.6 ± 0.1 × 10⁶ M⁻¹ s⁻¹), the rate constants were calculated as k₅₀ = k₅₀ ([cyt]/[Fe-SOD]₅₀). [Fe-SOD]₅₀ (the concentration of SOD that caused an inhibition fraction of 0.5) was obtained from the fitting of experimental data to a rectangular hyperbolic curve (27).

Kinetics Studies of *T. cruzi* Fe-SOD Reactions with Peroxynitrite

The kinetics of peroxynitrite (10 and 17 µM for Fe-SODA and Fe-SODB, respectively) decomposition was monitored at 302 nm (ε₉₀₂ = 1670 M⁻¹ cm⁻¹) in the presence or absence of control and NEM-treated Fe-SODA and Fe-SODB (0–15 µM) in a stopped-flow spectrophotometer (SX20, Applied Photophysics) with a mixing time of <2 ms. An initial rate approach was used to analyze the data (28); the first 0–0.15 s of peroxynitrite decomposition was fitted to a linear plot, and initial rates were calculated by dividing the slope of the absorbance time course by the peroxynitrite molar extinction coefficient at 302 nm and multiplying by a factor of 1.25 to account for the 20% fraction of peroxynitrite that is not deprotonated at pH 7.4 (because the absorption at 302 nm is due to the peroxynitrite anion). Second order rate constants (kₙ) were calculated according to the equation, kₙ = (k₁ × [Fe-SOD]₀ × [peroxynitrite]₀)/[Fe-SOD]₀ − k₁ × [Fe-SOD]₀ × [peroxynitrite]₀ were k₁ is the rate constant of proton-catalyzed peroxynitrite decomposition (28). Reported values are the average of at least seven separate determinations. Temperature was maintained at 37 °C, and the pH was measured at the outlet.

Measurements of Protein Thiol Content and Thiol Alkylation

Protein thiols were quantitated using the DTNB assay (29). Protein thiols were reduced by incubation with DTT (1 mM) for 30 min at room temperature. Alkylation of *T. cruzi* Fe-SOD thiols by NEM was performed by incubation of Fe-SODA or Fe-SODB (200 µM) with NEM (10 mM) for 2 h in phosphate buffer (50 mM, pH 7.4) at 4 °C. Excess DTT and NEM were removed immediately after incubation using HiTrap desalting columns (Amersham Biosciences) as described previously (30).
above. Peroxynitrite addition was done to control or NEM-treated enzymes in the presence or absence of GSH (10 mM), uric acid (100 μM), l-cysteine methyl ester (8 μM), bicarbonate (25 mM), PBN (50 mM), and DMOPO (100 mM). Peroxynitrite addition was also performed at different pH values (5.8 – 8.0), and T. cruzi Fe-SODs activity was measured as described above.

**Western Blotting, Protein Nitrotyrosine, and Immunospin Trapping Analysis**

After treatment, proteins were subjected to 15% SDS-PAGE, transferred to nitrocellulose membranes, and blocked in phosphate-buffered saline (PBS; 50 mM, pH 7.4) containing dry milk (5% w/v) for 1 h. Membranes were then probed with either rabbit polyclonal anti-nitrotyrosine antibody (1:2000 dilution raised in our laboratory (31)) or rabbit polyclonal anti-T. cruzi Fe-SODA or Fe-SODB (1:5000 dilution (32)) in PBS containing Tween 20 (0.1%, v/v) for 1 h. Membranes were washed and probed for 1 h with anti-rabbit-IgG (IR Dye-800- and IR Dye-680-conjugated (LI-COR Biosciences) or peroxidase-conjugated (Calbiochem), 1:15,000 dilution in PBS containing Tween 20 (0.1%, v/v)). After washing of the probed membranes, immunoreactive proteins were visualized with an infrared fluorescence detection system (Odyssey, LI-COR Biosciences) or using the Immun-Star™ chemiluminescence kit (Bio-Rad). For immune spin trapping, after exposure of T. cruzi Fe-SODA (5–20 μM) to peroxynitrite (5–20 μM) in the presence or absence of DMOPO (100 μM), protein samples were subjected to Western blot analysis, and protein-DMPO nitrene adducts were detected using a rabbit polyclonal anti-DMPO-nitrene primary antibody (rabbit anti-DMPO serum, 1:2000 dilution in PBS containing Tween 20 (0.1%, v/v) and bovine serum albumin (4%, v/v)) as described previously (33). Immunoreactive proteins were detected with the infrared system as described above.

**EPR Studies**

The EPR spectra were recorded at room temperature (25 °C) on a Bruker EMX EPR spectrometer. Wild type Fe-SODB or C83S single mutant (2 mm) were incubated with the spin trap PBN (50 mM) and exposed to peroxynitrite (500 μM). Immediately after oxidant addition, samples were transferred to a 200-μl flat cell, and the spectra were recorded within 1 min (15 spectrum acquisitions). Adducts between Fe-SODs and PBN were digested with Pronase (20 mg/ml) for 10 min, and spectra were recorded as above.

**Peptide Mapping Analysis of Peroxynitrite-treated T. cruzi Fe-SODs**

T. cruzi Fe-SODs (8 μM) were treated with peroxynitrite (0–300 μM) in potassium phosphate buffer (200 mM, pH 7.4) at 25 °C in the presence or absence of DMOPO (100 μM). Protein samples were separated by one- or two-dimensional gel electrophoresis. Commercially available IPG strips (7 cm, linear 3–10, GE Healthcare) were used for the first dimensional separation. Gels were stained with Colloidal Coomassie Blue G-250. Images of peptide standards (Applied Biosystems). Peptide sequences were confirmed by MS/MS analysis of selected ions.

**Nitrotyrosine Quantification**

For total 3-nitrotyrosine quantification, peroxynitrite in a wide concentration range (0.05–3000 μM) was added to Fe-SODs (8–16 μM) in sodium phosphate buffer (200 mM, pH 8–5.8). NO2− removal from the samples after peroxynitrite treatment was carried out by two subsequent protein precipitation steps with acetonitrile (1 volume) for 40 min at 4 °C following centrifugation at 14,000 g for 40 min at 4 °C. Proteins were resuspended in nanopure water (500 μl) containing the following internal standards: universal labeled tyrosine ([U-13C9]Tyr, 20 nmol); [NO2−13C6]Tyr (500 pmol); and [13C6]Tyr (35). Stable isotopically labeled precursors were used as internal standards for the quantification of total protein Tyr and NO2−Tyr ([U-13C9,15N1]Tyr and [NO2−13C6]Tyr, respec-
Peroxynitrite-mediated Inactivation of T. cruzi Fe-SODs

The crystal structure of the mitochondrial superoxide dismutase from T. cruzi (Fe-SOD) was determined at 2.23 Å resolution in the Protein Crystallography Facility of the Institut Pasteur de Montevideo (Protein Data Bank entry 4DVH). Fe-SOD crystals were grown in a hanging drop vapor diffusion setup, using the protein at 2.33 mg/ml in 50 mM sodium phosphate, pH 7.8. Protein (1 µl) was mixed in equal parts with mother liquor (1-ml reservoir), Tris-HCl (0.1 M, pH 8.5), and PEG 4000 (30%, w/v) and incubated at 291 K. Monoclinic crystals grew in a few days, were cryoprotected with mother liquor containing 25% (v/v) glycerol, and flash-frozen in liquid N2 until data collection. A complete data set was collected at 100 K with a copper rotating anode (Micromax-HF, Rigaku) and a Mar345DTB (Mar Research) image plate detector. Data were processed with MOSFLM (37) and Scala (38). The crystal structure was solved by molecular replacement with AMoRe (39) using the monomer of E. coli Fe-SOD (Protein Data Bank entry 2NYB) as a search probe. Restrained refinement was done with Buster-TNT (40) (BUSTER version 2.10.0, Global Phasing Ltd., Cambridge, UK), including a TLS model with one body per chain (one dimer in the asymmetric unit). Refinement was performed in iteration with manual model building using Coot (41). Structure validation was done with Molprobity (42).

**Molecular Dynamics (MD) Simulations**

**Starting Structures**—The crystal structure of the T. cruzi cytosolic wild type (WT) Fe-SOD was downloaded from the Protein Data Bank (Protein Data Bank entry 2GPC). Based on the WT structure, double mutant N187D/K189E was built in silico by replacing the corresponding residue side chains. Hydrogens were added using the Tleap module of the AMBER program package, considering standard protonation states for all titratable residues at physiological pH (Asp and Glu negatively charged, Lys and Arg positively charged, all of the rest neutral). Additionally, the tyrosyl radical (Tyr35-O•) and negatively charged cysteine (Cys83–S−, thiolate)-containing system (tyrosyl-radical-thiolate cytosolic Fe-SODB) was built by removing the corresponding phenolic and thiol hydrogens and changing the corresponding classical residue parameters (see below).

**MD Simulation Parameters**—Simulation of WT, mutant, and tyrosyl radical-thiolate cytosolic Fe-SODB homodimers was performed in an explicit solvent box (of ~513-nm3 volume and containing ~15,000 water molecules) using the TIP3P water model. Parameters for all standard residues were taken from the PARM99 force field (43). Classical parameters for Tyr-O• and Cys83–S− were taken from our previous work (44). Parameters for the Fe-SOD active site (the iron ion and its first sphere coordinating residues) were developed in the same way as those for Mn-SOD (20). All simulations were performed using the periodic boundary conditions approximation and the particle mesh Ewald summation method with a grid spacing of 1 Å for treating long range electrostatic interactions, whereas a direct cut-off distance of 8 Å was used for direct interactions. The Shake method was used to constrain the hydrogen atoms at their equilibrium distance, allowing the use of a 2-fs time step. The Berendsen thermostat was used to keep the temperature constant at 300 K (45). All MD simulations were performed with the AMBER program package (46). The equilibration protocol for all peptides consisted of slowly heating the optimized structures from 0 to 300 K during 0.1 ns while the volume of the system was kept constant (NVT). Next, a 0.2-ns-long density equilibrium simulation was performed using an NPT ensemble. Production simulations consisted of 10-ns-long NPT MD simulations for wild type and mutant cytosolic Fe-SODB and 20-ns-long NPT MD simulation for radical-thiolate cytosolic Fe-SODB. The stability of all simulations was assessed through root mean square deviation and root mean square fluctuation analysis (data not shown).

**Analysis of the Intramolecular Electron Transfer (IET) Pathway(s)**

IET pathways along the protein matrix were determined using the pathways algorithm developed previously (47, 48), with a specific set of parameters developed in our group to consider the Fe-SOD active site as well as recent observations of aromatic residues acting as stepping stones in long range electron transfer reactions (49–52). The method has been used successfully in previous work from our group (44, 51, 53). Pathways were computed between Cys83–S− and Tyr35–O• atoms located orbitals for 100 snapshots taken from the corresponding MD simulation. To estimate the pKₐ of the different Cys residues in WT and mutant cytosolic and mitochondrial Fe-SODs, the propKa pKₐ estimation software was used (54–57).

**Parasites**

T. cruzi epimastigotes (CL-Brener) were cultured at 28 °C in brain-heart infusion medium as described previously (12). T. cruzi CL-Brener (pTcINDEX-9E10) (Invitrogen) Fe-SODA overexpressers containing the bacteriophage T7 RNA polymerase, tetracycline (Tet) repressor genes, and the epitope (9E10) derived from the human c-Myc protein added to the C terminus of Fe-SODA were maintained as described previously in brain-heart infusion medium containing G418 and hygromycin (100 µg/ml) (12, 58). To induce Fe-SODA expression, epimastigotes were cultured in medium supplemented with tetracycline (2 µg/ml) for 3 days as described previously (12).

**Nitroxidative Treatment of T. cruzi Epimastigotes**

T. cruzi Fe-SOD overexpressers were used in order to identify, at the cellular level, peroxynitrite-dependent T. cruzi Fe-SOD nitroxidative modifications. For this, Fe-SODA parasites (5 X 10⁸ cells/ml in Dulbecco’s PBS, pH 7.4) were treated with the mitochondrial complex III inhibitor antimycin A (AA; 5 µM) in the presence of NOC-12 (5 mM, t½ = 100 min at pH 7.4), SIN-1 (5 mM), or peroxynitrite (300 µM in three subsequent additions of 100 µM) for 3 h at 28 °C under stirring (59). After
Peroxynitrite-mediated Inactivation of T. cruzi Fe-SODs

RESULTS

Purification and Characterization of Fe-SODA and Fe-SODB—The recombinant T. cruzi Fe-SODA and Fe-SODB containing one atom of iron per monomer were purified to homogeneity as active enzymes homodimers as established by SDS-gel electrophoresis and gel filtration chromatography analysis. The apparent monomer molecular mass was 23 and 22.6 kDa for Fe-SODA and Fe-SODB, respectively (Fig. 1, A and B), consistent with the predicted values according to their primary structures. All of the enzyme preparations used in this study, including the mutants generated, were of high purity (>99%) with specific activities of 2647 and 3551 units/mg for Fe-SODA and Fe-SODB, respectively. These specific activities compare well with previously reported T. brucei Fe-SODs (60).

Determination of the Rate Constant with O\textsubscript{2}\textsuperscript{-} and Kinetics of the Reaction of T. cruzi Fe-SODs with Peroxynitrite—A competition kinetic approach was used to determine the second order rate constant of the reaction of T. cruzi Fe-SODs with O\textsubscript{2}\textsuperscript{-} and it was found to be 4.5 ± 1.8 × 10\textsuperscript{4} M\textsuperscript{-1} s\textsuperscript{-1} for Fe-SODA and 7.6 ± 1.5 × 10\textsuperscript{4} M\textsuperscript{-1} s\textsuperscript{-1} for Fe-SODB in 50 mM sodium phosphate, pH 7.8 (ionic strength (μ) = 0.14), in agreement with values obtained for other iron-containing SODs (Fig. 1C and Table 1) (61).

The rate constant for peroxynitrite reaction with T. cruzi Fe-SODs was measured following the decay of peroxynitrite at increasing concentrations of enzyme (0–15 μM), as described previously (18). A linear correlation was obtained after plotting the initial velocity of peroxynitrite decomposition (ν\textsubscript{i}) (Fig. 2, inset) as a function of Fe-SOD concentrations (Fig. 2), with the y axis intercept reflecting the proton-catalyzed decomposition of peroxynitrite (as described under “Experimental Procedures”). By dividing the slope of the plot over peroxynitrite concentration, the second order rate constant was determined as 4.6 ± 0.2 × 10\textsuperscript{4} M\textsuperscript{-1} s\textsuperscript{-1} for Fe-SODA and 4.3 ± 0.4 × 10\textsuperscript{4} M\textsuperscript{-1} s\textsuperscript{-1} for Fe-SODB per monomer at pH 7.4 and 37 °C, in agreement with that obtained for other SODs (Table 1) (18, 62, 63). When the rate constant was determined in the presence of Fe-SOD in which the solvent-accessible cysteines were blocked by NEM alklylation, the same value was obtained (Table 1), indicating a negligible contribution of cysteine residues (k\textsubscript{onOOO-} = 6 × 10\textsuperscript{9} M\textsuperscript{-1} s\textsuperscript{-1} for free cysteine at pH 7.4 and 37 °C) to the global rate constant observed. Because the reactivity of cysteine toward peroxynitrite is typically the largest among all amino acids (28), the data support the existence of a fast direct reaction of peroxynitrite with the iron atom of the Fe-SODs.

Peroxynitrite-dependent Inactivation and Nitration of T. cruzi Fe-SODs—Peroxynitrite addition (0–1200 μM) to purified Fe-SODA and Fe-SODB (8 μM) at pH 7.4 led to a dose-dependent inhibition of the superoxide dismutase activity although with quite disparate susceptibilities (Fig. 3A). Indeed, Fe-SODB was largely resistant to peroxynitrite-mediated inactivation, in contrast with the highly sensitive Fe-SODA, an unexpected result taking into account the structural similarities between both isoforms (3, 4). Enzyme inactivation was associ-
Peroxy nitrite-mediated Inactivation of T. cruzi Fe-SODs

Rate constants with $O_2^\cdot$ and peroxynitrite

| Enzyme preparation | $O_2^\cdot$ $k$ | ONOO$^-$ $k$ |
|--------------------|---------------|----------------|
| Fe-SODA            | $4.5 \pm 1.8 \times 10^5$ | $4.6 \pm 0.2 \times 10^4$ |
| Fe-SODB            | $7.6 \pm 1.5 \times 10^5$ | $4.3 \pm 0.4 \times 10^4$ |
| NEM-treated Fe-SODB| ND            | $4.3 \pm 0.2 \times 10^4$ |

Fe-SODA and Fe-SODB B contain seven and nine tyrosine residues respectively, some of which are solvent-exposed and others of which are buried in the protein structure. Notably, Tyr$^{35}$ is part of the active site and the closest to the iron metal centers. Thus, in principle, tyrosine nitration could affect non-critical tyrosines as well as Tyr$^{35}$, with oxidative modification of the latter being responsible for enzyme inactivation. Under the experimental conditions of Fig. 3, in which biologically relevant micromolar concentrations of enzyme were used, there are two possible mechanisms by which peroxynitrite can generate nitrating intermediates to mediate Fe-SOD tyrosine nitration (64, 65): (i) the proton-catalyzed homolysis of peroxynitrous acid (ONOOH, $pK_a = 6.8$) to yield nitrogen dioxide ($NO_2^\cdot$) and hydroxyl radical ($OH^\cdot$) or (ii) the reaction of peroxynitrite anion with the iron center to yield the oxo-Fe complex and $NO_2^-$. To define which of the nitration mechanisms was better coupled to enzyme inactivation, we performed peroxynitrite exposures at different pH values and in the presence of CO$_2$ (Fig. 4), as in previous reports (66). Treatment of T. cruzi Fe-SODA with peroxynitrite (150 $\mu$M) at acidic pH values (where most peroxynitrite decays via homolysis) led to mild enzyme inactivation, whereas protein nitration was high; on the contrary, at basic pH values, enzyme inactivation was more pronounced, but the yields of protein nitration were less (Fig. 4, A and B). Thus,
overall, these data indicate that the reactive species leading to enzyme inactivation is peroxynitrite anion via its reaction with the metal center and the site-specific nitration of active site tyrosine (see below). In support of this contention, the presence of bicarbonate (25 mM, 1.3 mM CO2) protected Fe-SODA from peroxynitrite-mediated inactivation (Fig. 4C), as was previously seen for E. coli Mn-SOD (18). The reaction of peroxynitrite anion (ONOO−) with CO2 (k = 3 × 10^4 M^-1 s^-1 at pH 7.4 and 37 °C) yields the nitrosonitroxidocarboxylate adduct (ONOOCO2) that rapidly (<1 μs) decomposes by homolysis, resulting in the generation of NO2 and CO3 radicals (67, 68). Thus, CO2 competes with the metal of Fe-SOD for ONOO−, yielding the secondary derived radicals that may react with solvent-accessible non-critical protein tyrosine residues without causing enzyme inactivation (19, 69, 70).

Glutathione (10 mM) known to react with peroxynitrite (k = 1.35 × 10^3 M^-1 s^-1 (71)) and NO2 (72) and uric acid (100 μM), a known ‘OH and NO2 scavenger (k = 7.2 × 10^9 and 1.9 × 10^7 M^-1 s^-1, respectively) (73, 74) fully protected Fe-SOD from enzyme inactivation and nitration (not shown).

Peroxynitrite-dependent Inactivation of T. cruzi Fe-SOD by the Selective Nitration of Tyr15—In order to confirm the preferential nitration of Tyr15 after peroxynitrite treatment (0–300 μM) of T. cruzi Fe-SODs (8 μM), control and treated enzymes were separated by two-dimensional gel electrophoresis, and protein spots were analyzed by nano-LC-nano-SI-MS. One major spot for both Fe-SODA and Fe-SODB and other minor ones were identified in the native enzymes, indicating post-translational modifications that affect the protein isoelectric point. In the case of the peroxynitrite-treated Fe-SODs, the two-dimensional gels revealed the generation of several more acidic spots in both Fe-SODs with a more drastic shift in the case Fe-SODA, in agreement with its higher susceptibility to peroxynitrite (Fig. 5A). Peroxynitrite-treated proteins are
**Peroxy nitrite-mediated Inactivation of T. cruzi Fe-SODs**

![Image](https://example.com/image.png)

**FIGURE 5. Peptide mapping of T. cruzi Fe-SODs after peroxy nitrite treatment.**

**A.** T. cruzi Fe-SODs (8 μM) were treated with peroxy nitrite (150 μM) in sodium phosphate buffer (0.2 M) at pH 7.4 and 25 °C. Two-dimensional gel electrophoresis was performed as described under "Experimental Procedures." The arrowhead shows the selected Fe-SODB spot of pI 7.5 analyzed by mass spectrometry. **B.** MS/MS spectrum of triply charged ion at m/z 790.6 (MH^+ 2369.7, retention time = 30.3 min) from a tryptic digestion of peroxy nitrite-treated Fe-SODB spot (pI = 7.5, indicated with an arrow in A). The major N-terminal (b, red-labeled) and C-terminal (y, blue-labeled) fragment ions that allowed the sequence 31–52 assignment that includes a nitrated triple charged molecular ion, assigned to the sequence VTKLNAAAQTNSALATK^52, which contained major N-terminal (H11005) and C-terminal (H9262) fragment ions detected by full-scan MS/MS.

Expected to have more acidic pI values due to the change of the pK^*_a* of 3-nitrotyrosine (7.3) with respect to Tyr (10.3) (with the consequent gain of a negative charge) (21, 75), among other possible oxidative modifications. Mass spectrometry analysis of the major spot (pI = 7.5) of Fe-SODB generated after peroxy nitrite treatment (150 μM) revealed the presence of only one nitrated peptide (m/z = 790) corresponding to the sequence 3^1^HHQG^3^5YTKLNAAAQTNSALATK^5^2, which contained Tyr^35^ (Fig. 5B), revealing the preferential nitration of this residue under these experimental conditions. At higher concentrations of peroxy nitrite (600–1000 μM), other peptides containing Tyr^177^ (solvent-exposed) were also found nitrated (Table 2).

In the case of Fe-SODA, the critical Tyr (Tyr^36^ in the crystal structure; see below) was also preferentially nitrated after exposure to 100 μM peroxy nitrite, being detected on a dinitrated peptide containing Tyr^36^ and Tyr^35^ (Table 2).

Total 3-nitrotyrosine quantification was performed for both *T. cruzi* Fe-SODs isoforms after peroxy nitrite treatment (0–3000 and 0–100 μM peroxy nitrite for Fe-SODB and Fe-SODA, respectively) in order to accurately correlate enzyme inactivation with Tyr nitration. Control and peroxy nitrite-treated Fe-SODB were analyzed, and the results are shown in Table 3. Results are expressed as the ratio of 3-nitrotyrosine/SOD monomer, and theoretical 3-nitrotyrosine values were calculated assuming enzyme inactivation to be due to the sole nitration of one Tyr per Fe-SOD monomer (*i.e.* Tyr^15^ in Fe-SODB), and thus 100% inactivation must yield a 3-nitrotyrosine/SOD ratio of 1. For Fe-SODB treated with peroxy nitrite at pH 8, a linear correlation between the percentage of enzyme inactivation and total 3-nitrotyrosine quantification was observed, clearly indicating that peroxy nitrite-mediated enzyme inactivation is due to the selective nitration of Tyr^35^ (although the solvent-accessible Tyr^177^ was also found nitrated but had a small contribution to total 3-nitrotyrosine). In the case of Fe-SODA, a larger yield of tyrosine nitration and the extent of enzyme inactivation was observed at a smaller peroxy nitrite concentration at pH 8 in comparison with Fe-SODB (Table 3). Under this condition, the quantitated experimental value of 3-nitrotyrosine by LC-MS/MS was somewhat less than that predicted from the loss of activity, which may be due to the fact that other Tyr oxidative modifications were observed by MALDI-TOF/MS (Table 2) and may contribute to inactivation, a hypothesis that needs further confirmation. Interestingly, peroxy nitrite treatment of Fe-SODA at pH 5.8 revealed experimental 3-nitrotyrosine quantitation higher than the predicted one from the observed 16% of enzyme inactivation (Table 3), in good agreement with the nitration of non-critical solvent-exposed tyrosines via ‘NO2 and ‘OH formed in the bulk from ONOO· homolysis.

**Crystal Structure of T. cruzi Mitochondrial Fe-SOD and Differences from Cytosolic Fe-SOD**—In order to understand the contrasting behaviors of both Fe-SOD isoforms toward peroxy nitrite-mediated inactivation, we solved the crystal structure of the mitochondrial *T. cruzi* Fe-SODA (Table 4) and compared it with the previously reported structure of cytosolic Fe-SODB (Protein Data Bank entry 2GPC) (76). The Fe-SODA structure revealed a tightly bound dimer in the asymmetric unit (Fig. 6A), the two protomers related by a strong non-crystallographic 2-fold axis. The total buried area due to dimerization is high (~1900 Å²), consistent with the dimeric behavior of Fe-SODA in solution. The overall structure of Fe-SODA is similar to those of previously solved iron SODs (76, 77). The nomenclature of secondary structure elements is depicted on one of the Fe-SODA monomers in Fig. 6B. Each monomer binds one iron cation within the metal-binding pocket between the two domains. The metal is pentacoordinated to His^28^-Ne2 (in helix α1), His^79^-Ne2 (α3), Asp^163^-O62 (β3), His^167^-Ne2 (in the helical linker loop connecting β3 with α6), and a water molecule (HOH7 in chain A and HOH6 in chain B). As in other iron SODs, the metal coordination geometry is trigonal bipyramidal, with His^79^, His^167^, and Asp^163^ as equatorial ligands and the His^28^ and water oxygen axial. *T. cruzi* mitochondrial Fe-SODA is structurally very similar to cytosolic Fe-SODB with a root mean square deviation of 0.75 Å, superimposing one monomer.


Peroxynitrite-mediated Inactivation of T. cruzi Fe-SODs

TABLE 2
Peroxynitrite-modified tyrosine-containing peptides in T. cruzi Fe-SODs detected by MALDI-TOF mass spectrometry

MALDI-TOF/TOF mass spectrometry analysis was carried out in control and peroxynitrite-treated purified recombinant T. cruzi Fe-SODs. Fe-SODa and Fe-SODB were treated with increasing peroxynitrite concentrations (0–1000 µM), and enzyme activity was assayed. After treatment, control and treated enzymes were subjected to SDS-gel electrophoresis, and protein bands were in-gel digested with trypsin (sequence grade). Digested proteins were analyzed by MALDI-TOF mass spectrometry, and peptides were searched in the MASCOT database. Ox, oxidation; Mo, monoisotopic. Numbering corresponds to the position observed in the crystal structure of both T. cruzi Fe-SODs.

| Peptide | Ox(O) | Ox(M) | Mo(Ox) | Mo(M) | Assigned sequence |
|---------|-------|-------|--------|-------|------------------|
| 14DGACPVLSPQLEH2TYKHK44A4YVDRK40 + 2Nitro(Y) | | | | | |
| 15GLRPVFVTVDWEHA1692Y170YK171 + Ox(Y) | | | | | |
| 17RVD20YLKEKTVDFVSVSR127YEAMK132 + Ox(Y) + Ox(M) | | | | | |
| 40LNALAGAT43YDGK24T25MEVI1L26AND27SEK28 + Nitro(Y) | | | | | |
| 17RAA175YQTFWNVWV1193 + Nitro(Y) | | | | | |
| 17RAA175YQTFWNVWVWV1912 + Nitro(Y) | | | | | |
| 17RAA175YQTFWNVWV1193 + Nitro(Y) | | | | | |

Mo theoretical mass (M + H)2+ / Da

| Peroxynitrite | Mo observed mass (M + H)2+ / Da | Mo theoretical mass (M + H)2+ / Da | Assigned sequence |
|--------------|---------------------------------|---------------------------------|------------------|
| Fe-SODa      | 2156.00 2156.03 172K.NDRAA177YVQTFWNVWV188 | 2156.00 2156.03 172K.NDRAA177YVQTFWNVWV188 | 17RAA175YQTFWNVWV1193 + Nitro(Y) |
| Fe-SODb      | 2125.03 2125.04 155GLRPVFTVDVWEHA169Y170YK171 | 2125.03 2125.04 155GLRPVFTVDVWEHA169Y170YK171 | 17RAA175YQTFWNVWV1193 + Nitro(Y) |
| Fe-SODc      | 2884.37 2884.39 40LNALAGAT48YDGKT53MEDIIVALANDSEK66 | 2884.37 2884.39 40LNALAGAT48YDGKT53MEDIIVALANDSEK66 | 17RAA175YQTFWNVWV1193 + Nitro(Y) |

TABLE 3
3-Nitrotyrosine quantification of peroxynitrite-treated T. cruzi Fe-SODs by LC-MS/MS

After peroxynitrite treatment (0–1500 µM), T. cruzi Fe-SODs were hydrolyzed overnight at 116°C in HCl (6 N), and pellet was reuspended in formic acid (0.1%, v/v) and analyzed by LC-MS/MS as described under “Experimental Procedures.” Theoretical NO2-Tyr was calculated assuming that enzyme inactivation is due to the selective nitration of only one Tyr per enzyme monomer (i.e. Tyr35 in Fe-SODA or Tyr35 in Fe-SODA). Results are expressed as NO2-Tyr/SD molecule.

| Peptide | Experimental NO2-Tyr | Theoretical NO2-Tyr | Assigned sequence |
|---------|----------------------|---------------------|------------------|
| Fe-SODA | 100 56 0.26 0.43 | | |
| Fe-SODB | 100 3095.54 2868.39 2868.39 40LNALAGAT48YDGKT53MEDIIVALANDSEK66 | | |
| Fe-SODC | 100 3095.54 2868.39 2868.39 40LNALAGAT48YDGKT53MEDIIVALANDSEK66 | | |

TABLE 4
Crystal structure of mitochondrial Fe-SODA

| Space group | P21 |
|-------------|-----|
| Protein molecules per asymmetric unit | 2 |
| Solvent content (%) | 43.4 |
| Wavelength (Å) | 1.5418 |
| Data resolution (Å) | 24.17-2.23 (2.35-2.23) |
| Measured reflections | 133337 |
| Completeness (%) | 98.8 (95.7) |
| Rmerge (%) | 9.9 (43.2) |
| a, b, c (Å) | 18.5 (5.4) |
| β (degrees) | 96.8 |
| Refinement resolution (Å) | 24.17-2.23 |
| Rcryst (no. of reflections) | 0.168 (17,522) |
| Rfree (no. of reflections) | 0.219 (1368) |
| Root mean square, bonds (Å) | 0.01 |
| Root mean square, angles (degrees) | 1.1 |
| Protein non-hydrogen atoms | 3227 |
| Water atoms | 102 |
| Iron atoms | 2 |
| Mean B factor, overall: chain A/chains B (Å2) | 28/29 |
| Mean B factor, main chain: chain A/chains B (Å2) | 26/27 |
| Mean B factor, side chain: main chain A/chains B (Å2) | 30/31 |
| Mean B factor, waters (Å2) | 30 |
| Mean B factor, liganded iron (Å2) | 17.8 |
| Map versus model correlation coefficient (overall/local)2 (allowed/favored/outliers) | 0.889/0.919 |
| No. of residues in Ramachandran plot region (allowed/favored/outliers) | 402/390/90 |

Protein Data Bank code 4DVH

(843 atoms) and 1.14 Å aligning the whole dimer (1843 atoms). In particular, the identities and positions of the iron-binding residues in the active sites are absolutely conserved, including the axial water molecules. In the folded state of Fe-SOD, as represented by the crystal structure, the metal cation is seen completely buried within its binding pocket, with no connection to the solvent-accessible surface of the protein. An outer, second shell of residues with respect to the ones acting as direct iron ligands can be identified, interacting with the coordinating shell, and/or delimiting bulk solvent channels toward the metal pocket (Fig. 6C). These second shell residues are also positioned in the same way in both Fe-SOD isoforms, and they include critical Tyr35 (78) at 5.6 Å from the metal center. Further extending our structural analysis, there are differences between Fe-SODA and Fe-SODB, which concern Cys and Trp amino acids. Among the four cysteine residues present in Fe-SODA (Cys16, Cys85, Cys131, and Cys150) and the three in Fe-SODB (Cys85, Cys131, and Cys150), only one is conserved; Cys150 in Fe-SODA is structurally equivalent to Cys146 in Fe-SODB (Fig. 7). The four Cys side chains in mitochondrial Fe-SODA are...
solvent-accessible in the crystal structure, in agreement with the data obtained by sulphydryl titration (four DTNB-reactive thiols per monomer). In contrast, Fe-SODB displays only two of its Cys thiols accessible to bulk solvent (Cys83 and Cys146). Fe-SODB Cys159 is instead buried within the folded protein core, at 9.9 Å from the iron atom, again consistent with the thiol titration (2 DTNB-reactive thiols/monomer). Analysis of the Trp residues reveals that, apart from a conserved core of five tryptophans in both isoforms, key differences are uncovered in a critical region: the funnel entrance toward the metal-binding pocket, in the interface between helices α1 and α3, including also the rather long initial N-terminal loop that precedes α1. In the cytosolic Fe-SOD2 structure, Trp12 (substituted by Tyr11 in Fe-SODB) locates Trp12 (substituted by Tyr11 in Fe-SODB) within the N-terminal loop but pointing its side chain away with respect to the entrance channel. Overall, the structural differences in Cys and Trp residues comparing the two T. cruzi Fe-SOD isoforms...
could explain their different susceptibilities to peroxynitrite-mediated inactivation and nitration. The structural data are thus consistent with the existence of an IET mechanism in Fe-SODB involving Cys, Trp and the active site tyrosine residue, as observed previously for other proteins (33, 79, 80).

Insights for the Resistance of Cytosolic Fe-SOD to Peroxynitrite-mediated Inactivation—Cysteine residues can repair tyrosyl radicals in proteins via IET (44). The overall reaction with a peptide sequence can be exemplified as follows.

\[
\text{Cys}-X_n\text{-Tyr-O}^* + H^+ \rightarrow \text{Cys}-X_n\text{-Tyr-OH}
\]

(REACTION 1)

By this mechanism, the radical character initially located in a tyrosine residue is transferred to a cysteine residue, and therefore further tyrosine oxidative modifications, including tyrosine nitration (by the combination reaction of tyrosyl radical with \( \cdot \text{NO}_2 \)), are inhibited. In the case of Fe-SOD, the possible contribution of Cys residues to the resistance to peroxynitrite was explored at different levels. There are two solvent-accessible thiols (Cys^{83} and Cys^{146}) in Fe-SODB from a total of three (as measured with DTNB and by crystal structure analysis; Fig. 7). Blockage of the accessible thiols in Fe-SODB with NEM rendered the enzyme significantly more susceptible to peroxynitrite-mediated inactivation (Fig. 8A), whereas no alterations in sensitivity were observed for NEM-treated Fe-SODA (all four Cys residues in Fe-SODA are solvent-accessible as measured with DTNB and by crystal structure analysis; Fig. 7). These results support the participation of the Fe-SODB solvent-accessible Cys (Cys^{83} and/or Cys^{146}) in the resistance toward peroxynitrite. Previous mechanistic studies using Tyr-Cys-containing peptides (44, 80, 81) showed that IET (shown in Reaction 1) is mediated by a proton-coupled electron transfer mechanism that involves as a first key step the deprotonation of the Cys residue, which then in the thiolate form acts as the electron donor, followed by IET, yielding the final radical Cys-S\(^{-}\) (44).

To analyze the probability that any of the Cys residues present in \( T. \ cruzi \) Fe-SODs could participate in Tyr-O\(^{\bullet}\) repair involving an IET process, we first estimated each Cys p\( \text{Ka} \) value using the propKa software (54–57). The results obtained show that the predicted p\( \text{Ka} \) values of the Cys present in mitochondrial Fe-SODA (Cys^{16}, Cys^{85}, Cys^{131}, and Cys^{150}) and Fe-SODB (Cys^{146} and Cys^{159}) are similar to that observed for free Cys (8.2–8.3), making less likely the existence of deprotonated forms at pH 7.4. Interestingly, the p\( \text{Ka} \) value predicted for Fe-SODB Cys^{83} is approximately 2 pH units lower than the one obtained for the other Cys residues. This lower p\( \text{Ka} \) can be explained by the presence in its immediate environment of two positively charged residues, Lys^{188} and Arg^{192}. The Lys^{188}-N\(^{\epsilon} \) is located at 5.5 Å from the Cys^{83}-S\(^{-} \) and Arg^{192}-C\(^{\delta} \) at 8.2 Å, increasing the chance for the thiol group to be partially charged at pH 7.4. The Fe-SODB critical Tyr^{35} is located >20 Å away from Cys^{83}; thus, to repair Tyr^{35}-O\(^{\bullet}\), a long range IET needs to occur. In this case, relay amino acids (e.g. Trp^9 and Trp^79) in the electron transfer pathway are required to improve the efficiency of the process (82, 83).

To analyze possible IET pathways in cytosolic Fe-SODB, we performed 20-ns-long MD simulations of the corresponding homodimer, with Tyr^{35} described as radical, and Cys^{83} as thiolate (as predicted for its lower p\( \text{Ka} \)) (i.e. tyrosyl radical-thiolate Fe-SODB system). We then computed possible IET paths between both residues using the pathways algorithm as described under “Experimental Procedures” (44). The resulting most probable IET path comprises the participation of key residues that contributes to the process: Trp^79 and His^{32} (Fig. 8B). The presence of aromatic residues along the IET pathway (Trp^79 and Trp^9), absent in the Fe-SODA crystal structure) could increase the overall IET rate, as was recently shown (49, 52). In summary, the \( \text{in silico} \) analysis of the tyrosyl radical-thiolate Fe-SODB system strongly suggests that the partially charged Cys^{83}-S\(^{-}\) acts as the electron source to rescue critical Tyr-O\(^{\bullet}\) formed after peroxynitrite treatment, preventing nitration and inactivation of cytosolic Fe-SODB.

Detection of Cys^{83}-S\(^{-}\) after the Reaction of Fe-SODB with Peroxynitrite—If an IET mechanism is operative after peroxynitrite reaction with Fe-SODB, a protein radical corre-
Peroxy nitrite-mediated Inactivation of T. cruzi Fe-SODs

FIGURE 8. Participation of solvent-accessible Fe-SODB thiols in the resistance to peroxynitrite and role of intramolecular electron transfer. A, effects of sulfhydryl-blocked Fe-SODB in the reaction with peroxynitrite. Peroxynitrite (0–2000 μM) was added to control (empty circles) or NEM-blocked sulfhydryl Fe-SODB (filled circles) (8 μM) in sodium phosphate buffer (100 mM) at pH 7.4. Activity is expressed as percentage activity relative to the native enzyme or NEM-treated enzyme in the absence of peroxynitrite (100% activity). B, selected snapshot for Fe-SODB intramolecular electron transfer pathway from Cys159-S to Tyr35-O. The figure shows the path for IET as predicted with the pathways algorithm and taken from the explicit water MD simulation (see “Experimental Procedures” for details). Fe-SODB is shown as a black ribbon representation, and the residues Cys159, Met82, Trp9, Trp79, His32, and Tyr35 are shown as boldface sticks. The iron atom is shown as a pink sphere with the coordination residues as cylindrical representations. The predicted IET path is shown in orange and involves electron transfer starting on the Cys159-S atom sequentially to Met82, Trp9, Trp79, and His32 and ending in the aromatic ring of Tyr35-O. Parts of the pathway occur through the backbone, and others occur through space. Surrounding waters were omitted for clarity. Error bars, S.E.

FIGURE 9. Immunospin trapping of protein thyl radical in the peroxynitrite reaction with Fe-SODB. A, control or thiol-blocked NEM-treated Fe-SODB (50 μM) was exposed to peroxynitrite (0–20 μM) in sodium phosphate buffer (100 mM, pH 7.4) in the presence of the spin trap DMPO (100 mM). Immunoreactive proteins were detected with anti-DMPO nitrone antibody (green) and specific anti-Fe-SODB antibody (red). Merged bands are shown in yellow. B, two-dimensional gel electrophoresis of T. cruzi Fe-SODB (8 μM) treated with peroxynitrite (300 μM) in sodium phosphate buffer (0.2 M) at pH 7.4 and 25 °C in the presence and absence of DMPO (100 mM). Two-dimensional gel electrophoresis was performed as described under “Experimental Procedures.”

Peroxynitrite treatment (<7.2) was prevented in the presence of DMPO with a new more basic spot (pI = 7.8) probably corresponding to the Fe-SODB-DMPO adduct (Fig. 9B).

Furthermore, EPR spin trapping analysis of the reaction of Fe-SODB (2 mM) with peroxynitrite (500 μM) was performed in the presence of the spin trap PBN (50 mM). Again, reaction conditions were optimized to favor the direct reaction of peroxynitrite with Fe-SODB and to obtain detectable EPR signals. The addition of peroxynitrite to Fe-SODB led to the detection of an EPR spectrum characteristic of a strongly immobilized nitroxide adduct (Fig. 10A). The spin adduct was subjected to nonspecific proteolysis with Pronase, resulting in the conversion of the spectrum into an isotropic six-line spectrum characteristic of PBN-protein thyl adducts (Fig. 10B) (84–86). In order to confirm that the EPR signal observed was due to the formation of Cys83-S′ after peroxynitrite reaction, the Fe-SODB mutant C83S was generated. In this case, the PBN-protein radical adduct was almost completely inhibited (Fig. 10, C and D), demonstrating the generation of Cys83-S′.

The Role of Cys83 in the Inhibition of Tyr35 Nitration via IET; Studies with Mutant Fe-SODB and Free Thiols—In order to confirm the participation of Cys83 in the IET mechanism proposed, site-directed mutagenesis experiments were performed. First, our primary candidate for the IET process was the Cys159 located at 9.9 Å from the iron atom of the active site of Fe-
SODB and not present in the crystal structure of Fe-SODA. Mutation of Cys159 (C159S) did not affect the resistance of Fe-SODB to peroxynitrite-dependent inactivation, indicating that this residue was not participating in the IET process (Fig. 11B).

Importantly, mutation of the single Cys83 (C83S) rendered the enzyme more susceptible to peroxynitrite-mediated inactivation as compared with wild type Fe-SODB (Fig. 11C). Moreover, we also generated the double mutant N187D/K189E, which completely modifies the protein environment adjacent to Cys83 from a net positively charged to a negatively charged one. This change of microenvironment is expected to result in a significant increase in Cys83 pKa, with a consequent decrease of the amount of Cys-S- necessary to repair the critical Tyr35-O'.

Indeed, N187D/K189E Fe-SODB significantly lost the resistance against peroxynitrite treatment when compared with the wild type protein, reinforcing the role of Cys83 in the IET proposed mechanism (Fig. 11D).

It has been shown that thiols are the dominant “sink” for peroxynitrite and NO2 in cells (28, 87). The calculated rate constant of NO2 with GSH and Cys is 2 and 5 × 107 M−1 s−1, respectively (28). This scavenging reaction may prevent the NO2-dependent tyrosine nitration and thus enzyme inactivation (28, 72). In order to examine whether Cys83 could contribute to the inhibition of enzyme inactivation via direct NO2 scavenging, we performed experiments using the Fe-SODB C83S mutant in the presence of equimolecular amounts of the more reactive l-cysteine methyl ester (8 μM; pKa ~ 6.7 (88)).

The presence of this NO2 radical scavenger failed to protect Fe-SODB-C83S mutant from peroxynitrite inactivation, strongly suggesting that the protection observed in wild type Fe-SODB by Cys83 was mainly mediated by an IET and not by NO, free radical scavenging (Fig. 11C). It is important to note that although mutation of Cys83 in Fe-SODB renders the enzyme more susceptible to oxidant-dependent inactivation, it was still more resistant than Fe-SODA. This result indicates that other residues, probably Trp79 and Trp9, may be additionally participating in Tyr35-O' repair after peroxynitrite treatment.

Fe-SODA Modifications during Cellular Nitroxidative Stress Conditions to T. cruzi—Fe-SODA T. cruzi overexpressers (12, 58) were used to search for modifications of Fe-SOD occurring during nitroxidative stress conditions in living parasites (epimastigote stage). Following the induction of Fe-SOD expression by tetracycline (4–6-fold increase respect to wild type), parasites were incubated in the presence of antimycin A (complex III electron chain inhibitor) plus a NO donor, in order to specifically generate peroxynitrite at the mitochondrial cell compartment (59). Following treatment, parasites protein extracts were separated by two-dimensional electrophoresis and probed with anti-Fe-SODs antibodies. After AA/NO treatment, an important shift toward more acidic pH values in the pl of Fe-SODA was evident (Fig. 12). This pl shift was also observed during exogenous peroxynitrite challenge to parasites (Fig. 12A). The pl changes observed in Fe-SODA obtained from living parasites during exposure to either endogenous (AA/NO) or exogenous peroxynitrite were similar to those observed for the recombinant Fe-SODA after peroxynitrite treatment (Fig. 5A). Notably, Fe-SODB was not significantly altered under these cellular nitroxidative conditions, in agreement with its high resistance to peroxynitrite.

The pl changes in Fe-SODA during nitroxidative challenge to living parasites correlated reasonably well with the extents of protein tyrosine nitration (Fig. 12B). Indeed, endogenous and exogenous fluxes of peroxynitrite (AA/NO or the peroxynitrite donor SIN-1, respectively) caused nitration of parasite proteins.

Immunoprecipitation analysis of Fe-SODA revealed the presence of nitrated enzyme in the SIN-1-treated parasites, unambiguously revealing the reaction of nitrating species and subsequent oxidative posttranslational modifications of Fe-SODA in living parasites (Fig. 12C). Overall, the data in Fig. 12 confirm the feasibility of these biochemical events in Fe-SODA as biologically relevant processes.

DISCUSSION

T. cruzi mitochondrial and cytosolic Fe-SODs were purified to homogeneity as active enzymes with specific activities and O2 dismutation rates comparable with those of other Mn- and Fe-SODs. Similarly, both SODs readily reacted with peroxynitrite at comparable second order rate constants (~4.5 × 104 M−1 s−1) (Table 1) (18, 33, 62, 63) and were dose-dependently inactivated and nitrated by peroxynitrite (Fig. 3).

Peptide mapping by mass spectrometry analysis of the peroxynitrite-treated enzymes together with 3-nitrotyrosine quantification revealed that peroxynitrite-dependent inactiva-
tion of *T. cruzi* Fe-SODs is due to the selective nitration of the universally conserved Tyr35 located near the iron atom of the active site, as was previously observed for Mn-SOD and *E. coli* SODs (Fig. 5 and Table 2) (17, 18). The proximal reactive species was the anionic form of peroxynitrite (ONOO−), and the primary target at the enzyme was the active site iron atom, as was revealed by pH studies, cysteine alkylation, and CO2 competition experiments (Figs. 4 and 8 and Table 1).

In the most likely reaction mechanism, the metal-based Lewis adduct formed in the reaction (i.e. SOD-FeIII-OONO (89)) undergoes homolysis to yield ‘NO2 and the corresponding oxo-metal complex (SOD-FeIV=O) (89). This SOD-FeIV=O complex is strongly oxidizing and promotes the oxidation of Tyr35 to Tyr35-O that rapidly combines with NO2 generated in situ to yield a tyrosine-nitrated enzyme, as proposed in Scheme I. Thus, site specificity is provided by a combination of kinetic factors (fast reaction of peroxynitrite with the iron center in contrast to the much slower proton-catalyzed homolysis) and close structural relationships between the iron atom and the active site tyrosine. Once Tyr35 is nitrated, O2 dismutation is impeded by both a steric effect (as the nitro group located in the access channel impedes O2 diffusion) and the electrical charge repulsion provided by the ionization of the phenolic group in Tyr, by analogy with the recently reported data for mammalian Mn-SOD (20, 90).

Although both isoforms were inactivated by peroxynitrite, Fe-SODB was extremely resistant to nitration and inactivation as compared with its mitochondrial counterpart (Fig. 3). For example, only 20% of Fe-SODB became inactivated at pH 7.4 with high peroxynitrite concentrations (1500 μM), whereas most of the Fe-SODA activity was already lost at 200 μM peroxynitrite, a similar susceptibility to what was reported for *E. coli* Mn- and Fe-SODs and mammalian Mn-SOD (18, 91). Due to the apparent sequence and structural homology among Fe-SODs of different species, this disparate susceptibility on peroxynitrite-mediated inactivation of *T. cruzi* Fe-SODs was intriguing. Thus, in order to search for structural differences that may explain the high intrinsic resistance to oxidant inactivation of the Fe-SODB isoform, we solved the crystal structure of *T. cruzi* mitochondrial Fe-SODA at 2.2 Å resolution. Comparison of the crystal structures of both isoforms revealed a number of potentially relevant differences to explain the results obtained in our study. Two tryptophans (Trp9 and Trp79) and two cysteines (Cys83 and Cys159) are located near the active site of Fe-SODB and are absent in the crystal structure of the mitochondrial counterpart (Fig. 7). It was previously shown that the presence of a cysteine residue in the proximity of a tyrosine might enable radical transfer during one-electron oxidation processes, with the cysteine acting as electron donor (44). In this regard, following peroxynitrite reaction with the iron center of Fe-SODB, the Tyr35-O generated could be quickly reduced by a cysteine residue back to Tyr35 prior to the nitration step, therefore preventing enzyme inactivation. Of the three cysteines present in Fe-SODB, only Cys83, located
Peroxy nitrite-mediated Inactivation of T. cruzi Fe-SODs

SCHEME 1. Proposed reaction mechanisms for the reaction of T. cruzi Fe-SODs with peroxynitrite. Peroxy nitrite anion (ONOO−, 80% at pH 7.4) reacts with the FeIII atom of the Fe-SODs, yielding the corresponding oxo-metal complex (SOD-FeIV−O−) with NO2− generation (reaction I). SOD-FeIII−O oxidizes the active site Tyr (Tyr35 in Fe-SODB and Tyr36 in Fe-SODA) to its corresponding tyrosyl radical (SOD-FeIII−Tyr35−O•−) (reaction II). SOD-FeIII−Tyr35−O•− rapidly combines with NO2− (reaction III), yielding the nitrated and inactivated enzyme SOD-FeIII−Tyr35−NO2 (reaction III). In T. cruzi Fe-SODB (reaction IV), Cys83−S− repairs FeIII−Tyr35−O•−, regenerating FeIII−Tyr35−O•− through an IET process. The homolytic cleavage of ONOO− (reaction VI) and the nucleophilic addition of ONOO− to CO2 (reaction VII) (reviewed in Ref. 89) compete with reaction I and decrease the extents of Tyr35 nitration and enzyme inactivation.

In this work, using different experimental approaches, the Cys83−S− generated in the Fe-SODB after the reaction with peroxynitrite was identified (Figs. 9 and 10), demonstrating the participation of an IET pathway in the observed resistance to peroxynitrite. A further proof of the key role of Cys83 in modulating the redox chemistry at the active site is that the Fe-SODB C83S mutant significantly increased the sensitivity to peroxynitrite-mediated inactivation (Fig. 11C). Future experiments, including additional enzyme mutants, are needed in order to determine the precise amino acids involved in the IET pathway(s) and whether other amino acids can also participate in tyrosyl radical repair. From a biological perspective, it is conceivable that the solvent-accessible Cys83−S− can be repaired in living cells by low molecular weight reductants. For instance, molecules, such as glutathione, trypanothione, or ascorbate, present in the T. cruzi cytosol may provide a sustained protection to Fe-SODB at the expense of these “sacrificial” antioxidant molecules. In this regard, we were able to detect nitrated Fe-SODA in living parasites exposed to exogenous (SIN-1) or endogenous (AA/NO) fluxes of peroxynitrite. Whereas in the case of SIN-1, peroxynitrite will be formed and react in various cellular compartments, including cytosol and mitochondria, the AA/NO treatment leads to the mitochondrial formation of peroxynitrite. In both cases, only substantial post-translational oxidative modifications were observed in Fe-SODA, underscoring the capacity of Fe-SODB to resist peroxynitrite-mediated inactivation. Both an important shift in the pl of Fe-SODA and the detection of nitrated protein (after immunoprecipitation) are indicative of oxidative modifications due to peroxynitrite reactions in mitochondria (Fig. 12). Future experiments using Fe-SODA and Fe-SODB T. cruzi overexpressers in infections to macrophages and cardiomyocytes will allow us to evaluate in more detail the biological significance of our results. In particular, the peroxynitrite-mediated inactivation of Fe-SODA may be secondarily responsible for parasite programmed cell death during the infection process. Indeed, the inactivation of Fe-SODA would raise the intramitochondrial O2•− state concentration, which is an apoptotic signal in T. cruzi (12). On the other hand, the robust Fe-SODB would resist the oxidative challenge promoted by mammalian host cells and contribute to the neutralization of the oxidative challenge. Thus, as was observed previously for other T. cruzi antioxidant enzymes (7), T. cruzi Fe-SODs may function as virulence factors, a hypothesis that is currently under investigation in our laboratories both in the cellular and animal models of Chagas disease.

22 Å away from Tyr35, is predicted to have a lower pK of 6.8 value favoring the electron transfer process to Tyr35−O•− (44). Moreover, MD simulations of the tyrosyl radical-thiolate system (Tyr35−O•−−Cys83−S−) in Fe-SODB revealed Trp79 and His32 as key residues acting as “relay” amino acids or “stepping stones” for the electron transfer (Fig. 8D). Ultimately, this type of IET process generates a thiyl radical, as observed previously for the homolytic cleavage of ONOO− to CO2 (reaction VI) (reviewed in Ref. 89) compete with reaction I and decrease the extents of Tyr35 nitration and enzyme inactivation.

FIGURE 12. Cellular detection of Fe-SODA nitroxidative modifications. A, two-dimensional gel electrophoresis. T. cruzi Fe-SODA overexpressers were treated with AA (5 μM) plus NOC-12 (5 mM; t½ = 100 min at pH 7.4), SIN-1 (5 μM), or ONOO− (300 μM) for 3 h at room temperature. After treatment, samples were processed as described under “Experimental Procedures” and subjected to two-dimensional electrophoresis. Membranes were probed with the specific Fe-SODA and Fe-SODB antibodies. B, 3-nitrotyrosine detection in parasites. Membranes were probed with the specific anti-3-nitrotyrosine antibody. C, immunoprecipitation of nitrated Fe-SODA. Parasite extracts as above were incubated overnight at 4 °C in the presence of the monoclonal c-Myc antibody that recognized the 9E10 epitope of Fe-SODA in the presence of protein A/G-agarose as described under “Experimental Procedures.” Immunoprecipitated proteins were run in 15% SDS-gel electrophoresis, electrotroferred to nitrocellulose, and revealed using anti-c-Myc antibody and anti-3-nitrotyrosine antibody and as described under “Experimental Procedures.”
Peroxynitrite-mediated Inactivation of T. cruzi Fe-SODs

disease. From a chemistry-oriented point of view the data we are now reporting underscore that the extent of peroxynitrite-mediated oxidative modifications in genetically engineered proteins can be modulated taking advantage of long range intramolecular electron transfer processes.

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