Reaction of Peroxynitrite with Mn-Superoxide Dismutase

ROLE OF THE METAL CENTER IN DECOMPOSITION KINETICS AND NITRATION*

Received for publication, October 16, 2000, and in revised form, December 27, 2000
Published, JBC Papers in Press, January 4, 2001, DOI 10.1074/jbc.M009429200

Celia Quijano‡‡, Daniel Hernandez-Saavedra§§, Laura Castro**, Joe M. McCord¶¶, Bruce A. Freeman**, and Rafael Radi‡‡

From the ‡Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, 11800 Montevideo, Uruguay, the §Webb-Waring Institute, University of Colorado, Denver, Colorado 80262, and the-**Department of Anesthesiology and Center for Free Radical Research, University of Alabama, Birmingham, Alabama 35233

Manganese superoxide dismutase (Mn-SOD), a critical mitochondrial antioxidant enzyme, becomes inactivated and nitrated in vitro and potentially in vivo by peroxynitrite. Since peroxynitrite readily reacts with transition metal centers, we assessed the role of the manganese ion in the reaction between peroxynitrite and Mn-SOD. Peroxynitrite reacts with human recombinant and Escherichia coli Mn-SOD with a second order rate constant of $1.0 \pm 0.2 \times 10^9$ and $1.4 \pm 0.2 \times 10^8 \text{M}^{-1} \text{s}^{-1}$ at pH 7.47 and 37 °C, respectively. The E. coli apoenzyme, obtained by removing the manganese ion from the active site, presents a rate constant $<10^4 \text{M}^{-1} \text{s}^{-1}$ for the reaction with peroxynitrite, whereas that of the manganese-reconstituted apoenzyme (apo/Mn) was comparable to that of the holoenzyme. Peroxynitritedependent nitration of 4-hydroxyphenylacetic acid was increased 21% by Mn-SOD. The apo/Mn also promoted nitration, but the apo and the zinc-substituted apoenzyme (apo/Zn) enzymes did not. The extent of tyrosine nitration in the enzyme was also affected by the presence and nature (i.e. manganese or zinc) of the metal center in the active site. For comparative purposes, we also studied the reaction of peroxynitrite with low molecular weight complexes of manganese and zinc with tetrakis(4-benzoic acid) porphyrin (tbap). Mn(tbap) reacts with peroxynitrite with a rate constant of $6.8 \pm 0.1 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ and maximally increases nitration yields by 350%. Zn(tbap), on the other hand, affords protection against nitration. Our results indicate that the manganese ion in Mn-SOD plays an important role in the decomposition kinetics of peroxynitrite and in peroxynitrite-dependent nitration of self and remote tyrosine residues.

Manganese-superoxide dismutase (Mn-SOD) is the SOD isoform found in the mitochondrial matrix of eukaryotes and in a variety of prokaryotes (1–3). Mn-SODs from different organisms are homologous and have a manganese ion in the active site. Whereas the human mitochondrial enzyme is a homotetramer (~88 kDa) (4), Escherichia coli Mn-SOD (45.8 kDa) is a dimer (3). Mitochondria are essential organelles where most of the cell superoxide (O$_2^-$) is produced (5, 6), and therefore, Mn-SOD plays an active role detoxifying the cell from this species. In addition, pharmacological agents and cytokines that promote intracellular reactive oxygen species production, like paraquat (7) and tumor necrosis factor-α (8), induce Mn-SOD expression. Experiments with knock-out mice shed further light on the relevance of this enzyme, with Mn-SOD-deficient mice surviving only up to 3 weeks of age (9, 10) and presenting many features of mitochondrial disease associated with reactive oxygen species toxicity (11).

Nitric oxide (NO) is a relatively unreactive free radical formed by nitric oxide synthase (12). However, fast reaction of nitric oxide with superoxide gives rise to peroxynitrite anion (ONOO$^-$), a potent oxidant (13–15). Peroxynitrite is formed during sepsis, inflammation, excitotoxicity, and ischemia-reperfusion of tissues, conditions under which the cellular production of nitric oxide and superoxide increase (12, 16–18), and participates in reactions related with the pathological expression of these processes. Recent reports regarding the presence of nitric oxide synthase in the mitochondria (19–21), along with the easy diffusion of nitric oxide through membranes (22), make the intramitochondrial formation of peroxynitrite possible and highlight the relevance of its interactions with intramitochondrial targets.

Mn-SOD inhibits peroxynitrite formation in mitochondria, but it may be oxidatively inactivated by excess peroxynitrite. Indeed, peroxynitrite-mediated nitration of tyrosine 34 of human Mn-SOD results in enzyme inactivation in vitro (23, 24). The presence of a nitrated and dysfunctional enzyme in rejected human renal allografts (25) strongly supports the relevance of this process in vivo. Mn-SOD inactivation, due to tyrosine 34 nitration, would lead to an increase in peroxynitrite formation that would in turn impair mitochondrial energy metabolism (26) and signal apoptotic cell death (27).

Peroxynitrite decomposition is catalyzed by a variety of enzymes, including Mn-SOD (28). The reaction with Mn-SOD is endothermic with a GS value of 21.6 kcal/mol (29). Peroxynitrite is a potent oxidant and single electron donor that can react directly with Mn-SOD (30). Peroxynitrite decomposition is catalyzed by a variety of enzymes, including Mn-SOD (28). The reaction with Mn-SOD is endothermic with a GS value of 21.6 kcal/mol (29). Peroxynitrite is a potent oxidant and single electron donor that can react directly with Mn-SOD (30).

The abbreviations used are: Mn-SOD, manganese-superoxide dismutase; SOD, superoxide dismutase; apo, apoenzyme; apo/Mn, manganese-reconstituted apoenzyme; apo/Zn, zinc-substituted apoenzyme; BSA, bovine serum albumin; HPA, para-hydroxyphenylacetic acid; tbap, tetrakis(4-benzoic acid) porphyrin; HPLC, high performance liquid chromatography; DTPA, diethylenetriaminepentaaetic acid; EDTA, ethylenediaminetetraacetic acid; Me$_3$SO, dimethyl sulfoxide; GSH, glutathione; hrMn-SOD, human recombinant Mn-SOD.
Lewis acids (28). These are electron-accepting compounds like H⁺ (29, 30), carbon dioxide (31), and transition metals (29) that favor the cleavage of the O–O bond and lead to the formation of nitrosating species. In the case of H⁺, peroxynitrous acid (ONOÖH) undergoes homolysis to hydroxyl radical and nitrogen dioxide with yields up to 30% (28, 32), resulting in nitration yields in the range of 6–10% (29, 30). In the case of transition metal-containing compounds, such as metalloproteins and Mnporphyrin SOD mimetics, higher nitration yields are obtained (29, 33–35). These facts led us to consider that the manganese-free enzyme was active on the site, which could react with the critical tyrosine 34.

In this work, we studied the role of the manganese metal center in the decomposition kinetics of peroxynitrite and in peroxynitrite-dependent nitration of the enzyme and non-protein aromatic residues. These studies shed light on the nature of peroxynitrite reaction with Mn-SOD and provide further rationale to account for the toxic actions that peroxynitrite may have in vivo.

**Experimental Procedures**

**Chemicals**—Manganese-superoxide dismutase of *E. coli*, potassium phosphate (mono- and di-basic), diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), sodium nitrate, hydrogen peroxide, manganese dioxide, manganese dioxide (MnO₂), xanthine, cytochrome c, poro-hydroxyphenylacetic acid (HPA), glutathione, sodium bicarbonate, desferrioxamine, dimethyl sulfoxide, Trizma (Tris base), 8-hydroxyquinoline, guanidinium hydrochloride, manganese chloride, zinc chloride, sucrose, bovine serum albumin (BSA), Tween 20, sodium chloride, bicinchoninic acid reagents, and all electrophoretic reagents were purchased from Sigma. HPLC-grade methanol was obtained from J. T. Baker Inc. Tetrakis-(4-benzoic acid) porphyrins, (Mn(tbap)) and Zn(tbap)), were synthesized and generously supplied by Ines Batiníć-Haberle and Irwin Fridovich (Duke University).

A rabbit polyclonal antibody against nitrotirosine was raised with nitratated keyhole limpet hemocyanin and purified in our laboratory by affinity chromatography as described elsewhere (36). The rabbit polyclonal antibody against human Mn-SOD was a kind gift from Dr. Ling-Yi Chang (University of Colorado). The donkey monoclonal antibody against rabbit IgG, linked to horseradish peroxidase, nitrocellulose (0.45 μm pore size, Hybond C extra), and luminol-enhanced chemiluminescence detection kit (ECL) were obtained from Amersham Pharmacia Biotech.

Peroxynitrite was synthesized in a quenched flow reactor as described previously (37), and excess hydrogen peroxide was removed by treatment with MnO₂. Peroxynitrite concentrations were determined spectrophotometrically at 302 nm (ε₅₅₀ = 1670 M⁻¹ cm⁻¹) (38).

The reagents for Mn-SOD expression and purification were the following: tryptone and yeast extract for LB medium were obtained from Difeo, and methyl viologen (pararquat), ampicillin, Tris-Cl, cytochrome c, CaCl₂, DNase, RNase, Sephadex G-25 and CM-Sepharose were purified from Pharmacia BioSepra. The reagents for Mn-SOD expression and purification were the following: tryptone and yeast extract for LB medium were obtained from Difeo, and methyl viologen (pararquat), ampicillin, Tris-Cl, cytochrome c, CaCl₂, DNase, RNase, Sephadex G-25 and CM-Sepharose were purified from Pharmacia BioSepra.

**Expression and Purification of Human Recombinant Mn-SOD—E. coli sodAssD** strain QC774 lacking Mn-SOD and Fe-SOD was transformed with the pGBl expression vector (39) containing the coding sequence of wild type human recombinant Mn-SOD (hrMn-SOD), minus that encoding residues 2–24 (i.e. minus the mitochondrial targeting sequence). The cells were grown in LB medium supplemented with 0.2 mM MnCl₂, 50 μg/ml ampicillin, and 30 μg/ml chloramphenicol. The cultures were incubated with a shaking rate of 200 rpm and 37°C. The cell pellets were harvested using a centrifuge and were broken by UltraTurrax on ice. The crude extracts were treated with 100 units/ml DNase, 10 mg/ml RNase, 10 mM MgCl₂, 10 mM CaCl₂, 0.15 M NaCl in 10 mM KPO₄, pH 7.0, at room temperature for 1 h. Mn-SOD was precipitated by (NH₄)₂SO₄ fractionation between 65 and 80% saturation. The precipitate obtained at 80% saturation was salted out by gel filtration chromatography on Sephadex G-25 in 20 mM potassium acetate buffer, pH 6.6. The proteins were then applied to a CM-Sepharose column and eluted in a gradient of 0–100 mM KCl. The yield of purified hrMn-SOD was 72%.

**SOD Activity**—SOD activity was determined measuring the inhibition of the reduction of cytochrome c by the xanthine-xanthine oxidase system (40). The concentrations of hrMn-SOD and hrMn-SOD were measured by absorbance at 282 nm (ε₅₅₀ = 8.67 × 10³ M⁻¹ cm⁻¹ (3)) and 280 nm (ε₅₅₀ = 1.81 × 10³ M⁻¹ cm⁻¹ (41, 42)) respectively, and by the bichinchoninic acid method obtaining concordant results. Enzyme preparations of hrMn-SOD and E. coli Mn-SOD, used in the different assays, typically had specific activities of 2500 and 3200 units/mg, respectively.

**Removal of Manganese from the Active Site of Human Recombinant Mn-SOD—** 25 ml of hrMn-SOD (200 μg/ml) were dialyzed 48 h at 4°C against 400 ml of 200 mM Tris-HCl buffer, pH 3.8, containing 1.5 mM guanidinium hydrochloride, 20 mM 8-hydroxyquinoline, and 0.5 mM sucrose. The apo-enzyme produced was then dialyzed 48 h against 200 ml of 200 mM Tris-HCl, pH 8.8, containing 0.5 mM sucrose and either 0.6 mM MnCl₂ or 0.6 mM ZnCl₂, with a change of buffer after 24 h. Finally, the samples were again dialyzed 48 h against 200 ml of 200 mM Tris-HCl, pH 8.8, containing 0.5 mM sucrose, changing the buffer each 12 h. The apo-enzyme and the reconstituted enzymes were assayed for SOD activity and protein content as described above. Recovered specific activities as percentages of original activity were apo-enzyme 0%, apo/Mn enzyme 21%, and apo/Zn enzyme 20%.

**Metal Analyses—** Zinc and manganese content of the enzymes were determined with a graphite furnace atomic absorption spectrometer (Spectra 20, Varian Instruments, Victoria, Australia). Calibration curves of each element were made from dilutions in deionized water of atomic absorption standards. The enzyme samples were diluted with water and metal ion content calibrated against standards.

**Results and Discussion—** The nitrating properties of peroxynitrite decomposition in absence and presence of enzyme were studied in a stopped-flow spectrophotometer (Applied Photophysics, SF.17MV) with a mixing time of less than 2 ms, at 302 nm. An initial rate approach was used to analyze the data; the first 0.1 s were fit to a linear plot, and the rate constant was determined as the ratio between the slope and the difference between the initial and final absorbance (Aₙ – A₁). To ensure the accuracy of the rate constant determinations, 200 absorbance measurements were acquired during the initial part of the reaction (first 0.2 s) and 200 further points were acquired until more than 99.9% peroxynitrite had decomposed (0.2–10 s) (45).

**Kinetics of HPA nitration by peroxynitrite** were studied under pseudo-first order conditions with peroxynitrite in excess over the porphyrin, following absorbance changes on the porphyrin, at 468 and 421 nm, respectively, as described previously (34). Data obtained in the first 0.03–0.2 s were fit to single exponential and pseudo-first order rate constants determined. Reactions were performed at 37.0 ± 0.1°C, and the final pH of the mixture was measured at the outlet.

**Kinetics of HPA nitration by peroxynitrite** were studied using an initial rate approach, at 430 nm.

**Nitrination of HPA**—Nitrination of HPA by peroxynitrite was assessed spectrophotometrically. After the reaction had taken place, the pH of the solution was adjusted to 10–11 with 6 N NaOH, and absorbance was recorded at 430 nm. Absorbance of a control containing everything except peroxynitrite was subtracted before determining the-nitro-HPA concentration (ε₅₅₀ = 4400 M⁻¹ cm⁻¹) (29). Percent yield was calculated with respect to initial peroxynitrite concentration.

**Nitrination of HPA** was also studied by high performance liquid chromatography (HPLC)-based techniques. Standards and samples of HPA and nitro-HPA were separated using a Gilson 306 pump (Wilson Medical Electronics, Inc.) and a C₈-derivatized silica column. Samples were eluted with 50 mM potassium phosphate, pH 3, and HPLC-grade meth-
RESULTS

Kinetics of Peroxynitrite Reaction with Mn-SOD—The decay of peroxynitrite (0.1 mM) was followed in absence and in presence of hrMn-SOD (10 µM) (Fig. 1). At these concentrations pseudo-first order conditions are not achieved, and the kinetic traces of peroxynitrite decay in the presence of the enzyme did not follow a single exponential function (Fig. 1, inset). Indeed, the rate of peroxynitrite decomposition in the presence of hrMn-SOD was initially faster, reflecting the reaction of peroxynitrite with the enzyme (Fig. 1, inset).

To obtain the rate constant of peroxynitrite reaction with hrMn-SOD, the decay of peroxynitrite (0.2 mM) was followed in the absence and in presence of different concentrations (2.5–15 µM) of hrMn-SOD tetramer, obtaining plots such as presented in Fig. 1. The apparent rate constant of peroxynitrite decomposition ($k_{obs}$) was determined by measuring the initial rate of peroxynitrite decay (i.e. during the first 100 ms), as reported recently (45). The plot of the apparent rate constants of peroxynitrite decomposition as a function of Mn-SOD concentrations was linear (Fig. 2). The slope of such plot rendered the second order rate constant ($k_{diss}$) for the reaction of Mn-SOD with peroxynitrite of 1.0 ± 0.04 × 10^8 M^−1 s^−1 per tetramer and 2.5 × 10^8 M^−1 s^−1 per monomer, at pH 7.47 and 37 °C. This constant correlates well with that obtained for other metallo-proteins (46–50) and strongly suggests a role for the manganese ion in the decomposition kinetics of peroxynitrite. The second order rate constant for the reaction of E. coli Mn-SOD (2.5 µM) with peroxynitrite (0.2 mM) was determined, consider-
Peroxy nitrite Reaction with Mn-SOD

Table I

| Enzyme          | Protein | Manganese | Zinc | % SOD activity |
|-----------------|---------|-----------|------|----------------|
| Holoenzyme      | 0.36    | 0.7       | ND   | 100            |
| Apoenzyme       | 0.35    | 0         | ND   | 1              |
| Apo/Mn          | 0.38    | 0.5       | ND   | 76             |
| Apo/Zn          | 0.38    | 1.5       | ND   | 0              |

Table II

Role of the metal center in peroxynitrite decomposition kinetics

The time course of the decomposition of peroxynitrite (0.2 mM) in absence and presence of either E. coli Mn-SOD, apoenzyme, apo/Mn, or apo/Zn (2.5 μM) was studied as in Fig. 1. The kinetic constant was determined considering the k_{obs} and enzyme concentration.

| Enzyme          | k [μM⁻¹ s⁻¹] | % SOD activity |
|-----------------|--------------|---------------|
| Holoenzyme      | 1.4 ± 0.2 x 10⁴ | 100           |
| Apoenzyme       | <4.0 x 10⁴    | 0             |
| Apo/Mn          | 5.2 ± 0.7 x 10⁴ | 40            |
| Apo/Zn          | 2.6 ± 0.5 x 10⁵ | 0             |

FIG. 3. Role of the metal center in enzyme nitration. Immunochemical detection of nitrotyrosine was performed after peroxynitrite (0.1 mM) exposure to either E. coli Mn-SOD, apoenzyme, apo/Mn, or apo/Zn (5 μM). The blot was scanned and analyzed by densitometric techniques. Nitration is expressed as relative to the native enzyme exposed to peroxynitrite. Activity is expressed relative to the native enzyme incubated in absence of peroxynitrite.

The apoenzymes, apo/Mn, and apo/Zn enzymes were obtained as described under “Experimental Procedures.” Protein concentration, metal content, and specific activity were determined. Percentage activity was expressed relative to the native enzyme-specific activity. ND, not determined.

Zinc was considered to be a good candidate for the substitution of manganese and evaluation of peroxynitrite reactions with Mn-SOD. On one hand, the Zn(II) and Mn(II) ions have similar sizes (ionic radius of 0.74 and 0.80 Å, respectively (52)), and zinc is reported to bind to Mn-SOD in a stoichiometric amount displacing manganese-from the active site (51), so the apo/Zn conformation would probably be similar to the native one. On the other hand, zinc is not capable of rendering high oxidation states (53), such as those proposed to participate in tyrosine nitration in the case of manganese and iron (i.e. oxo-manganese (O=–Mn(IV)) and oxo-iron (O=–Fe(IV))) (34, 35), so the apo/Zn enzyme would not be an efficient promoter of nitration reactions.

The apo, apo/Mn, and apo/Zn forms of hrMn-SOD were obtained in 0.5 m sucrose. When the disaccharide was extracted by dialysis, these enzymes largely precipitated (60–70%). The different stability of the human and E. coli preparations may be due to their different quaternary structures, tetrameric and dimeric, respectively.

Role of the Metal Center in Peroxy nitrite Decomposition Kinetics—Peroxy nitrite decomposition kinetics in the presence of the apo, apo/Mn, and apo/Zn forms of the enzyme was assessed as described in Fig. 1. Table II reports the second order rate constants determined for the decomposition of peroxynitrite in the presence and absence of 2.5 μM enzyme. The apoenzymes did not affect peroxynitrite decomposition kinetics in a detectable way, so the product of its second order rate constant and the concentration of enzyme is less than 10% of that determined for peroxynitrite alone (k_{apo} x [apo] < 0.1 x k_{apoMnSOD}). These considerations indicate that the second order rate constant of the apoenzyme must be smaller than 4 x 10⁸ M⁻¹ s⁻¹ and probably reflects the reaction rate of peroxynitrite with the enzyme amino acids (45). The apo/Mn rate constant was 37% that of the holoenzyme, in agreement with the data obtained for the SOD-specific activity, which is in turn proportional to the manganese content (51). Thus it is reasonable to assume that apo/Mn preparations with the same manganese content as the holoenzyme will recover 100% of the rate constant value. The apo/Zn presented an unexpectedly high rate constant, approximately twice that obtained for the holoenzyme. These results support the idea that metal center plays a central role in peroxynitrite decomposition kinetics.

Removal of Manganese from the Active Site and Reconstitution with Manganese or Zinc Ions—To study the role of the metal center in peroxynitrite reaction with Mn-SOD, the metal was removed from the active site of the E. coli enzyme, and the apoenzyme (apo) was obtained. The apoenzyme was then reconstituted with manganese (apo/Mn) or substituted with zinc (apo/Zn). The activity and metal content of these enzymes is shown in Table I. The native enzyme presented 0.7 manganese-atoms per monomer, in agreement with that reported for the E. coli enzyme (3). The manganese-content of the holoenzyme, apo, apo/Mn, and apo/Zn enzymes correlated with the activity, in agreement with the literature (51). The apo/Zn enzyme presented slightly more than one atom of zinc per monomer, implying the existence of unspecific binding of the metal to the enzyme.
tive, apo/Mn, and apo/Zn enzymes, which must be related to a larger surface exposure and accessibility to tyrosine residues in the apoenzyme, due to the denaturalization and metal extraction process.

Mass spectrometry studies of the native enzyme and the apoenzyme showed that incubation with peroxynitrite resulted in the formation of a species with a molecular mass 47 Da higher than the control enzyme (22,944 to 22,991 Da), consistent with the addition of a single nitro group to both enzymes (Fig. 4). Integration of the area below the peaks showed that the nitration yields with respect to the protein were higher in the apoenzyme than in the holoenzyme, in agreement with the results obtained by immunoblot techniques.

The exposure of the holoenzyme to peroxynitrite under these conditions resulted not only in nitration but also in a small degree of dimerization of the enzyme. Densitometric analysis of Western blots, using an antibody against human Mn-SOD, revealed that ~3–5% of the native enzyme was present as dimer after the exposure to peroxynitrite (not shown).

**Mn-SOD and Peroxynitrite-dependent Nitration of Phenols**—4-Hydroxyphenylacetic acid (5 mM) was exposed to peroxynitrite (1 mM) in the absence and in the presence of increasing concentrations of *E. coli* Mn-SOD (Fig. 5A). While in absence of enzyme 10.3% of the added peroxynitrite was recovered as nitro-HPA, and in presence of the *E. coli* Mn-SOD, nitration yields increased with the concentration of enzyme, fitting a hyperbolic profile. A maximum of 12.5% nitration yield could be predicted from these data. Considering the maximum nitration yield (%$R_{\text{max}}$) and that obtained in absence of enzyme (%$R_0$) (see Equation 1) the maximum increase in nitration yield was calculated to be 21%.

$$\% \text{ increase} = \left( \frac{R_{\text{max}} - R_0}{R_0} \right) \times 100 \quad (\text{Eq. 1})$$

The Mn-SOD-promoted increase in nitration yields was also assessed by HPLC techniques. HPA (5 mM) was incubated with peroxynitrite (1 mM) in the absence and presence of *E. coli* Mn-SOD (5 mM). Mn-SOD was extracted from the samples by filtration, and HPA and nitro-HPA were separated using a reverse phase chromatography column, presenting elution times of 19.7 and 24.1 min, respectively. Nitro-HPA was quantified measuring the peak area obtained at 360 nm. In the absence of the enzyme a nitration yield of 10.9 ± 0.7% was obtained, whereas in the presence of Mn-SOD this increased to 12.6 ± 0.4% (not shown), in complete agreement with the data obtained by spectrophotometric techniques.

Then the role of the metal center, in promoting peroxynitrite-dependent nitration of HPA, was assessed. HPA (5 mM) was exposed to peroxynitrite (1 mM) in the presence of holo, apo, apo/Mn, and apo/Zn enzymes (5 mM) (Fig. 5B), and percentage increases in nitration yields were determined to be 14.3, 0, 10.6, and 0.6%, respectively. These results unambiguously show that the manganese-ion is responsible for the increase in
HPA nitration yields observed in presence of Mn-SOD.

Kinetics of Peroxynitrite Decomposition in the Presence of Mn(tbap) and Zn(tbap)—Due to the surprisingly high rate constant obtained for the reaction of peroxynitrite with the apo/Zn, compared with that obtained for the native and apo/Mn, and to obtain a better comprehension of peroxynitrite reactivity with these metals, we studied the reaction of peroxynitrite with low molecular weight complexes of manganese and zinc with a substituted porphyrin (tbap).

The reaction rate of the Mn(tbap) and Zn(tbap) (8 mM) with peroxynitrite was followed at 468 and 421 nm, the respective Soret peak wavelengths of these porphyrins. Peroxynitrite was present in 5–50-fold excess (0.04–4 mM) over the porphyrin achieving pseudo-first order conditions. The kinetic traces of the Mn(tbap) reaction with excess peroxynitrite displayed a biphasic pattern as follows: a first order descent (Fig. 6A), followed by a slow recovery of the absorbance values. A total absorbance recovery was observed for Mn(tbap), similar to that described for the reaction of other manganese-porphyrins (34). In the case of Zn(tbap), the kinetics were more complex. An initial rapid descent (Fig 6A, inset) was followed by a slower one. This second descent was independent of peroxynitrite concentration and had a $k_{obs}$ value similar to that of the proton-catalyzed decomposition of peroxynitrite, suggesting reactions between peroxynitrite-derived radicals and the porphyrin moiety. This latter idea is consistent with the fact that Zn(tbap) recovered its initial absorbance only partially. The observed rate constants determined from the exponential fit in the first 30–200 ms (Fig. 6A, inset) were plotted in function of peroxynitrite concentration (Fig. 6B). From the slope of these plots, second order rate constants for the reactions of Mn(tbap) and Zn(tbap) with peroxynitrite of $6.8 \pm 0.1 \times 10^4$ and $4.9 \pm 0.1 \times 10^5$ M$^{-1}$ s$^{-1}$ at pH 7.2 and 37 °C were obtained, respectively. The ratio between the rate constants obtained from the Mn- and Zn-porphyrins is in good agreement with that obtained with the native enzyme and zinc-substituted apoenzyme.

Mn(tbap) and Zn(tbap) Catalysis of Peroxynitrite-dependent Nitration of HPA—Mn(tbap) (1–20 mM) increased peroxynitrite (1 mM)-dependent yield of nitration of HPA (5 mM) in a dose-dependent fashion. These results fit a hyperbolic plot, and a maximum increase in nitration yields of 350% was determined, in agreement with previous reports (33). On the other hand, Zn(tbap) did not promote peroxynitrite-dependent nitration of HPA (Fig. 7), and in fact at high concentrations of Zn(tbap) (20 mM) a small decrease in nitration yields was observed, in agreement with recent reports (35). These results are in agreement with those obtained with the native enzyme and zinc-substituted apoenzyme, supporting the role for the active site manganese ion in promoting the peroxynitrite-dependent nitration of low molecular weight phenols. At the same time it is clear that either the environment of the manganese ion in the enzyme or the poor accessibility of HPA to the active site makes the enzyme a less efficient promoter of peroxynitrite-dependent...
nitrination of phenols, compared with the manganese-porphyrin.

The fast reaction of Zn(tbap) with peroxynitrite in conjunction with a marginal effect on nitrination yields supports the idea that zinc is behaving like a Lewis acid, resulting in nitrination yields similar to those of H+–catalyzed nitrination. This hypothesis was further evaluated by experiments showing that Zn(tbap) (10–50 μM) accelerated peroxynitrite (1 mM)-dependent HPA (5 mM) nitrination and diminished peroxynitrite nitrination yields in presence of carbon dioxide (0.2 mM) (not shown).

Effect of Scavengers on Mn-SOD Nitration and Inactivation—Different compounds known to interact either with peroxynitrite or with hydroxyl radical or nitrogen dioxide were assessed for their ability to inhibit or increase the nitrination and inactivation of E. coli Mn-SOD (5 μM) by peroxynitrite (0.5 mM) (Fig. 8A). Coincubation of Mn-SOD with 1 mM glutathione (GSH) and HPA largely prevented both nitrination and inactivation. Dimethyl sulfoxide (Me2SO) (10 mM), a well known hydroxyl radical scavenger, was a weak inhibitor of inactivation and nitrination, implying a modest role for the radical pathway in the nitrination of the holoenzyme. Most interestingly, bicarbonate (HCO3−) (1 mM carbon dioxide) also rendered partial protection at this enzyme concentration.

At higher concentrations of Mn-SOD, HCO3− enhanced the nitrination and inactivation of the enzyme by peroxynitrite, whereas HPA and GSH (5 mM) achieved total protection, the latter both in absence and in presence of HCO3−.

**DISCUSSION**

Peroxynitrite reacts with human recombinant and E. coli Mn-SOD in a direct reaction with second order rate constants of 1.0 ± 0.2 × 105 and 1.4 ± 0.2 × 105 M−1 s−1 at pH 7.47 and 37 °C, respectively. The rate constant for the reaction with the E. coli apoenzyme was at least 1 order of magnitude smaller, whereas that of the apo/Mn was comparable with that of the holoenzyme, confirming that the reaction between Mn-SOD and peroxynitrite largely depends on the presence of the metal in the active site. The reactions of peroxynitrite with metalloproteins are typically fast; therefore, these are likely to be major targets in vivo. In this context, the reactivity of peroxynitrite with Mn-SOD is similar to that previously reported for mitochondrial aconitase (47), cytochrome c2+ (48), alcohol dehydrogenase (46), and peroxidases (49).

The surprisingly high rate constant determined for the reaction between peroxynitrite and the zinc-substituted apoenzyme suggested that the decomposition kinetics of peroxynitrite could also be affected by the nonredox metal zinc (53). Indeed, kinetic rate determinations for the reaction of peroxynitrite with Mn(tbap) and Zn(tbap), revealed that the zinc-substituted porphyrin reacts with peroxynitrite faster than its manganese counterpart. The decomposition of peroxynitrite in the presence of Mn-SOD and Mn(tbap) is attributed to a redox reaction which involves the oxidation of the metal ion (34, 54). However, in the presence of the zinc-substituted apoenzyme or Zn(tbap), peroxynitrite decomposition must proceed through a different pathway that may involve the utilization of the zinc ion as a Lewis acid.

Peroxynitrite reaction with Mn-SOD leads to the formation of nitrating species, capable of modifying low molecular weight aromatic compounds in a manganese-dependent process (Fig. 5). In addition, and as reported for the human recombinant Mn-SOD (23, 24), peroxynitrite promoted the inactivation of the E. coli enzyme mainly by the nitration of one tyrosine residue. While in the apoenzyme peroxynitrite-derived hydroxyl radical and nitrogen dioxide-mediated tyrosine nitrination, the manganese ion played an important role in tyrosine nitrination in the holoenzyme (Fig. 3). The nitrating species could be either a nitronium ion (NO2+) bound to the metal (29, 55) or an oxo-manganese complex plus nitrogen dioxide (34, 54). Nevertheless, the kinetic traces of peroxynitrite decomposition in the presence of Mn-SOD do not fit single exponential kinetics indicating that the enzyme is being consumed in the reaction with peroxynitrite, so even though it enhances nitrination yields, it is not a true catalyst. In all, metal cofactors prone to undergo redox transitions, such as manganese, iron, or copper, are likely to promote site-specific nitration of protein aromatic residues; this idea is consistent with previous data on prosta-
cyclophilase synthesis and Cu,Zn-SOD as well (55–57).

E. coli Mn-SOD tyrosine residues have different degrees of solvent accessibility, as revealed by the analysis of the native structure using the Swiss PDB Viewer program. Tyrosine 34 is less accessible to the solvent than Tyr-2, Tyr-9, and Tyr-11 and equally accessible as Tyr-173, Tyr-174, and Tyr-184, but tyrosine 34 is the residue located closest to the active site, only at 5 Å from the manganese ion. The attraction of peroxynitrite to the active site, probably by the basic residues in the channel entrance, and its reaction with the manganese ion leading to the formation of nitratating species provide a reasonable explanation to the fact that tyrosine 34 is the tyrosine residue most susceptible to nitration by peroxynitrite (23, 24).

Considering the specific activity of purified Mn-SOD (4000 ± 1000 units/mg (1, 4, 58, 59)) and that observed in mitochondria (10 ± 2 units/mg, in heart and liver mitochondria),2 as well as the enzyme molecular mass (88 kDa) and mitochondrial volume (1.2 µL/mg), a concentration of Mn-SOD inside the mitochondria of 20 ± 10 µM (80 µM subunits) was estimated. In concentrations similar to those found in the mitochondria, GSH protected Mn-SOD from nitration and inactivation both in the absence and presence of carbon dioxide (Fig. 8). These data underscore the role of GSH as a mitochondrial antioxidant and may ultimately signal cell death. Strategies directed to attenuate nitration of Mn-SOD tyrosine 34 should provide a reasonable explanation to the fact that tyrosine 34 is the tyrosine residue most susceptible to nitration by peroxynitrite (23, 24).

Since Mn-SOD is a critical mitochondrial antioxidant, its nitration represents a severe hazard that will promote oxidative damage and may ultimately signal cell death. Strategies directed to attenuate nitration of Mn-SOD tyrosine 34 should result in a better mitochondrial and cellular outcome under conditions of excess peroxynitrite formation.

Acknowledgements—We thank Marion Kirk and Stanley Digerness for their assistance in the mass and absorption spectrometry studies, respectively.

REFERENCES
1. Weisiger, R. A., and Fridovich, I. (1973) J. Biol. Chem. 248, 3592–3592
2. Keele, B. B., Jr., McCord, J., and Fridovich, I. (1970) J. Biol. Chem. 245, 6176–6181
3. Beyer, W., Imlay, J., and Fridovich, I. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8655–8658
4. Matsuda, Y., Higashiyama, S., Kijma, Y., Suzuki, Y., Kawano, K., Akiyama, M., Katowa, S., Tanini, S., Deutsch, H. F., and Taniguchi, N. (1990) Eur. J. Biochem. 194, 713–720
5. Turrens, J. F., Freeman, B. A., Levitt, J. G., and Crapo, J. D. (1982) J. Biol. Chem. 257, 7207–7295
6. Li, Y., Huang, T.-T., Carlson, E. J., Melov, S., Utsel, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., Chan, P. H., Wallace, D. C., and Epstein, C. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9782–9787
7. Lewrox, R. M., Zhang, H., Vogel, H., Cartwright, J., Sr., Dienne, L. N., Huang, S., and Matzuw, M. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11070–11075
8. Li, Y., Huang, T.-T., Carlson, E. J., Melov, S., Utsel, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., Chan, P. H., Wallace, D. C., and Epstein, C. J. (1995) J. Biol. Chem. 270, 376–381
9. Melov, S., Cookson, P., Patel, M., Tuinstra, R., Cottrell, B., Jun, A. S., Zastawny, T. H., Dizdaroglu, M., Goodman, S. I., Huang, T., Mizokoto, H., Epstein, C. J., and Wallace, D. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 846–851
10. Gross, S. S., and Wolin, M. S. (1995) Annu. Rev. Physiol. 57, 737–769

2 C. Quijano, and R. Radi, unpublished data.