Peroxynitrite-Mediated Inactivation of Manganese Superoxide Dismutase Involves Nitration and Oxidation of Critical Tyrosine Residues†

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ABSTRACT: Previous studies from our laboratory have demonstrated that the mitochondrial protein manganese superoxide dismutase is inactivated, tyrosine nitrated, and present as higher molecular mass species during human renal allograft rejection. To elucidate mechanisms whereby tyrosine modifications might result in loss of enzymatic activity and altered structure, the effects of specific biological oxidants on recombinant human manganese superoxide dismutase in vitro have been evaluated. Hydrogen peroxide or nitric oxide had no effect on enzymatic activity, tyrosine modification, or electrophoretic mobility. Exposure to either hypochlorous acid or tetranitromethane (pH 6) inhibited (approximately 50%) enzymatic activity and induced the formation of dityrosine and higher mass species. Treatment with tetranitromethane (pH 8) inhibited enzymatic activity 67% and induced the formation of nitrotyrosine. In contrast, peroxynitrite completely inhibited enzymatic activity and induced formation of both nitrotyrosine and dityrosine along with higher molecular mass species. Combination of real-time spectral analysis and electrospray mass spectroscopy revealed that only three (Y34, Y45, and Y193) of the nine total tyrosine residues in manganese superoxide dismutase were nitrated by peroxynitrite. Inspection of X-ray crystallographic data suggested that neighboring glutamate residues associated with two of these tyrosines may promote targeted nitration by peroxynitrite. Tyr34, which is present in the active site, appeared to be the most susceptible residue to peroxynitrite-mediated nitration. Collectively, these observations are consistent with previous results using chronically rejecting human renal allografts and provide a compelling argument supporting the involvement of peroxynitrite during this pathophysiologic condition.

We have recently demonstrated that the mitochondrial antioxidant protein, manganese superoxide dismutase (Mn-SOD)[1], is both nitrated and inactivated during human kidney allograft rejection (*1*). Nitric oxide (NO) has been implicated as a mediator in rejection of many organs (*2*), although the precise pathological role of NO is not clear. One mechanism of toxicity may involve NO reacting with superoxide to form peroxynitrite (*3*). Peroxynitrite is a potent oxidant and nitrating agent that leads to a host of potentially injurious events including lipid peroxidation, depletion of cellular antioxidant defenses, inactivation of enzymes, and nitration of tyrosine residues in proteins that may adversely affect their function as well as affecting signal transduction processes (*4*−*8*). Nitration and inactivation of MnSOD would dramatically increase mitochondrial superoxide levels leading to generation of additional peroxynitrite, thereby further amplifying nitration/oxidation of other mitochondrial proteins.

Recent reports involving SOD knockouts have revealed that MnSOD is essential for life whereas Cu,ZnSOD is not. Cu,ZnSOD knockout mice appear normal and exhibit differences only after traumatic injury, whereas MnSOD knockouts do not survive past 3 weeks of age (*9, 10*). MnSOD knockout mice exhibit several organ-level pathologies including myocardial injury, fatty liver, and anemia as well as more generalized types of injury such as lipid peroxidation and severe mitochondrial damage, which could result in injury to any tissue.

MnSOD is a homotetramer (96 kDa) containing one manganese atom per subunit that cycles from Mn(III) to Mn(II) and back to Mn(III) during the two-step dismutation of superoxide. The enzyme contains nine tyrosine residues, one of which (Tyr34) is highly conserved phylogenetically and is located only a few angstroms from the manganese atom in the active site (*11*). To better understand the particular susceptibility of MnSOD to oxidative inactivation, we treated recombinant human MnSOD with various oxidants in vitro and subjected the treated enzyme to a number of biochemical assays.

The primary oxidants used in these studies included peroxynitrite, tetranitromethane (TNM), hypochlorous acid (HOCl), and hydrogen peroxide (H_2O_2). Peroxynitrite has been shown to attack nucleophilic amino acids including

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[1] Abbreviations: MnSOD, manganese superoxide dismutase; ONOO, peroxynitrite; TNM, tetranitromethane; HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; NO, nitric oxide; NO_2, nitrite; tyr, tyrosine.

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lysine (unpublished observations), cysteine, methionine, tyrosine, and tryptophan (*12*−*15*). TNM is an oxidant and nitrating agent that has been used for many years to assess the role of tyrosine residues in protein function (*16*−*20*). Oxidative activity of TNM predominates at pH 6.0 whereas it is almost exclusively a tyrosine nitrating agent at pH 8.0; this pH-dependent reactivity can be used to discriminate between oxidation and nitration reactions. HOCl is a potent two-electron oxidant and chlorinating agent produced by myeloperoxidase (*21*). It has been reported that HOCl reacts with nitrite to form a tyrosine-nitrating species (*22*, *23*). Thus, HOCl can be a strong oxidant and/or nitrating species, depending on experimental conditions. Hydrogen peroxide has been shown to oxidize methionine residues within proteins (*24*), which could alter protein function.

Both peroxynitrite and HOCl have been shown to modify tyrosine residues through formation of dityrosine (3,3′-dityrosine), a stable covalent modification resulting from the addition of two tyrosyl radicals, leaving a product with a fluorescent emission distinct from tyrosine itself (*25*, *26*). Our earlier studies suggested that not only was MnSOD inactivated but also appeared to form higher molecular mass species during chronic rejection of human renal allografts (*1*). Results presented here provide a compelling argument that both nitration and oxidation of tyrosine residues can account for the inactivation of MnSOD.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma unless otherwise specified. Peroxynitrite was synthesized from sodium nitrite and acidified H_2O_2 and quantified as described previously (*27*).

Expression and Purification of MnSOD. Escherichia coli harboring a novel expression vector (*28*) containing the human MnSOD cDNA were used to produce the recombinant protein. The human MnSOD used here lacks the 24 amino acid mitochondrial targeting leader sequence and is numbered starting with the N-terminal residue of the mature protein. Bacteria were grown in Luria broth, and induction was performed by adding paraquat (20 mM) and $MnCl_2$ (200 mM) for 3 h at 23 °C. Bacteria were harvested by centrifugation (4000*g* for 10 min at 4 °C), resuspended in 20 mM Tris-HCl at pH 7.8, and frozen overnight. Following freeze/thaw, solutions were sonicated, treated with 0.05% (w/v) polyethylenimide to remove DNA, and centrifuged at 18000*g*. Supernatants were precipitated with 80% (w/v) ammonium sulfate and pellets resuspended in 10 mM KPi, pH 7.4, and dialyzed against buffer containing $MnCl_2$ (2 mM). Final purification was performed by strong anion-exchange chromatography (Biocept Inc.); MnSOD was eluted using a 0−0.25 M NaCl gradient in 5 mM KPi, pH 8.0. MnSOD activities of column fractions were determined by the cytochrome *c* reduction method (*29*), and specific activity calculations were made based on protein values determined using the Bradford Assay (Pierce). Atomic absorption spectroscopy confirmed the presence of 0.9 Mn atom per monomeric subunit. Pure MnSOD (96 kDa tetramer) was concentrated to 25−50 mg/mL using ultrafiltration (10 000 MWCO), fast frozen in liquid nitrogen, and stored at −80 °C until use.

Oxidant Treatment of MnSOD. (a) Peroxynitrite Treatment. Peroxynitrite was added (23 °C) at the final indicated concentrations to recombinant human MnSOD (15 μM) in 0.1 M potassium phosphate (KPi), pH 7.4, while vortexing. Decomposition products of peroxynitrite were removed using four sequential rounds of ultrafiltration (5000 MWCO) prior to further analysis. Working solutions of peroxynitrite were prepared by diluting stocks in 100 mM NaOH prior to use.

(b) NO Treatment. MnSOD (15 μM) in 0.1 M KPi, pH 7.4, was treated (2 h, 37 °C) with 1 mM authentic NO. The saturated aqueous NO solution was prepared as previously described (*30*). Briefly, 75 mL of pure water was added to a 100-mL glass vial sealed with a silicon rubber stopper. The solution was deoxygenated via argon bubbling and then bubbled with nitric oxide gas that had been passed through an aqueous solution of 0.1 N NaOH to remove traces of nitrogen dioxide and nitrite. The saturated nitric oxide solution (1.9 mM) was sampled with a gas-tight Hamilton syringe and added directly to the stirred MnSOD solution.

(*c*) *Tetranitromethane Treatment.* MnSOD (15 μM) in 0.1 M KPi, pH 6 or 8.0, was treated (2 h, 37 °C) with 0.5 mM TNM. Unreacted TNM was removed by ultrafiltration (5000 MWCO).

(d) HOCl and H_2O_2 Treatment. MnSOD (15 μM) was treated with either HOCl (1 mM) alone or in combination with 1 mM sodium nitrite ($NaNO_2$) in 0.1 M KPi buffer, pH 7.4 (2 h, 37 °C). H_2O_2 (1 mM) was incubated (2 h, 37 °C) with MnSOD (15 μM) in 0.1 M KPi buffer, pH 7.4. Unreacted oxidants were removed by ultrafiltration (5000 MWCO) prior to further analysis. The concentrations of oxidants were measured spectrally: $E_{290\ nm} = 350\ M^{-1}\ cm^{-1}$ for HOCl (*31*) and $E_{240\ nm} = 39.4\ M^{-1}\ cm^{-1}$ for H_2O_2 (*32*).

Amino Acid Analysis. Both untreated and peroxynitrite-treated MnSOD were dialyzed extensively against 10 mM potassium phosphate buffer, pH 7.4. Proteins were hydrolyzed under standard conditions (6 M HCl at 110 °C for 24 h). Hydrolysates were derivatized with *o*-pthalaldehyde (OPA) and quantified by reversed-phase HPLC as compared to OPA-derivatized amino acid standards. Sulfhydryl content of MnSOD was determined using dithiobis(nitrobenzoic acid) (DTNB) in the presence of 6 M guanidine hydrochloride as described previously (*27*). Lysine content of MnSOD was determined using fluorescamine in the presence of 6 M guanidine hydrochloride as described previously (*33*).

Spectral Nitrotyrosine Assay. Peroxynitrite was added sequentially to a stirred MnSOD solution (15 μM) in 0.1 M KPi buffer, pH 7.4, at 37 °C. Each peroxynitrite concentration shown in Figure 4 represents sequential bolus additions; complete reaction of peroxynitrite occurred (∼30 s) prior the next addition. Nitrotyrosine content of MnSOD treated with peroxynitrite was monitored spectrally at 430 nm. The extinction coefficient ($E_{430\ nm} = 4400\ M^{-1}\ cm^{-1}$) under these conditions was determined using commercial nitrotyrosine. HPLC analysis of hydrolyzed nitrotyrosine-containing proteins has been used to validate the nitrotyrosine extinction coefficient (*34*). Data are expressed as nitrotyrosine/MnSOD subunit by dividing total nitrotyrosine content by the molar concentration of MnSOD monomer.

Electrospray Mass Spectroscopy. MnSOD (15 μM) was treated with peroxynitrite as in the spectral assay above (cumulative dose of 0.4−1 mM) or with a single bolus addition of peroxynitrite (0.4−1 mM), denatured with argon-

purged 6 M guanidine hydrochloride in 50 mM Tris, pH 8 (2 h; 37 °C in the dark), reduced with 20 mM dithiothreitol (2 h; 37 °C), and alkylated with 0.12 M iodoacetamide (2 h; 23 °C in the dark). Samples were exhaustively dialyzed (two buffer changes/day) for 3 days against 100 mM ammonium bicarbonate, pH 8, and then cleaved with trypsin or chymotrypsin (50 μg, 12 h; 37 °C). Alternatively, MnSOD was treated with peroxynitrite (either as in the spectral assay or with a bolus addition of peroxynitrite), cleaved with chymotrypsin (50 μg, 12 h; 37 °C), and subjected to RP−HPLC. Peptides were eluted (1 mL/min) from a 15-cm C-18 reverse-phase column using an increasing linear gradient (30 mL) from 5% (w/v) to 80% (w/v) acetonitrile mixed with 50 mM ammonium formate, pH 4. Tyrosine-nitrated fragments were collected based on the absorbance at 365 nm (*34*). Mass spectroscopy was performed using an LC mass spectrometer containing three mass spectrometers (Perkin-Elmer, Sceiex) operating in tandem. The first mass spectrometer was tuned to feed specific mass peaks (determined by previous MS of purified peptides) into the entrance of the second mass spectrometer. A fine stream of argon atoms was mixed with selected fragments from the first spectrometer, which randomly fragmented the peptides at the relatively weak peptide bond between amino acids. These fragments were then separated in the third spectrometer, making it possible to deduce the sequence and sites of nitration on the peptide. Trypsin cleaves after lysyl and arginyl residues, leaving positively charged residues at the carboxy terminus. Chymotrypsin primarily cleaves after aromatic residues but can cleave after the hydrophobic residues leucine or isoleucine. The amino terminus is also positively charged under the acidic conditions used. Thus, both the N and C peptides contained one positive charge and could be resolved as the so-called b and y fragments. Because nitrotyrosine is stable enough to survive fragmentation, the sites of nitration in a nitrated peptide can be readily determined. This technique allows for the identification of the nitrated tyrosine residue(s) by searching for a mass/charge (*m*/*z*) ratio shift of 45 (addition of $-NO_2$ minus $-H$).

Western Blot Analysis. Western blot analysis was performed as recently described (*1*). Briefly, following separation by 12% (w/v) SDS−PAGE, proteins were transferred electrophoretically to nitrocellulose membranes and blocked with 5% (w/v) nonfat milk in 50 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% (v/v) Tween-20 (TBS/T). MnSOD was detected by incubating blots with a polyclonal rabbit anti-rat MnSOD antibody (kindly provided by Dr. Naoyuki Taniguchi). Nitrotyrosine-containing proteins were detected by incubating blots with a mouse monoclonal anti-nitrotyrosine antibody (kindly provided by Dr. Joe Beckman). Probed membranes were washed three times, and immunoreactive proteins were detected using enhanced chemiluminescence. Blocking experiments were done by preincubating (23 °C; 30 min) the nitrotyrosine antibody with 10 mM 3-nitrotyrosine, a process that completely eliminated antibody binding. Blots were "stripped" of primary antibody by incubation (30 min, 50 °C) in buffer containing 63 mM Tris, pH 6.7, 2% SDS (w/v), and 100 mM 2-mercaptoethanol. Blots were washed extensively in TBS/T followed by reprobing with fresh primary antibody.

Determination of Dityrosine. Protein solutions were analyzed for the presence of dityrosine using a Perkin-Elmer fluorescence spectrometer (LS-50). Aliquots of oxidant-treated or untreated MnSOD were added to solutions of 0.1 M KPi buffer (pH adjusted to 9.0), and the fluorescent emission was scanned from 350 to 500 nm at a fixed excitation wavelength of 325 nm. Dityrosine has a characteristic emission spectrum that is pH-dependent (*25, 26*) and quite distinct from tyrosine. Tyrosine and tyrptophan are the only fluorescent, naturally occurring amino acids but do not fluoresce at these wavelengths. Dityrosine standard was prepared using the HRP-catalyzed oxidation of tyrosine by H_2O_2 (*35*). For HPLC measurement of dityrosine, MnSOD (15 μM) was digested using Proteinase K (10 mg/mL, Calbiochem) for 24 h at 37 °C in 50 mM phosphate buffer, pH 8.0. Protease-treated MnSOD was ultrafiltered (5000 MWCO), and aliquots of the filtrates (5−20 μg) were injected directly into the HPLC system using dual diode-array UV (190−365 nm) and fluorometric detection at E_x = 325 nm, E_m = 410. Peptides were eluted (1 mL/min) from a 15-cm C-18 reverse-phase column using an increasing linear gradient (30 mL) from 5% (w/v) to 80% (w/v) acetonitrile mixed with 50 mM ammonium acetate, pH 5.

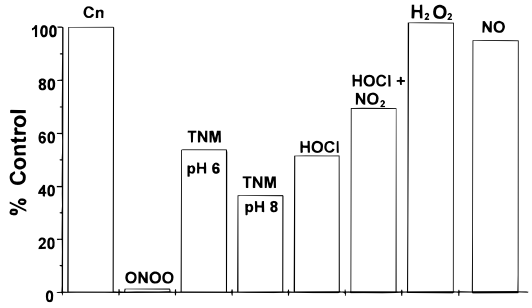

FIGURE 1: Manganese superoxide dismutase activity. Treatment of recombinant MnSOD (15 μM) with ONOO (peroxynitrite cumulative dose of 0.5 mM), TNM (tetranitromethane 0.5 mM) pH 6, TNM (0.5 mM) pH 8, HOCl (hypochlorous acid 1 mM), HOCl (1 mM) with NO_2 (nitrite 1 mM), H_2O_2, pH 7.4 (1 mM), or with authentic NO (1 mM) was assessed for affects on dismutase activity. Assays were performed in triplicate, and each experiment was performed three times. Data are presented as percent of control MnSOD activity (Cn).

RESULTS

Superoxide Dismutase Activity. Exposure of recombinant human MnSOD (15 μM) to a total of 0.5 mM peroxynitrite (using successive additions as in the spectral assay) resulted in complete inactivation (Figure 1). Treatment of MnSOD with TNM (0.5 mM, 2 h, 37 °C) at pH 6.0 resulted in a 46% loss of activity whereas treatment at pH 8.0 inhibited activity by 67% (Figure 1). Treatment of MnSOD with the potent two-electron oxidant HOCl (1 mM, 2 h, 37 °C) resulted in a 49% loss of activity. A combination of HOCl (1 mM) and nitrite (NO_2^-, 1 mM) inhibited MnSOD activity by 32%, an effect somewhat less than HOCl alone. Exposure of MnSOD to authentic NO (1 mM) or H_2O_2 at pH 7.4 (1 mM, 2 h, 37 °C) had no effect on activity.

Nitrotyrosine and MnSOD Immunoblots. MnSOD samples treated with peroxynitrite, NO, HOCl plus nitrite, or HOCl as described above were separated on SDS−PAGE and transferred to nitrocellulose. MnSOD immunoreactivity was confined to a single band in the untreated sample while additional higher molecular mass bands (∼46 and 60 kDa) were apparent in samples treated with peroxynitrite, HOCl,

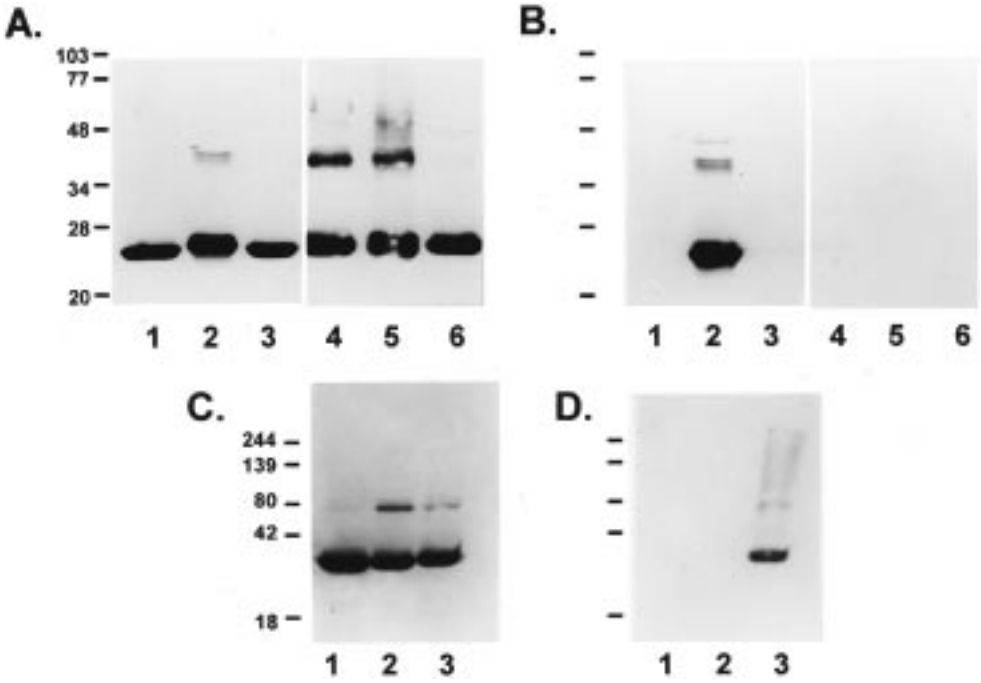

FIGURE 2: Western analysis of recombinant MnSOD following oxidant treatment. MnSOD (10 μg) was treated with ONOO, NO, HOCl with NO_2, HOCl, or H_2O_2 (panels A and B; lanes 2−6, respectively) and either TNM, pH 6, or TNM, pH 8 (panels C and D; lanes 2 and 3, respectively) as described in Figure 1, resolved on a 12% (w/v) reducing SDS−PAGE and examined by immunoblot analysis. Panels A and C represent a MnSOD immunoblot using the polyclonal MnSOD antibody (1:2000). Panels B and D represent a nitrotyrosine immunoblot of the same blot following stripping of the first antibody, using the monoclonal nitrotyrosine antibody (1:1500). Untreated MnSOD served as a positive control (lane 1). Prestained molecular weight markers were used to estimate approximate sizes (kDa) as shown on left.

or HOCl plus NO_2 (Figure 2A). Immunoblotting of MnSOD samples treated with TNM at pH 6 or pH 8 demonstrates that TNM treatment resulted in the formation of a higher molecular mass band predominately at pH 6 but also to a lesser degree at pH 8 (Figure 2C). The blots shown in Figure 2A,C were stripped of antibody and reprobed with the anti-nitrotyrosine antibody. Immunoblotting confirmed the presence of nitrotyrosine only in the peroxynitrite-treated enzyme (Figure 2B, lane 2) and the TNM (pH 8) -treated enzyme (Figure 2D, lane 3). Exposure of MnSOD to authentic NO (1 mM) or H_2O_2 at pH 7.4 (1 mM, 2 h, 37 °C) resulted in no detectable aggregate formation or tyrosine nitration (Figure 2A,B; lanes 3 and 6).

Protein-Bound Dityrosine. The presence of higher molecular mass MnSOD immunoreactive bands following reducing SDS−PAGE both here (Figure 2) and following nitrotyrosine immunoprecipitation from chronically rejecting human kidneys (*1*) strongly suggested that covalent cross-links were being formed during oxidation. Fluorescent spectroscopy of peroxynitrite-treated MnSOD revealed an emission spectrum characteristic of dityrosine (410 nm peak) (*25*, *26*) when excited at 325 nm (Figure 3A), which was not seen in untreated enzyme. The 410-nm emission peak was also seen following treatment with HOCl and, to a lesser extent, with TNM-treated MnSOD at pH 6.0 (not shown) but not with TNM, pH 8.0, or H_2O_2. Oxidant-treated MnSOD samples were proteolyzed with Proteinase K, and the resulting peptides were analyzed by HPLC with dual diode-array UV and fluorescent detection (Figure 3B). The protein-derived fluorescent peaks were slightly more retained than authentic dityrosine, suggesting that additional amino acid residues were present in the fluorescent peptides following protease digestion (Figure 3B, arrow). HPLC analysis of completely hydrolyzed (6 M HCl at 105 °C for 12 h) peroxynitrite-treated MnSOD revealed a fluorescent peak with approximately the same retention as authentic dityrosine (1.8 min vs 1.9 min, Figure 3C). The integrated areas of all 410-nm fluorescent peaks were summed and compared to the total fluorescence of untreated MnSOD (Table 1). The values in Table 1 revealed 26-fold more dityrosine in peroxynitrite-treated MnSOD. Significant amounts of dityrosine were also present in HOCl- and TNM-treated (pH 6.0) enzyme but not in TNM-treated (pH 8.0) or H_2O_2-treated samples.

Peroxynitrite-Induced Modifications of Amino Acids. Cysteine residues were measured as total reduced sulfhydryl content of MnSOD using Ellman's reagent under denaturing conditions (6 M HCl). The total sulfhydryl content of untreated MnSOD (4 μM) was 9.1 μM and did not change significantly following peroxynitrite treatment (8.8 μM). Methionine content was not determined; however, treatment of MnSOD with H_2O_2 at pH 7.4, which is reported to oxidize methionines (*24*), had no effect on MnSOD activity. Lysine content was also unchanged following peroxynitrite treatment as measured using the amine-labeling agent fluorescamine under denaturing conditions (*33*). Amino acid analysis of MnSOD treated with peroxynitrite (1 mM) confirmed that only tyrosine residues were altered. Therefore, peroxynitrite-induced modifications in MnSOD appear to be restricted to tyrosine residues.

Peroxynitrite-Induced Tyrosine Nitration. The nitrotyrosine content of MnSOD as a function of added peroxynitrite was determined using a real-time spectral assay (Figure 4). Bolus additions of peroxynitrite were made sequentially to a stirred solution of 0.36 mg/mL (15 μM subunit) MnSOD. Titration with peroxynitrite resulted in a maximum observed yield of 2.2 nitrotyrosines per enzyme subunit. However, the fitting of titration data to a double exponential association

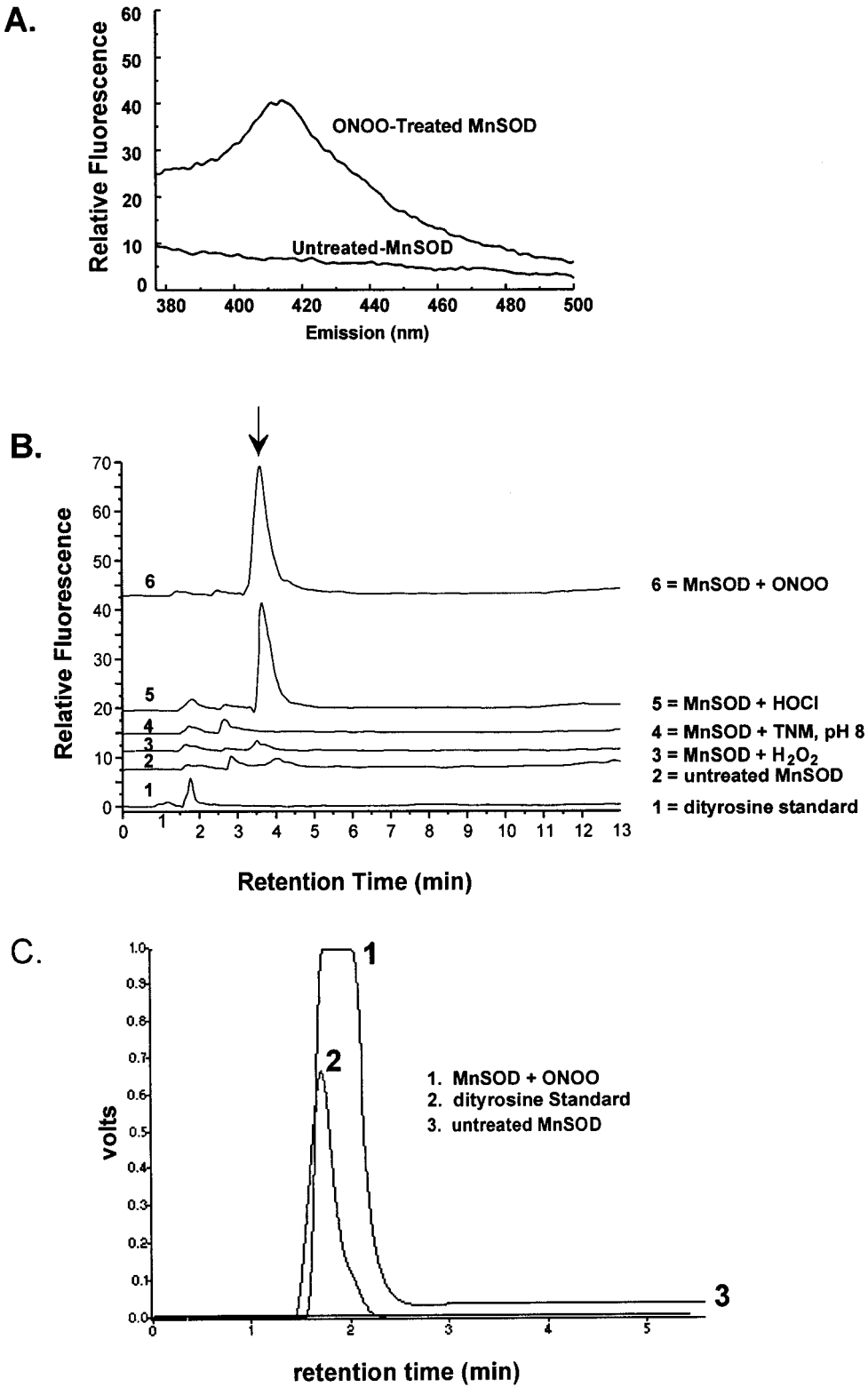

FIGURE 3: Fluorescence spectroscopy and HPLC detection of dityrosine formation in MnSOD. (Panel A) Fluorescence spectroscopy of intact proteins. Aliquots of peroxynitrite (ONOO)-treated or untreated MnSOD were added to solutions of 0.1 M potassium phosphate (pH adjusted to 9.0), and the fluorescent emission was scanned from 350 to 500 nm at a fixed excitation wavelength of 325 nm. Dityrosine has a characteristic emission spectrum at 410 nm. (Panel B) HPLC detection of partially proteolyzed proteins. Oxidant treated (curves 3−6) or untreated MnSOD (0.36 mg/mL; curve 2) samples were digested using proteinase K, and aliquots of the filtrates (5−20 μg) were injected directly into the HPLC system using dual diode-array UV (190−365 nm) and fluorometric detection at E_x = 325 nm, E_m = 410 nm. Peptides were separated by reversed-phase chromatography (see Methods) and compared to a control dityrosine standard (curve 1). Conditions of individual oxidant treatments are as described in Figure 1. The arrow represents the dityrosine-containing peptides. (Panel C) Acid hydrolysis of authentic dityrosine (1 μM), untreated MnSOD (15 μM), and peroxynitrite-treated MnSOD (15 μM). Samples were hydrolyzed with 6 M HCl at 105 °C for 12 h and separated by reverse-phase chromatography using fluorometric detection at E_x = 325 nm, E_m = 410 nm (see Methods).

Table 1: Relative Amounts of Dityrosine following Oxidant Treatment of MnSOD[a]

MnSOD	oxidant concn (mM)	relative dityrosine
untreated		1.0
+ $ONOO^-$	1.0	25.9
+ HOCl	1.0	17.4
+ TNM, pH 6	0.5	5.7
+ TNM, pH 8	0.5	0.7
+ H_2O_2	1.0	0.6

[a] Relative amounts of dityrosine in MnSOD treated with various oxidants as measured using the described HPLC fluorometric detection method. The integrated areas of all fluorescent peaks, as shown in Figure 3B, were summed and compared to the total fluorescence of untreated MnSOD.

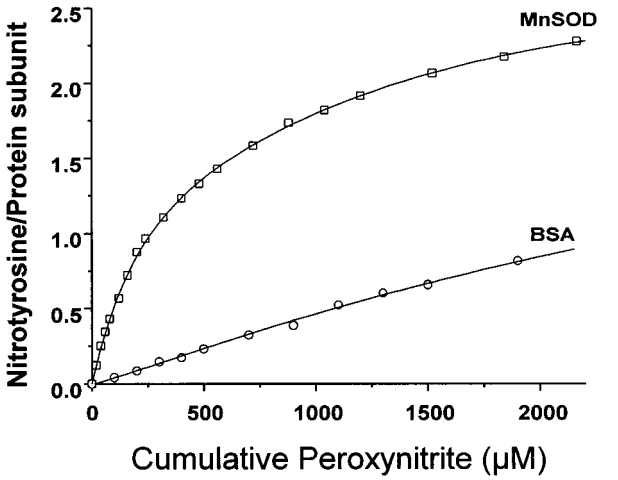

FIGURE 4: Spectral nitrotyrosine assay for nitrotyrosine formation. Peroxynitrite was added sequentially to a stirred protein solution (MnSOD, 0.36 mg/mL, or BSA, 0.52 mg/mL) in 0.1 M potassium phosphate buffer, pH 7.4 at 37 °C. Each point represents a bolus addition of peroxynitrite; complete reaction of peroxynitrite occurred (~30 s) prior the next addition. Nitrotyrosine content of proteins treated with peroxynitrite was measured by absorbance at 430 nm using an extinction coefficient ($E_{430\ nm}$ = 4400 M^{-1} cm^{-1}) determined under these conditions using commercial nitrotyrosine. Data are expressed as nitrotyrosine/protein subunit by dividing total nitrotyrosine content by the molar concentration of either the MnSOD monomer or BSA.

equation extrapolated a maximum yield of 2.6 nitrotyrosines per subunit and revealed an apparent differential susceptibility for tyrosine nitration by peroxynitrite. Specifically, the half-maximal (ED_{50}) concentration of peroxynitrite required for nitration of 0.8 tyrosine per MnSOD subunit was 155 μM peroxynitrite, while 1.2 mM peroxynitrite was required for half-maximal nitration of an additional 1.8 tyrosines. By comparison, nitration of the same molar concentration of bovine serum albumin (BSA) by peroxynitrite was essentially linear (Figure 4) and produced ~0.9 nitrotyrosine residues per mole at 2.2 mM peroxynitrite despite the fact that BSA has twice as many tyrosine residues as MnSOD. MnSOD, first treated with HOCl to induce formation of dityrosine, was still capable of being nitrated to a similar extent by peroxynitrite (not shown), suggesting that tyrosine nitration and dityrosine formation are independent oxidations involving different tyrosine residues.

Identification of Nitrated Residues. Mass spectrometric analysis of proteolyzed fragments recovered from MnSOD (15 μM) treated with peroxynitrite (0.5 mM) revealed that three residues (Tyr45, Tyr193, and Tyr34) were nitrated (Figure 5). The MS/MS spectra of trypsin fragments (parent ions) 822 (control) and 867 (peroxynitrite-treated) are shown in Figure 5A. The right panel of Figure 5 shows the masses and deduced sequence of each peak, including both the b and y ions. The underlined text indicates the masses corresponding to the fragments containing the nitrated tyrosine (mass increased by 45). This sequence matched residues 45−51 of the mature human MnSOD. The spectra from the chymotryptic fragment corresponding to residues 187−193 revealed that Tyr193 was also nitrated (Figure 5B). Peroxynitrite-treated (0.5 mM) MnSOD (42 μM) was cleaved with chymotrypsin followed by HPLC purification (pH 4) of tyrosine-nitrated proteolytic fragments based on the characteristic absorbance at 365 nm. A single predominant peak was observed (365 nm) and purified for subsequent evaluation (Figure 5C, box). MS/MS analysis of this collected nitrated fragment (parent ion 506) corresponded to residues 31−34 in MnSOD, identifying Tyr34 as the most susceptible target for nitration by peroxynitrite (Figure 5C). Interestingly, this ion was an unpredicted chymotryptic fragment resulting from cleavage between two histidine residues (30 and 31). All other tyrosine-containing proteolytic fragments were analyzed, and there was no evidence for any additional nitration.

DISCUSSION

Tyrosine nitration and dityrosine formation were the only amino acid modifications detected following peroxynitrite treatment of recombinant MnSOD, and loss of dismutase activity was correlated with these modifications. Treatments that resulted in formation of tyrosine nitration but not dityrosine (TNM at pH 8) caused only partial inhibition just as treatments that produced dityrosine but no tyrosine nitration (HOCl ± nitrite or TNM at pH 6). H_2O_2 and NO did not modify tyrosines in MnSOD and had no effect on enzyme activity. Peroxynitrite totally inhibited enzyme activity and produced both dityrosine and nitrotyrosine. Thus, partial activity loss can be attributed to nitration of tyrosine residues and to dityrosine formation with the two modifications being additive.

The spectral assay for nitrotyrosine demonstrated that only three of the nine total tyrosine residues in the MnSOD subunit were nitrated even at high cumulative concentrations of peroxynitrite, and the presence of only three nitrated residues was corroborated by mass spectrometry. The peroxynitrite titration data (Figure 4) suggested a differential susceptibility for tyrosine nitration; approximately 1 tyrosine per subunit was nitrated with an ED_{50} of 155 μM peroxynitrite whereas nitration of the additional two tyrosine residues required almost 10-fold higher peroxynitrite concentrations. Previous studies determined that the IC_{50} for inactivation of MnSOD occurred at 10 μM peroxynitrite (*1*), consistent with the notion that enzyme inactivation is correlated with nitration of only one, and most likely the more susceptible, tyrosine residue. The higher ED_{50} seen here is related to the fact that a 30-fold higher concentration of MnSOD was used. Studies with other enzymes have revealed that the amount of peroxynitrite required for inactivation decreases as the target enzyme concentration decreases to more physiological levels (*27*, *36*).

Mass spectroscopy analysis of proteolyzed MnSOD that had been maximally nitrated with peroxynitrite (as in Figure

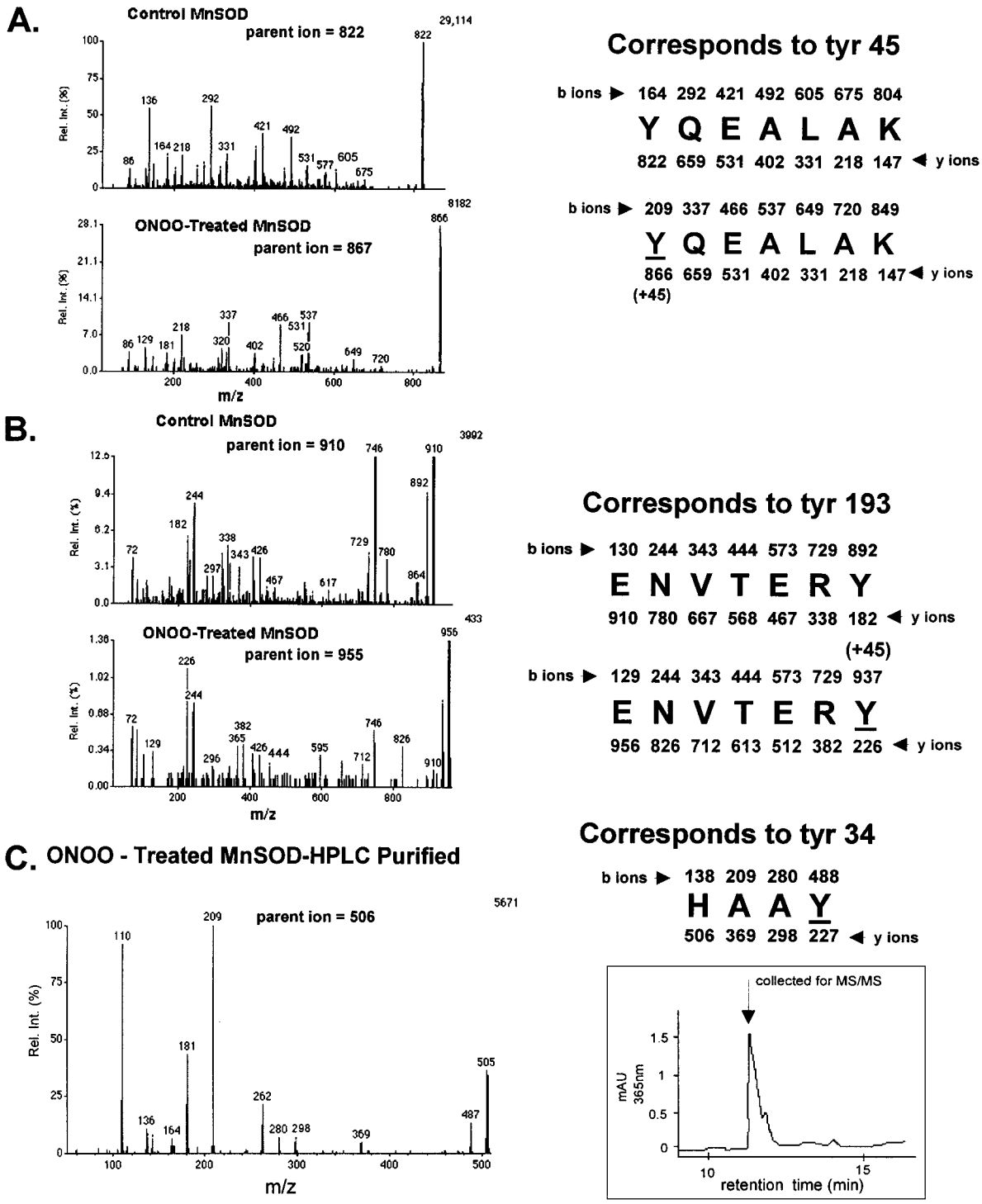

FIGURE 5: Identification of nitrated-tyrosine residues using electrospray mass spectroscopy. Recombinant MnSOD was either untreated (control) or treated with 0.45 mM peroxynitrite (ONOO-treated) and prepared for mass spectroscopy analysis. (Panel A, left) Spectra of selected tryptic fragments were subjected to mass spectroscopy. The top spectra refer to MS/MS of the control MnSOD *m/z* parent ion = 822. The bottom spectra refers to MS/MS of the peroxynitrite-treated MnSOD *m/z* parent ion = 867. (Panel A, right) The sequence of the unknown peptide is readily deduced from the pattern of the mass fragments. The text to the right of ONOO-treated MnSOD refers to the *b*-ions, which have an additional 45 mass units, due to the nitrated tyrosine residue (*m/z* 209). This tyrosine corresponds to Tyr45 in recombinant MnSOD. (Panel B, left) Spectra of selected chymotryptic fragments were subjected to mass spectroscopy. The top spectra refers to MS/MS of the control MnSOD *m/z* parent ion = 910. The bottom spectra refers to MS/MS of the peroxynitrite-treated MnSOD *m/z* parent ion = 955. (Panel B, right) The sequence of the unknown peptide is readily deduced from the pattern of the mass fragments. The text to the right of ONOO-treated MnSOD refers to the *y*-ions which have an additional 45 mass units, due to the nitrated tyrosine residue (*m/z* 227). This tyrosine corresponds to Tyr193 in recombinant MnSOD. (Panel C) Recombinant MnSOD (42 μM) was treated with peroxynitrite (0.5 mM) and cleaved with chymotrypsin. Nitrated peptides were HPLC-purified (pH 4) based on characteristic absorbance at 365 nm (inserted box). (Panel C, left) Spectra of collected nitrated chymotryptic fragment using RP−HPLC (365 nm) subjected to mass spectroscopy. The spectra refer to MS/MS of peroxynitrite-treated MnSOD *m/z* parent ion = 506. (Panel C, right) The sequence of the unknown peptide is readily deduced from the pattern of the mass fragments. This tyrosine corresponds to Tyr34 in recombinant MnSOD.

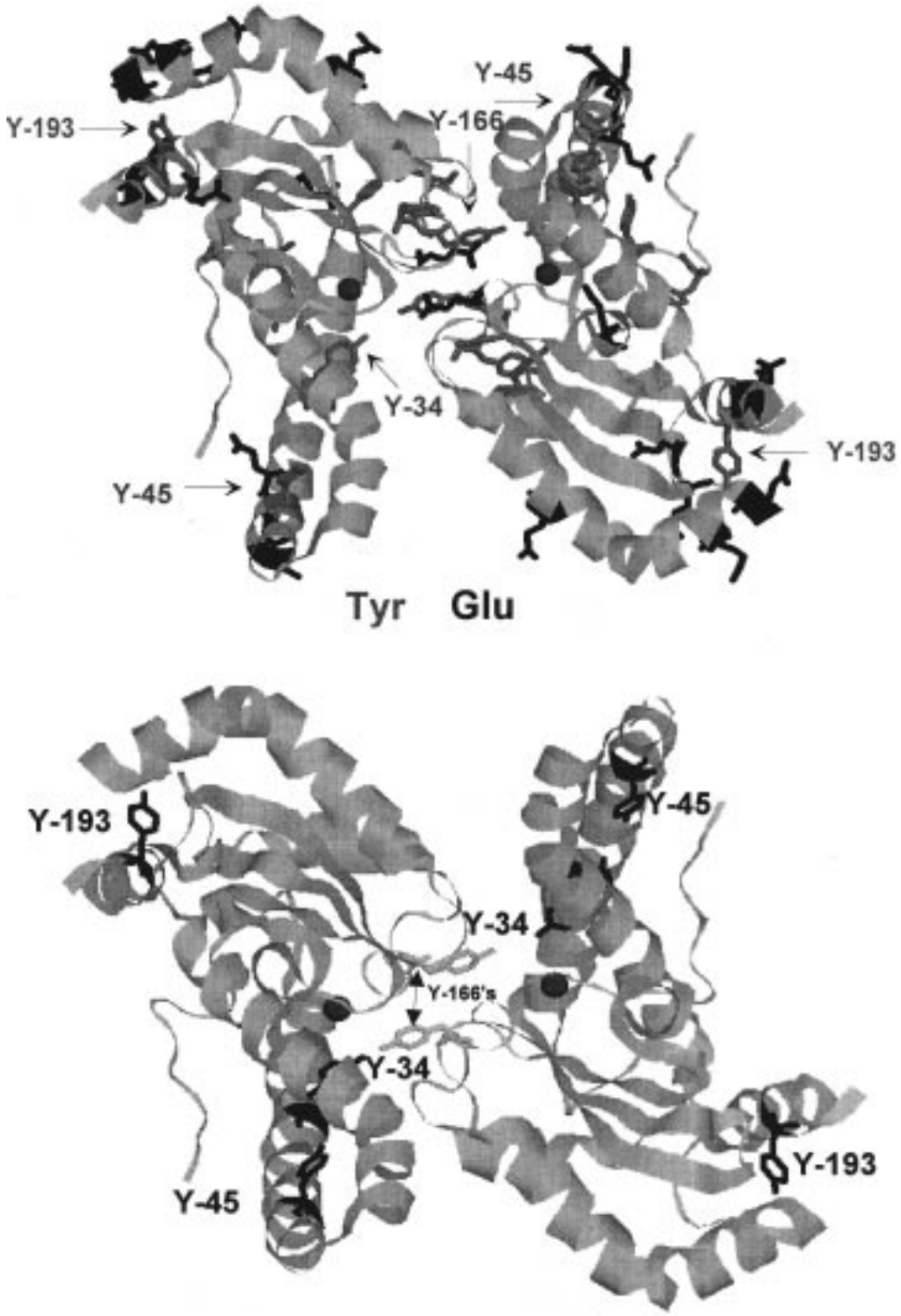

FIGURE 6: Diagram showing the X-ray crystal structure of MnSOD. (Top) Ribbon structure of MnSOD homodimer illustrating all tyrosine (labeled Y, in gray) and glutamate (labeled black) residues. The manganese atom is represented by a black ball. Note that only Y-193 and Y-45 are surrounded by glutamate residues. Native MnSOD is a homotetramer; however, the enzyme crystallized as a homodimer (*11*). (Bottom) Ribbon structure of dimeric MnSOD illustrating the three nitrated tyrosine residues (Y-45, Y-193, and Y-34) of each subunit and the putative dityrosine cross-link at Y-166. Drawings were generated using the Rasmol program.

5) demonstrated that tyrosines 34, 45, and 193 were nitrated. The crystal structure of human MnSOD (*11*) reveals that tyrosines 45 and 193 are nested within virtual clusters of glutamate residues (Figure 6). The apparent ability of glutamate to direct peroxynitrite-mediated nitration to specific tyrosine residues has been seen with other proteins and is the subject of ongoing investigation. Mass spectroscopy studies with the structural protein neurofilament-L have revealed that the four tyrosine residues that are susceptible to nitration by peroxynitrite are located within one or two residues of glutamates (*8*). Tyr34 has no proximal glutamate residues yet was still nitrated. It is possible that the positively charged manganese atom may either alter the ionization state of Tyr34 and thereby make it more susceptible to electrophilic attack or simply attract the negatively charged peroxynitrite to the active site. The ability of cations such as iron and zinc to enhance thiol oxidation by peroxynitrite has been previously reported (*27*, *36*).

Within MnSOD, the only other tyrosine residue with a proximal glutamate is Tyr166. We have no evidence for

nitration of Tyr166; however, the X-ray crystal structure of human MnSOD demonstrates that Tyr166 in one subunit is aligned within a few angstroms of Tyr166 in the other subunit of the homodimer (Figure 6). Thus, Tyr166 seems a likely site for subunit cross-linking via dityrosine formation. In addition, Tyr165 may form a intrachain cross-link with Tyr166 which, in combination with interchain cross-links, may explain the higher molecular mass species observed by electrophoretic analysis (Figure 2).

Oxidants including HOCl and hydroxyl radical generators have been shown to promote the formation of dityrosine (*37*−*39*). Peroxynitrite has also been shown to form dityrosine from free tyrosine in solution (*40*). To our knowledge this is the first demonstration of dityrosine formation in a protein following peroxynitrite treatment. Tetranitromethane may promote dityrosine formation via a mechanism that also involves radical intermediates.

There are a number of potential mechanisms by which tyrosine nitration and dityrosine formation could inhibit enzyme activity, and it is currently not possible to precisely distinguish between them. However, some reasonable predictions can be made from the crystal structure. Because Tyr34 resides within a few angstroms of the active site manganese, this was the first tyrosine examined for nitration. Initially, we detected no nitrated chymotryptic fragments corresponding to Tyr34. However, when the major nitrated chymotryptic fragments were collected using RP−HPLC and subjected to MS/MS, nitrated Tyr34 was identified. MS/MS of the collected fragment also revealed that it was not an expected chymotryptic fragment of MnSOD, since cleavage was between two histidine residues. Atypical proteolytic activity appears to be associated with MnSOD structure rather than a consequence of tyrosine nitration, since (a) peroxynitrite treatment did not alter generation of predictable proteolytic fragments corresponding to Tyr45 and Tyr193 and (b) MS/MS analysis identified the same atypical chymotryptic fragment (corresponding to the nonnitrated fragment) in untreated MnSOD. In any event, the combination of HPLC and MS/MS analysis suggested that the active site tyrosine (Tyr34), ostensibly the most critical for enzyme activity, was indeed nitrated and the most susceptible residue to be modified by peroxynitrite (Figure 5C).

Tyr45 and Tyr193 may also contribute to the inactivation of MnSOD following peroxynitrite treatment. Examination of the crystal structure of MnSOD shows that Tyr166 and Tyr45 lie on opposite sides of the channel traversed by superoxide as it approaches the active site. Covalent linkage of the Tyr166 residues from adjacent subunits could conceivably distort the alignment of the active site channel and thereby position negatively charged residues within the channel, which would repel the superoxide anion; X-ray crystallographic studies of peroxynitrite-treated MnSOD aimed at addressing these issues are under way. On the other hand nitration decreases the pK_a of the tyrosine hydroxyl group from 10 to 7.5 (*41*). Thus, nitration of Tyr45 would introduce a permanent negative charge alongside the active site. Tyr193 is far removed from the active site but is quite close to the negatively charged Glu92 and the positively charged Lys197. Introduction of a negative charge to Tyr193 via nitration could alter protein conformation by interacting with these proximal charged residues. This type of charge attraction/repulsion is analogous to the process whereby tyrosine phosphorylation induces conformational changes and subsequent activity changes in susceptible enzymes. Current studies utilizing site-directed mutations of MnSOD are underway to determine precisely which tyrosine residues are critical for inactivation of MnSOD by peroxynitrite.

Bovine Cu,ZnSOD contains a single tyrosine residue and has been shown to catalyze its own nitration via the redox active copper atom at the active site (*14*). Thus, it seemed reasonable that the redox active manganese atom of MnSOD, which catalyzes the same dismutase reaction as Cu,ZnSOD, might also catalyze nitration. However, we saw no enhancement of nitration of a tyrosine-containing peptide in the presence of MnSOD (not shown). Peroxynitrite could lead to inactivation of MnSOD by oxidation of the manganese atom. However, no EPR signal was detected with either native and peroxynitrite-treated MnSOD, consistent with the presence of catalytically active Mn(III) at the active site (J. Peterson and L. A. MacMillan-Crow, unpublished observations).

HOCl is a potent two-electron oxidant that is also capable of chlorinating tyrosine residues (*21, 23*). We did not directly assess chlorotyrosine content or its potential effect on activity; however, indirect measures suggested that either it was not formed or, if formed, did not significantly alter enzyme activity. For example, HOCl inactivated MnSOD by the same amount as did TNM (pH 6), which cannot chlorinate. Also, HOCl-treated MnSOD was capable of being nitrated to the same extent by peroxynitrite as untreated enzyme. Because nitration and chlorination both occur ortho to the hydroxyl group on the tyrosine ring, the two modifications are almost certainly mutually exclusive.

It has been reported that a combination of HOCl and nitrite forms a nitrating species (*22, 23*). However, in our experiments a sensitive immunologic detection method revealed no nitration by HOCl plus nitrite (Figure 2). Recent studies have shown that a peroxidase is required for efficient nitration in the presence of HOCl and nitrite and that myleoperoxidase (MPO) is by far the most efficient catalyst (Sampson et al., manuscript submitted). A requirement for HOCl (which is produced only by MPO) and MPO itself, which is present only in neutrophils, makes this a viable nitration mechanism in vivo only when neutrophilic infiltration can be demonstrated. Immunohistochemical staining of rejected human kidney revealed virtual overlap of MnSOD and nitrotyrosine immunoreactivity in tissue sections devoid of any inflammatory cells (*1*). Furthermore, it would be extremely unlikely that the highly reactive MPO/HOCl complex would reach the mitochondrial compartment to nitrate MnSOD. Given the constitutive production of superoxide within mitochondria and the ability of uncharged nitric oxide to readily diffuse across membranes, it is more plausible that peroxynitrite is formed directly within the mitochondrion.

We have shown previously that MnSOD isolated from rejecting human kidney homogenates was both nitrated and inactivated (*1*). In addition, higher molecular mass MnSOD immunoreactive bands were seen on blots of proteins immunoprecipitated with nitrotyrosine antibody, suggesting that the higher molecular mass complexes were also nitrated. Covalent binding of two tyrosine residues to yield 3,3′-dityrosine is generally considered to arise via the combination of two tyrosyl radicals—a process that would be facilitated by immobilization of tyrosine residues in close proximity

such as occurs with Tyr166 from each subunit (Figure 6). Mass spectroscopic studies are underway to determine whether Tyr166 is involved in dityrosine formation and, thus, could account for subunit cross-linking and higher molecular mass complexes on reducing gels. However, these in vitro studies indicate that peroxynitrite was the only oxidant that produced both nitrotyrosine and dityrosine in purified Mn-SOD. The similarity of these findings to those seen previously with MnSOD immunoprecipitated from chronically rejecting human kidneys (*1*) provides compelling evidence that MnSOD undergoes concurrent oxidative modifications in vivo under pathological conditions and that oxidation and inactivation is peroxynitrite-dependent.

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