J. Biochem. **138**, 57–69 (2005)
DOI: 10.1093/jb/mvi095

Nitrated and Oxidized Products of a Single Tryptophan Residue in Human Cu,Zn-Superoxide Dismutase Treated with Either Peroxynitrite-Carbon Dioxide or Myeloperoxidase-Hydrogen Peroxide-Nitrite

Fumiyuki Yamakura[1,*], Takashi Matsumoto[2], Keiichi Ikeda[1,3], Hikari Taka[4], Tsutomu Fujimura[4], Kimie Murayama[4], Eiji Watanabe[1], Makoto Tamaki[5], Takeo Imai[6] and Kenji Takamori[3,7]

[1]Department of Chemistry, Juntendo University School of Medicine, Inba, Chiba 270-1695; [2]Department of Food Science and Nutrition, Showa Women's University, Setagaya-ku, Tokyo 154-8533; [3]The Institute for Environmental and Gender-Specific Medicine, Juntendo University Graduate School of Medicine, and [7]Department of Dermatology, Juntendo University Urayasu Hospital, Urayasu, Chiba 279-0021; [4]Division of Proteome and Biomolecular Science, BioMedical Research Center, Juntendo University Graduate School of Medicine, Bunkyo-ku, Tokyo 113-8421; [5]Department of Chemistry, Faculty of Science, Toho University, Miyama, Funabashi, Chiba 274-8510; and [6]Department of Life Science and Graduate School of Life Science, Rikkyo (St. Paul's) University, Toshima-ku, Tokyo 171-8501

Received March 14, 2005; accepted April 20, 2005

We reported previously that a single tryptophan residue, Trp32, in human Cu,Zn-superoxide dismutase is specifically modified by peroxynitrite-CO_2 [Yamakura *et al.* (2001) *Biochim. Biophys. Acta* 1548, 38–46]. In this study, we modified Cu,Zn-superoxide dismutase by using a combination of myeloperoxidase, hydrogen peroxide, and nitrite. The modified enzyme showed no loss of copper and zinc, and 15% less enzymatic activity. Trp32 was the only significant amino acid lost. After trypsin digestion of the modified SOD with peroxynitrite-CO_2 and the myeloperoxidase system, six newly appearing peptides containing tryptophan derivatives were observed on microLC-ESI-Q-TOF mass analyses and HPLC with a photodiode-array detector. The derivatives of the tryptophan residue exhibiting mass increases of 4, 16 (2 peaks), 32, 45 (major), and 45 Da (minor) were identified as kynurenine, oxindole-3-alanine and its derivatives, dihydroxytryptophan, 6-nitrotryptophan and 5-nitrotryptophan, respectively. We further identified 6-nitrotryptophan from the ^{1}H-NMR spectrum for the pronase-digested product and calculated the yield of 6-nitrotryptophan as being about 30% for each of the modification methods. The tryptophan residue in the modified human Cu,Zn-superoxide dismutase gave the same spectra for the products including 6-nitrotryptophan as the major nitrated product with the two different modification systems.

Key words: Cu,Zn-superoxide dismutase, myeloperoxidase, 6-nitrotryptophan, peroxynitrite, tryptophan residue.

Abbreviations, ESI, electrospray ionization; FALS, familial amyotrophic lateral sclerosis; HPLC, high performance liquid chromatography; LC, liquid chromatography; MPO, myeloperoxidase; MS, mass spectrometry; Q-TOF, quadrupole time-of-flight; SOD, superoxide dismutase.

Reactive nitrogen species, such as peroxynitrite and nitrogen dioxide ($^{\bullet}NO_2$), have been implicated as causes of various pathophysiological conditions, including inflammation, neurodegenerative and cardiovascular diseases, and cancer (*1*, *2*). Peroxynitrite has the potential to cause DNA-strand breaks, lipid peroxidation, and oxidation and nitration of nucleotides and proteins (*1*, *2*), or diverse modifications of small biomolecules, such as the oxidation of thiols or methionine, and the oxidation and nitration of tyrosine, serotonin (*3*), or tryptophan (*3–6*). A specific nitration product of tyrosine, 3-nitrotyrosine, has been widely utilized as a biomarker for reactive nitrogen species production in a wide range of human and animal diseases (*1*, *2*). Furthermore, peroxynitrite has been shown to react with a physiological concentration of carbon dioxide to form a peroxynitrite-carbon dioxide adduct (nitrosoperoxycarbonate, $ONOOCO_2^-$), which also exhibits a very high reactivity to nitrate free and protein-bound tyrosine, and this may be the ultimate chemical species *in vivo* (*7*). In general, the nitration reaction is enhanced but the oxidation reaction is diminished by this adduct, compared with the use of peroxynitrite (*8*, *9*). Another nitrating agent that could be involved in biological nitration is $^{\bullet}NO_2$. This species is produced through one-electron oxidation of nitrite that can be accumulated under conditions when NO is overproduced (*10*, *11*). Oxidation of nitrite to $^{\bullet}NO_2$ can be catalyzed by peroxidases in the presence of hydrogen peroxide *via* either a com-

*To whom correspondence should be addressed. Tel: +81-476-98-1001, Fax: +81-476-98-1011, E-mail: yamakura@sakura.juntendo.ac.jp

pound I– or II–catalyzed reaction with nitrite (*12*, *13*) according the following reactions, 1–3:

$$\text{MPO} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I} + \text{H}_2\text{O} \quad (1)$$

$$\text{Compound I} + \text{NO}_2^- \rightarrow \text{Compound II} + {}^\bullet\text{NO}_2 \quad (2)$$

$$\text{Compound II} + \text{NO}_2^- \rightarrow \text{MPO} + {}^\bullet\text{NO}_2 \quad (3)$$

Sampson *et al.* (*14*) have reported that MPO utilizes nitrite and hydrogen peroxide as substrates to catalyze tyrosine nitration in proteins *in vitro* and that it may also do so *in vivo*. Recently, by using MPO gene knockout mouse (*15*), it has been proposed that the formation of 3-nitrotyrosine in extracellular proteins can be ascribed to at least 50% of the MPO system *in vivo*. However, Ichimori *et al.* (*16*) have reported the opposite result, a high nitration product being observed in MPO-deficient leukocytes. Further studies are required to clarify these discrepancies.

Superoxide dismutase [EC 1.15.1.1] (SOD) catalyzes the disproportionation of the superoxide anion to hydrogen peroxide and oxygen. Three types of SOD have been found in human tissues: Cu,Zn-containing SOD, Mn-containing SOD, and extracellular SOD. We have reported that exclusive nitration of Tyr-34 in human mitochondrial Mn-SOD is responsible for inactivation of the enzyme by peroxynitrite (*17*). Ischiropoulos *et al.* (*18*) have reported that Tyr 108 in bovine Cu,Zn-SOD, which has no tryptophan residue, was modified by peroxynitrite without inactivation of its enzymatic activity. On the other hand, human Cu,Zn-SOD has no tyrosine residue and only one tryptophan residue in the entire molecule. Therefore it is an ideal enzyme for analyzing the reaction of reactive nitrogen species with tryptophan residue in proteins. In addition, this enzyme is critical for antioxidant processes in human cells. We have reported that human Cu,Zn-SOD reacted with peroxynitrite in the presence of sodium bicarbonate, resulting in the loss of the single tryptophan residue and 30% reduced enzyme activity (*19*). Recently, Alvarez *et al.* (*20*) also reported that human Cu,Zn-SOD was inactivated by peroxynitrite. We suggested the formation of 6-nitrotryptophan from the difference between the spectra of the modified enzyme and the control enzyme (*19*). Herold *et al.* (*21*) have reported that the formation of 4-, 5-, and 6-nitrotryptophan was observed on the addition of peroxynitrite-CO_2 to apo-, oxy-, and met myoglobin, but that only 5- and 6-nitrotryptophan were formed in oxy- and methemoglobin. Furthermore, Suzuki *et al.* (*22*) have also reported the formation of 6-nitrotryptophan in bovine serum albumin on the addition of myeloperoxidase, NO_2^-, Cl^-, and H_2O_2. However, no study on the entire products of the reactions has been reported. It is very important to know all the reaction products of a tryptophan residue reacted with each of the peroxynitrite-CO_2 and MPO-H_2O_2-NO_2^- systems in order to evaluate the pathophysiological and physiological significance of the tryptophan residue modification by reactive nitrogen species.

In this study, we analyzed the reaction products of a tryptophan residue with peroxynitrite-CO_2 adducts or a combination of MPO-H_2O_2-NO_2^-, and obtained almost the same spectra of the products including 6-nitrotryptophan as the major nitrated product with other nitrated and oxidized products. This is the first detailed analysis of the total products of a single tryptophan residue reacted with the peroxynitrite-CO_2 and MPO-H_2O_2-NO_2^- systems.

MATERIALS AND METHODS

Materials—Peroxynitrite, synthesized through the reaction of sodium nitrite and hydrogen peroxide, was purchased from Dojindo Laboratory Co. (Kumamoto, Japan). Excess hydrogen peroxide in this reagent had been removed with MnO_2. The concentration of peroxynitrite was determined using a molar extinction coefficient of 1670 M^{-1} cm^{-1} at 302 nm in 0.1 M NaOH. Human recombinant Cu,Zn-SOD was cloned, expressed in *Escherichia coli*, and highly purified (*23*) by Nippon Kayaku Co. (Tokyo, Japan), and generously donated by them. We further purified the enzyme for the modification study as described previously (*19*). 6-Nitrotryptophan was prepared according to the procedure of Moriya *et al.* (*24*). MPO from human polymorphonuclear leukocytes was purchased from Calbiochem Co., CA, USA. All other reagents were of the highest possible quality and obtained from commercial sources.

Modification of Cu,Zn-SOD with the Peroxynitrite-CO_2 or MPO-H_2O_2-NO_2^- System—The reaction of peroxynitrite (1.9 mM, final conc.) with Cu,Zn-SOD (1 mg/ml) was carried out by the successive addition of 0.2 mM peroxynitrite at 30 s intervals at pH 7.4 in the presence of 25 mM sodium bicarbonate at 37°C as described previously (*19*). As a control experiment, the same amount of peroxynitrite was added to the reaction mixture 1 min before the addition of Cu,Zn-SOD. The reaction mixtures were dialyzed against 1 liter of 10 mM Tris-HCl buffer, pH 7.8, at 4°C overnight. The reaction of Cu,Zn-SOD with the combination of MPO, hydrogen peroxide, and sodium nitrite was carried out as follows. Cu,Zn-SOD (1 mg/ml) was treated with 0.3 mM hydrogen peroxide, 0.6 mM sodium nitrite, and 0.4 μM MPO in 1.0 ml of 50 mM potassium phosphate buffer (pH 7.2), containing 0.1 mM diethylenetriamine pentaacetic acid. Two reaction mixtures without MPO (control [1]) and without sodium nitrite (control [2]), respectively, were used as controls. The reaction was started by the addition of a concentrated H_2O_2 solution. The reaction mixtures were incubated at 25°C for 10 min, and then an additional 0.3 mM hydrogen peroxide was added after 10 min and 20 min incubation of the reaction mixture. In order to stop the reaction, 10 μg of catalase was added after 25 min. Each reaction mixture was concentrated to 0.1 ml by ultra-filtration (Microcon YM-10; Millipore, MA, USA). The modified Cu,Zn-SOD was separated from MPO, catalase, and the remaining nitrite and hydrogen peroxide by using a Superdex 75 HR column (10 × 300 mm; Amersham Bioscience, NJ, USA). The SOD was eluted after small peaks of MPO and catalase in this step. For determination of the pH-dependency of the tryptophan modification by fluorescence measurement, essentially the same concentrations of each component of the reaction mixture was used, except that the total volume of the mixture was 0.1 ml and the reaction was stopped after 10 min incubation by the addition of 2 μg of catalase.

Identification of the Tryptic Peptides Containing Trp Derivatives for the Modified SODs Using a MicroLC-Q-TOF Mass Spectrometer and an HPLC-Photodiode Array Detector—The modified enzymes (1 mg) were dialyzed against ultra-pure water (Milli Q) and concentrated by freeze-drying. The freeze-dried samples were dissolved in 0.8 ml of nitrogen gas-purged 0.5 M Tris-HCl buffer, pH 8.5, containing 7 M guanidine hydrochloride and 10 mM EDTA for denaturation, reduced with 10 mg dithiothreitol for 30 min at 55°C, and then reacted with 25 mg of monoiodoacetamide for 30 min in the dark at 4°C. The protein solution was dialyzed extensively against Milli Q water at 4°C and then freeze-dried. A sample was digested with trypsin (sequence grade; Promega, WI, USA; substrate:enzyme = 65:1, mol:mol) in 50 mM ammonium bicarbonate (pH 8.0) at 37°C for 12 h. After freeze-drying of the sample, the tryptic peptides were analyzed with an ABI QSTAR Pulsar hybrid mass spectrometer system (Applied Biosystems, CA, USA), which consisted of an electrospray and a high performance quadrupole time-of-flight (ESI-Q-TOF) combined with a micro liquid chromatograph (Magic 2002; Michrom Bioresources Inc., CA, USA). The peptides were separated on a Magic C18 column (0.2 mmID × 50 mm). The solvent systems consisted of (A) 0.1% formic acid and (B) 0.1% formic acid/90% acetonitrile. The initial step program comprised 5 and 3% B in the $ONOO^-$-modified and MPO system modified samples, respectively, for 5 min, a gradient of 2.1%/min for 45 min, and 100% for 5 min. The flow rate was 2.5 μl/min. The conditions for MS analysis were as follows: ion spray voltage, 3.8 kV; voltage for electron multiplier, 2,200 V; and curtain gas nitrogen, 10 psi. The conditions for MS/MS analysis were as follows: collision energy 15–55 electron voltage; and nitrogen as the collision gas. Isolation and characterization of the tryptic peptides containing the Trp derivatives were carried out using a reverse-phase HPLC (Hitachi L-6200, Tokyo, Japan) equipped with a photodiode-array detector (Waters 990J, MA, USA). The column was a C8 (4.6 × 250 mm; Shiseido Capcell Pak, Tokyo, Japan), the same gradient system as that mentioned above being used. The flow rate was 0.5 ml/min.

NMR Spectra for 6-Nitrotryptophan—For measurement of NMR spectra of nitrated tryptophan, a modified SOD was dialyzed against 0.1 M ammonium bicarbonate and then extensively digested with pronase (Roche, Switzerland; substrate:enzyme =100:1, wt:wt) for 16 h at 37°C. The digested products were analyzed by reverse-phase HPLC as described above. The yellow colored fractions eluted at 21 min, which showed the same absorption spectrum as that of authentic 6-nitrotryptophan, were collected and analyzed as to the NMR spectrum. ^1H NMR spectra were recorded with a JEOL ECP-400 spectrometer (Tokyo, Japan) operating at 399.78 MHz Larmor frequency at 298 K. The solvent for authentic 6-nitrotryptophan and the pronase-digested sample was exchanged with a 30 mM NaOD solution, which was prepared by exchanging of 30 mM NaOH/H_2O to NaOD/D_2O by three times addition of the same volume of D_2O, with freeze-drying each time. Six hundred microliters of an authentic 6-nitrotryptophan solution (1.5 mM, pH 12.5) was measured using a standard 5 mm tube. 200 μl of the pronase-digested sample (pH 11.1) was measured by using a micro cell (Shigemi BMS-005, Tokyo, Japan). All proton chemical shift values were referenced to 3-(tri-methylsilyl)-propionic-acid-d_4 sodium salt.

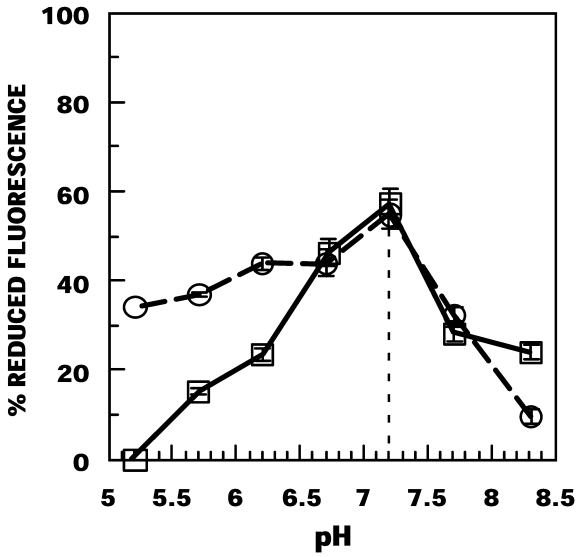

Fig. 1. **pH-dependent disappearance of the fluorescence at 350 nm of human Cu,Zn-SOD after 10 min incubation in a reaction mixture containing MPO, nitrite, and hydrogen peroxide.** The reaction mixture comprised 31.6 μM Cu,Zn-SOD in 0.1 ml of the reaction mixture described in the text. After 10 min incubation, the reaction mixture was diluted by the addition of 0.9 ml of 10 mM Tris-HCl buffer, pH 7.2. The excitation wavelength was 278 nm. circles, with 25 mM $NaHCO_3$; squares, without $NaHCO_3$. Error bars represent ± SD for three determinations.

Analytical Methods—Superoxide dismutase activity was measured as the inhibition of cytochrome *c* reduction by xanthine oxidase at pH 7.8, with reduction of the final volume of the assay system from 3 to 0.75 ml (*25*). The absorption spectra and fluorescence spectra of the enzyme were obtained with a Hitachi U-3000 recording spectrophotometer and a Shimadzu RF-1500 spectrofluorophotometer (Kyoto, Japan), respectively. Metal contents were determined by atomic absorption spectrometry with a Hitachi Z-8100 Polarized Zeeman Flame/Furnace Atomic Absorption Spectorophotometer. The protein concentrations of peroxynitrite-modified Cu,Zn-SOD were estimated using a molar absorption coefficient at 265 nm of 19,880 M^{-1} cm^{-1} as described in our previous paper (*19*). The protein concentration of the modified SOD with the MPO system was estimated using bicinchonic acid (Pierce Co., Il, USA) with bovine serum albumin as a standard (*26*). Amino acid analyses were performed with a Hitachi L-8500 amino acid analyzer. The enzyme modified with the MPO system was applied to a C18 column (Shiseido Capcell Pak, 4.6 × 250 mm) and eluted with a linear gradient from 10% acetonitrile in 0.1% TFA to 60% acetonitrile in 0.1% TFA. After freeze-drying of the enzyme samples, air in the samples was extensively evacuated with a rotary pump. The samples were hydrolyzed with 4 M methane sulfonic acid containing 0.2% tryptamine for 10, 20, 30 min for the control [2] sample and 10, 30 min for the modified sample, respectively, at 160°C. Hen egg-white lysozyme was used as a

Table 1. **Metal contents and specific activities of the control and modified Cu,Zn-SODs with the MPO system.**

Sample	Metal contents (mol/mol of subunit)		Specific activity (units/mg protein/mol of Cu/mol of subunit)
	Cu	Zn	
Control [1] SOD	0.93 ± 0.02	1.18 ± 0.013	3,890 ± 280[a]
Control [2] SOD	0.95 ± 0.01	1.20 ± 0.006	3,750 ± 210[b]
MPO-modified SOD	1.00 ± 0.02	1.15 ± 0.096	3,290 ± 290[c]

[a,b,c]Differences are statistically significant ($p \leq 0.01$) between a and c, and b and c, but not significant between a and b.

standard protein to correct the yields of tryptophan and cysteine + cystine with this method.

RESULTS

pH-Dependency of the Modification of Cu,Zn-SOD with the MPO-System—We have observed that a single tryptophan residue in human Cu,Zn-SOD was modified by peroxynitrite in the presence of sodium bicarbonate (*19*). In this study, we examined the reaction of human Cu,Zn-SOD with another major nitration system, *i.e.*, a combination of MPO, hydrogen peroxide, and nitrite. In order to determine the pH dependency of the reaction, Cu,Zn-SOD was reacted with the combination of MPO (0.2 μM), hydrogen peroxide (0.2 mM), and sodium nitrite (0.2 mM) in the presence or absence of 25 mM sodium bicarbonate. Modification of the tryptophan residue in the enzyme was monitored as the disappearance of the fluorescence emission maximum at 350 nm with excitation at 276 nm (*19*). As shown in Fig. 1, the optimum pH of the fluorescence decrease of the enzyme was 7.2, the decreased percentage being the same regardless of the presence or absence of sodium bicarbonate. However, the fluorescence decrease percentage was higher in the acidic region with sodium bicarbonate. According to this finding, we chose pH 7.2 and no addition of sodium bicarbonate.

Specific Activity, Metal Contents, and Amino Acid Analysis of the MPO System–Modified Enzyme—Table 1 shows the specific activity and metal contents of the MPO system–modified enzyme and the control enzymes. The values represent the averages of two different experiments. The zinc content of the modified enzyme was the same as those of the control enzymes. Although the copper contents seem rather higher in the control enzymes than that in the modified enzyme (Table 1), the difference is not significant. The specific activity of the MPO-modified enzymes, which is based on the copper content, showed 15 and 12% decreases compared with that of the control [1] (-MPO) and [2] (-nitrite), respectively. Although the decrease in the enzymatic activity was not pronounced, it was statistically significant. Table 2 shows the amino acid compositions of the MPO system–modified and control [2] enzymes. Essentially the same results as those of control [2] enzyme were obtained using control [1] enzyme. A decrease of 0.91 moles of tryptophan per mole of subunit was observed for the modified enzyme. No statistically significant differences in the other amino acid compositions, except the serine contents, were observed between the modified and control enzymes.

Table 2. **Amino acid compositions of the control and MPO-modified Cu,Zn-superoxide dismutases.**[a]

Amino acid	Control [2] SOD (residues/subunit)	Modified-SOD (residues/subunit)	Difference (residues/subunit)
Asx	18.20 ± 0.20 (18)[b]	18.01 ± 0.06	–0.19
Thr[c]	7.65 (8)	7.40	–0.25
Ser[c]	9.99 (10)	9.09	–0.90
Glx	12.80 ± 0.08 (13)	12.63 ± 0.02	–0.17
Pro	4.70 ± 0.02 (5)	4.66 ± 0.01	–0.04
Gly	24.80 ± 0.29 (25)	24.51 ± 0.13	–0.29
Ala	10.20 ± 0.18 (10)	10.15 ± 0.01	–0.05
Val[d]	13.12 (14)	13.01	–0.11
Cys[e]	3.61 ± 0.19 (4)	3.41 ± 0.01	–0.20
Met	0 (0)	0	–
Ile[d]	7.12 (9)	7.14	0.02
Leu	9 (9)	9	–
Tyr	0 (0)	0	–
Phe	3.85 ± 0.02 (4)	3.92 ± 0.21	0.07
Lys	10.94 ± 0.05 (11)	10.86 ± 0.09	–0.08
His	7.70 ± 0.36 (8)	7.71 ± 0.29	0.01
Arg	4.29 ± 0.22 (4)	4.18 ± 0.11	–0.11
Trp	0.96 ± 0.17 (1)	0.05 ± 0.002	–0.91[f]

[a]The number of residues was calculated from the number of leucines, assumed to be 9, and on the basis of the subunit molecular weight of 15,804. Values are given as means of 10, 20, and 30 min analyses for the control [2] SOD, and 10 and 30 min analyses for the modified SOD ± SD at 160°C. [b]The values in parentheses were calculated from the total amino acid sequence. [c]Based on extrapolation to zero time of hydrolysis. [d]The values at 30 min of hydrolysis. [e]Combined amount of cysteine and cystine. [f]Difference is statistically significant ($p < 0.05$).

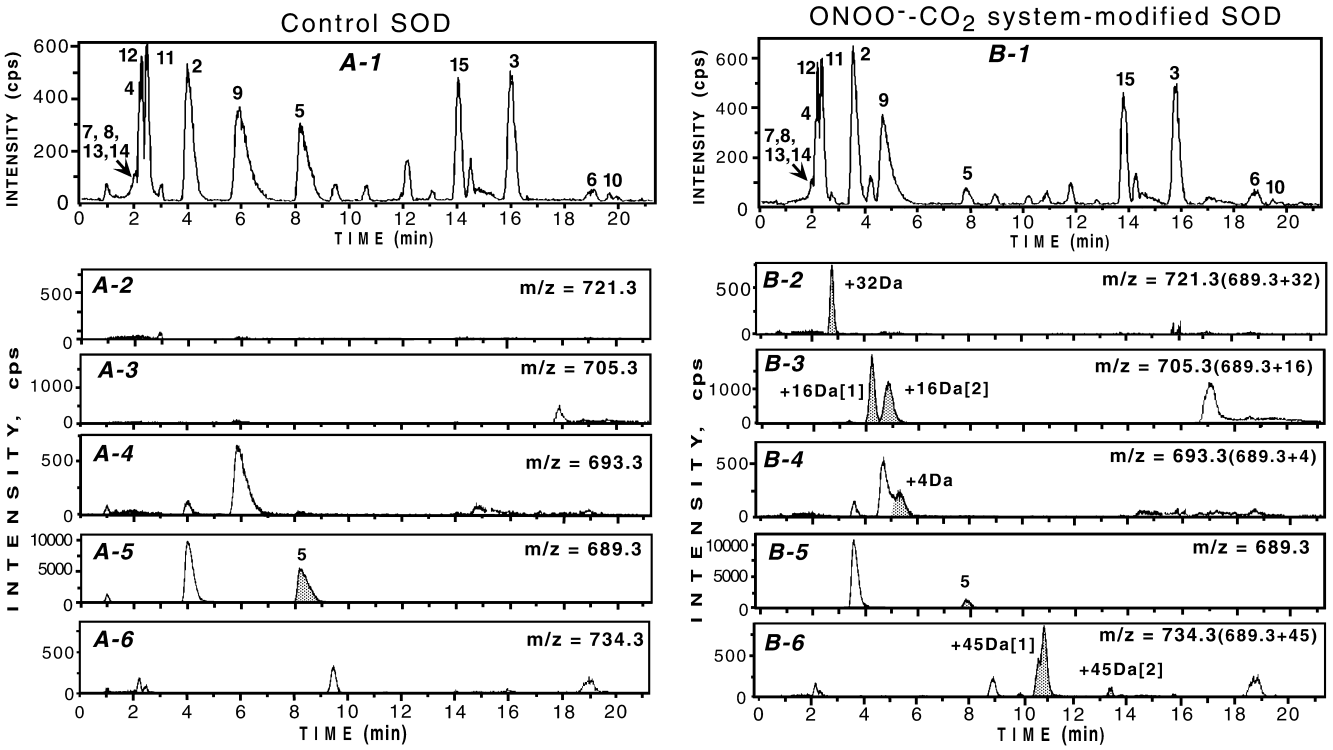

Fig. 2. **Identification of tryptic peptides of the peroxynitrite-CO_2–modified Cu,Zn-SOD by microLC-ESI-Q-TOF mass analysis.** A-1–A-6 represent the control enzyme, which was obtained as shown in the text. B-1–B-6 represent the modified enzyme. A-1 and B-1, total ion chromatograms. The peaks indicated by numbers correspond to the expected peptides on trypsin digestion of human Cu,Zn-SOD, as shown in Table 3. Peak 5 is a single tryptophan-containing peptide, Val31-K36 (A-5 and B-5). A-2–A-6 and B-2–B-6, selected ion monitoring chromatograms of the m/z values of Trp and the modified peptides. These are aligned with the sequence of the elution times of newly appearing peaks. In addition to peak 5, newly appearing peaks are shown in gray. The gray peaks in B-2, B-3, B-4, and B-6 were identified as peptides with increases in mass of 32 (B-2), 16 (2 peaks) (B-3), 4 (B-4) 45 Da (2 peaks) (B-6), respectively.

Identification of the Tryptic Peptides of the Control and Modified SODs Using a microLC-ESI-Q-TOF Mass Spectrometer—A single tryptophan residue in human Cu,Zn-SOD is the residue only significantly modified by the reactions with the MPO system, as shown in this study, and with the peroxynitrite-CO_2 system, as shown in the previous study (*19*). In order to identify the derivatives of the tryptophan residue, the control and modified enzymes with the two systems were digested with trypsin and then analyzed by microLC-ESI-Q-TOF mass spectrometry. Figure 2, A-1 and B-1 shows typical mass chromatograms for the tryptic peptides of the control and peroxynitrite-CO_2–modified SODs. Fifteen peptides are theoretically produced on complete trypsin digestion, as shown in Table 3. Most of the peptides (97.4%) can be seen in Fig. 2, A-1 and B-1. The N-terminal tri-peptide and inner Lys residue position 70 are missing (Table 3 and Fig. 2, A-1 and B-1). The same result as in A-1 was observed for the purified (untreated) Cu,Zn-SOD (data not shown). Peak 5 was attributed to a peptide consisting of Val31 to Lys36 (m/z ([M+H]$^+$) = 689.3), which includes Trp32. This peak decrease drastically in the modified SOD (Fig. 2, A-1 and B-1). The amino acid sequences of the peptides were confirmed by MS/MS analysis (data not shown). Figure 2, A-2–A-6 and B-2–B-6, shows selected ion monitoring (SIM) chromatograms with the

Table 3. **Expected fragments of unmodified Cu,Zn-SOD on trypsin digestion.**[a] All cysteine residues were modified with iodoacetamide to form carbamiomethyl-cysteine.

Fragments	Sequence	Mass	Fragments	Sequence	Mass
(1)	1–3	318.2	(2)	4–9	688.4
(3)	10–23	1,500.8	(4)	24–30	729.4
(5)[b]	31–36	688.4	(6)	37–69	3,518.6
(7)	71–75	494.3	(8)	76–79	547.2
(9)	80–91	1,224.6	(10)	92–115	2,504.2
(11)	116–122	824.5	(12)	123–128	617.3
(13)	129–136	820.4	(14)	137–143	661.3
(15)	144–153	1,000.6			

[a]Lys70 is expected to be released as a free amino acid. [b]Tryptophan residue is included in this fragment (VWGSIK).

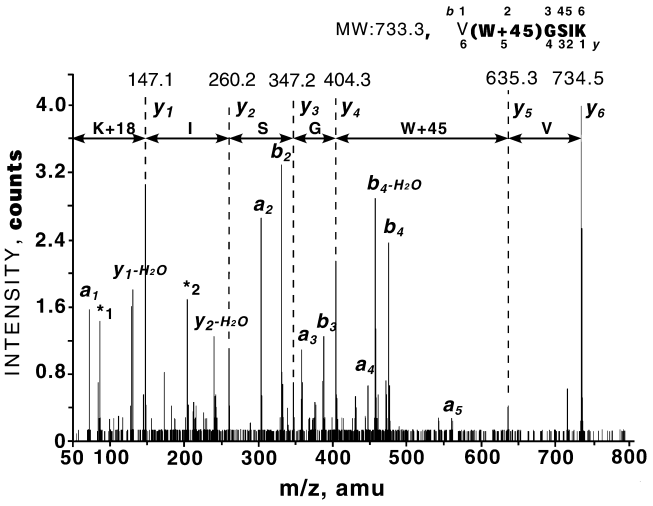

Fig. 3. **Tandem mass spectrum of the *m/z* = 734.3 fraction of the peroxynitrite-CO_2–modified Cu,Zn-SOD, which was eluted at 11 min from a C18 column.** The sequence of the tryptic peptide, Val31–Lys36, with an increase in mass of 45 Da as to Trp32. The y, b, and a ion nomenclature conforms to that proposed by Biemann (*46*). *1 and *2 represent imonium ions of glycine and tryptophan, respectively. Masses of the y-series ions of predicted fragment are shown. y_5 and all observed b ions exhibit a +45 mass shift in the MS/MS spectrum, which confirms Trp 32 is the site of nitration.

m/z values of Trp and the modified peptides, which newly appeared in the mass chromatogram of the modified SOD. We found six newly appearing peptides; B-2, *m/z* 721.3 (Trp+32 Da); B-3, *m/z* 705.3 (Trp+16 Da) (2 peaks, [1] and [2]); B-4, *m/z* 693.3 (Trp+4 Da); B-6, *m/z* 734.3 (Trp+45 Da) (2 peaks, [1] and [2]). They were only observed for the modified SOD (B-2, 3, 4, and 6). The peptides that were found in both A-4 and B-4, and A-5 and B-5 in Fig. 2, having retention times of around 5–6 min and 4 min, are not specific for the modified enzyme and can be attributed to doubly charged peptide 9 and peptide 2 (Table 3), respectively.

In order to confirm the modified amino acids in these modified peptides, MS/MS analyses of each peptide were performed. Figure 3 shows the results of MS/MS chromatography of the major peak [1] of the Trp+45 Da peptide (Fig. 2, B-6, 45 Da [1]). Y_5 and all b ions, except b_5, which was not observed in this experiment, exhibit a +45 mass shift in the MS/MS spectrum. This evidence confirms that Trp 32 is the site of nitration. The sequence of the other amino acids of the peptides was consistent with that of the tryptophan-containing peptide. We obtained the same MS/MS results for the other newly appearing peptides, with molecular weight increases of 4, 32, 16, 16, and 45 Da for the tryptophan residue, respectively (data not shown).

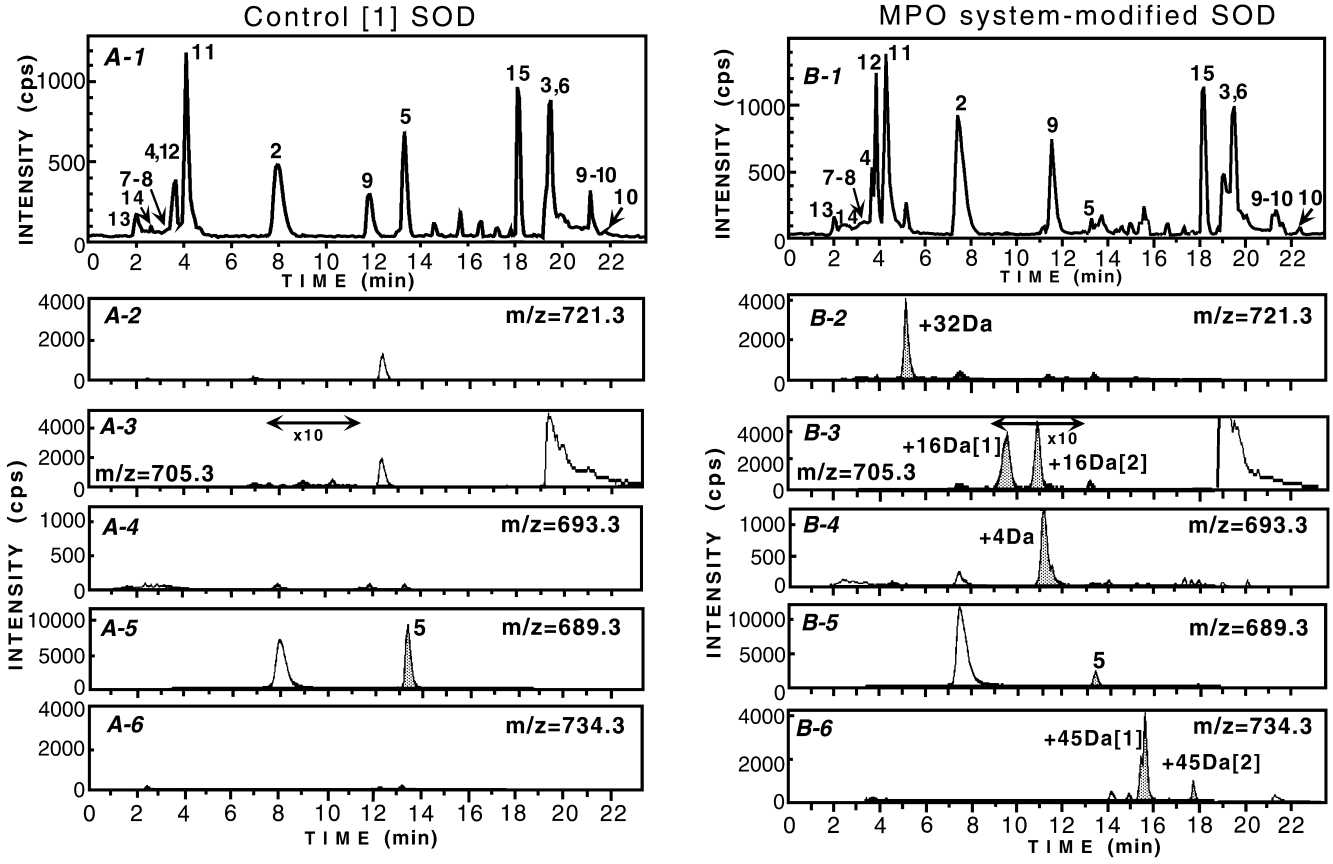

Fig. 4. **Identification of tryptic peptides of the MPO/H_2O_2/NO_2^- system-modified Cu,Zn-SOD by microLC-ESI-Q-TOF mass analysis.** A-1–A-6 represent the control [1] enzyme, which was prepared as shown in the text. B-1–B-6 represent the modified enzyme. The peaks indicated by numbers correspond to the expected peptides on trypsin digestion of human Cu,Zn-SOD, as shown in Table 3 (A-1 and B-1). The newly appearing peaks are shown in gray and were identified as peptides with increases in mass of 32 (B-2), 16 (2 peaks) (B-3), 4 (B-4) 45 Da (2 peaks) (B-6), respectively. Intensities are magnified 10 times within the area indicated by arrows in A-3 and B-3.

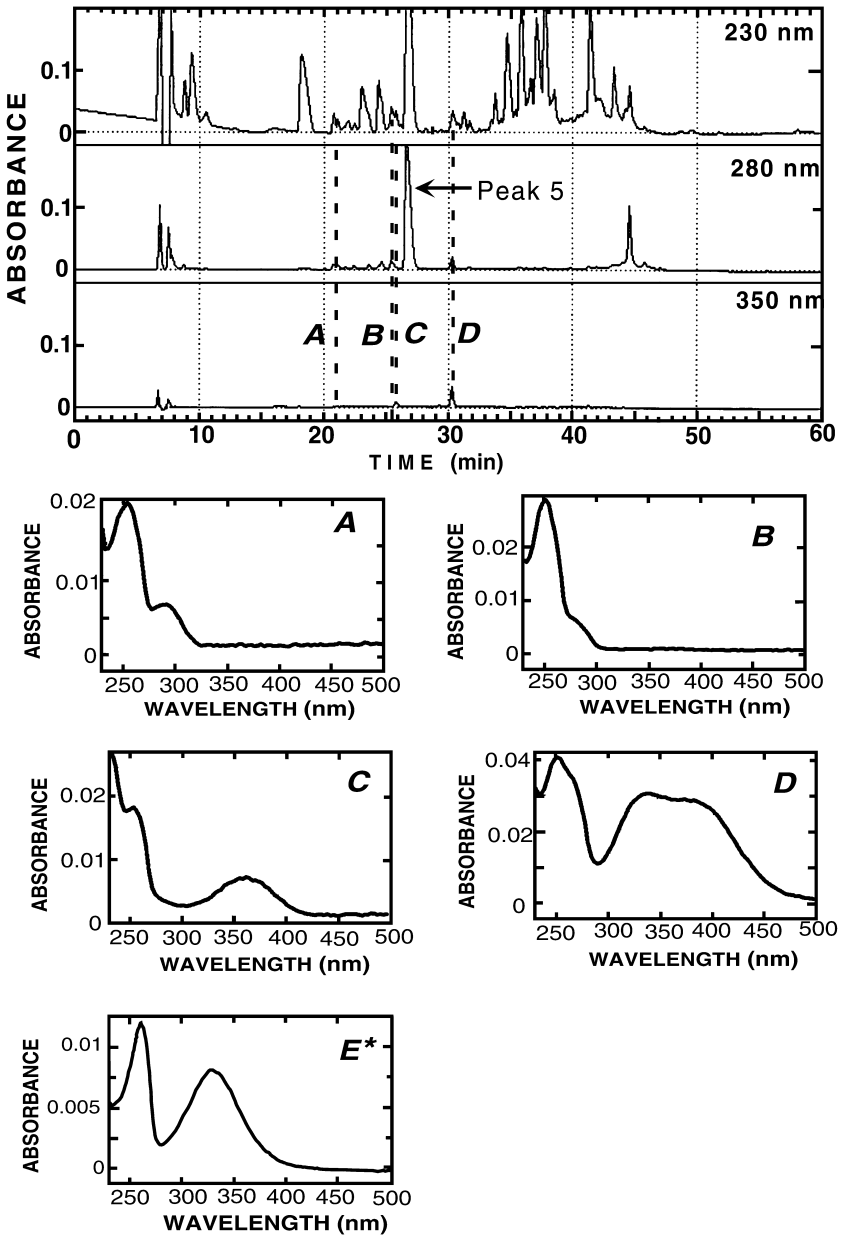

Fig. 5. **HPLC chromatograms of the products of Cu,Zn-SOD reacted with peroxynitrite-CO_2 after digestion with trypsin.** Chromatograms: upper, measured at 230 nm; middle, measured at 280 nm; lower, measured at 350 nm. A–D: Absorption spectra of the peaks exhibiting absorption over 250 nm, which were eluted from the C 8 column at 21.1 min (A), 25.4 min (B), 25.7 min (C), and 30.3 min (D). Peak 5 represents the tryptophan-containing fragment, which may correspond to peak 5 on LC/MS analysis. Absorption spectrum (E*) was obtained for a peak at 34 min from the C 8 column with the other preparation.

Figure 4 shows the total ion chromatograms for the tryptic peptides of the control [1] (-MPO) and modified Cu,Zn-SOD with the MPO-H_2O_2-NO_2^- system. The same result as in A-1 was obtained for the purified (untreated) Cu,Zn-SOD (data not shown). Although the intensities of the tryptic peptides derived from the control and modified SODs were almost the same, peak 5 containing the single tryptophan residue was markedly decreased for the modified SOD (Fig. 4, A-1 and B-1). Figure 4, A2–A6 and B2–B6, shows selected ion monitoring (SIM) chromatogram with the m/z values of Trp and the modified peptides, as in Fig. 2. The same six peaks, which had the same relative elution positions and m/z values corresponding to the tryptic peptides derived from the peroxynitrite-CO_2–modified SOD, were also observed for the SOD modified with the MPO system. MS/MS analysis of these peptides showed that the increased molecular weights were associated with tryptophan residues in each of the peptides (data not shown).

The increase in mass of 32 Da corresponds to the addition of two oxygen atoms, therefore either *N*-formylkynurenine or dihydroxytryptophan is a possible product of the modifications with the peroxynitrite-CO_2 and MPO-H_2O_2-NO_2^- systems. The increase in mass of 4 Da corresponds to kynurenine as a possible product of tryptophan. The increased mass of 16 Da corresponds to the addition of one oxygen atom, therefore a hydroxytryptophan (including oxindole-3-alanine) is a possible product of the modification. The modified tryptophan residue exhibiting the increase in mass of 45 Da corresponds to substitution of a single nitro group.

HPLC Separation and Photodiode-Array Detection of the Tryptic Peptides—In order to further identify the modified products of the tryptophan residues in the tryptic peptides with both the modification systems, we sepa-

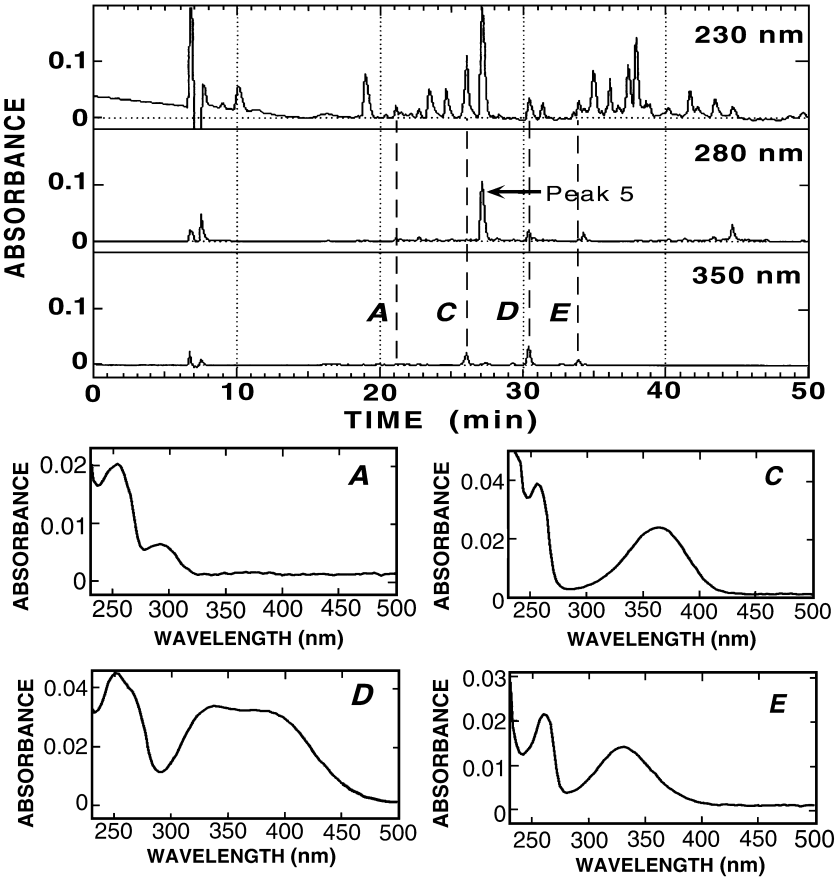

Fig. 6. **HPLC chromatograms of the products of Cu,Zn-SOD reacted with the MPO system after digestion with trypsin.** Chromatograms: upper, measured at 230 nm; middle, measured at 280 nm; lower, measured at 350 nm. A–E: Absorption spectra of the peaks exhibiting absorption over 250 nm, which were eluted from the C8 column at 21.1min (A), 26 min (C), 30.4 min (D), and 33.9 min (E).

rated the tryptic peptides with a reverse-phase HPLC system and obtained UV-visible absorption spectra for them with a photodiode-array detector. Figure 5 shows typical elution profiles of peroxynitrite-CO_2–modified SOD monitored at 230, 280, and 350 nm. The absorption spectra of fractions exhibiting absorption over 250 nm were monitored with a photodiode-array and are shown in Fig. 5. The fraction at 20.8 min gave a weak absorption spectrum with a peak at 255 nm and a shoulder at around 295 nm, as shown in Fig. 5A. This spectrum does not resemble that of *N*-formylkynurenine [absorption maxima at 260 and 322 nm (*27*)] but resembles that of 5,7-dihydroxytryptamine, which has two absorption maxima at 264 nm and 295 nm. Therefore it might be a dihydroxytryptophan. We observed that the fraction at 25.4 min exhibited the same absorption spectrum as that of oxindole-3-alanine (tautomer of 2-hydroxytryptophan) (*28*), but different from those of other hydroxytryptophans (4-, 5-, 6-, and 7-) (*28*) (Fig. 5B). Therefore, the compound in the fraction at 25.4 min is most probably oxindole-3-alanine. In addition, we observed the same absorption spectrum as for kynurenine (*29*) at the elution position of 25.7 min (Fig. 5C). The fraction at 30.3 min had a very similar absorption spectrum to that of authentic 6-nitrotryptophan [Fig. 5D (*30*)]. Although we did not detect another nitrotryptophan in the chromatogram of this $ONOO^-$-modified preparation, we found a small peak eluted at around 34 min, which had the spectrum shown as Fig. 5E*, for the other preparation. This spectrum resembles that of 5-nitrotryptophan (*30*). We found no other fractions exhibiting significant absorbance over 250 nm. In order to link these results with the results of LC-MS analysis, we collected these fractions on the reverse-phase HPLC and subjected these fractions to LC-MS analysis. The fraction eluted at 20.8 min (Fig. 5A) gave a peak with m/z = 721.3, which is the same that of +32 Da peptide on mass analysis (Fig. 2, B-2). This result supports the conclusion that one of the modified products is a dihydroxytryptophan. The fraction eluted at 25.4 min (Fig. 5, B) consisted of two peaks with m/z= 705.3, which were the same that of +16 Da [1] and +16 Da [2] peptides on mass analysis (Fig. 2, B-3). We could not explain why the oxindole-3-alanine fraction was divided into two peaks on LC-MS analysis, but the presence of a tautomer form (oxindole-3-alanine ⇔ 2-hydroxytryptophan) and/or an unknown modification of oxindole-3-alanine could be the reason. The fractions eluted at 25.7 min and 30.3 min gave the peaks of the +4 Da peptide (Fig. 2, B-4) and +45 Da [1] peptide (Fig. 2, B-6) on mass analysis, respectively. These results are consistent with the products, kynurenine and 6-nitrotryptophan, identified from these spectra.

The same analyses were carried out on the tryptic peptides of Cu,Zn-SOD modified with MPO-H_2O_2-NO_2^-. Essentially the same fractions with the same retention times and the same absorption spectra were observed as in the case of peroxynitrite-CO_2 modification (Fig. 6, A, C, D, and E), except for fraction B in Fig. 5. Fractions A (21.1 min), C (26.0 min), D (30.4 min), and E (33.8 min) seemed to correspond to +32, +4, +45 [1], and +45 Da [2] in microLC-ESI-Q-TOF mass (Fig. 4), respectively, on the basis of the similarity of the relative elution positions and coincidence of molecular weights. We did not observe

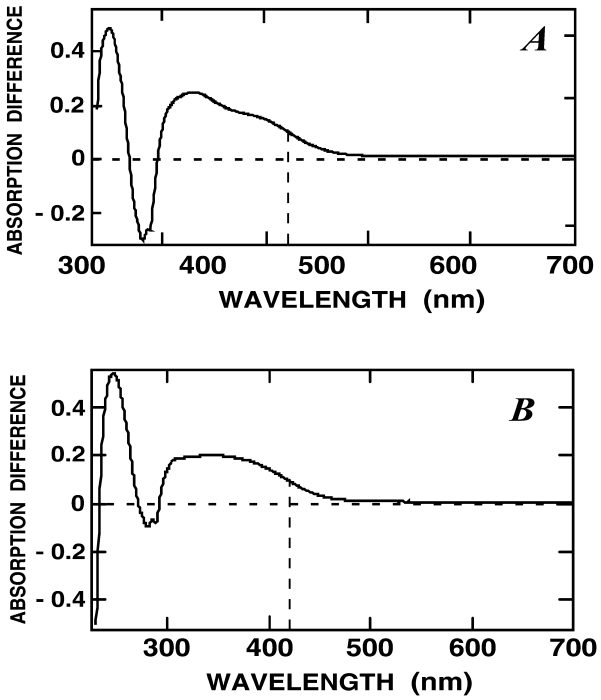

Fig. 7. **Difference spectra for the modified and the control enzymes.** A, between the modified enzyme with the MPO system and the control [1] enzyme. B, between the modified Cu,Zn-SOD with the peroxynitrite-CO_2 system and the control enzyme (*19*). The concentration of each enzyme was 3.2 mg/ml in 10 mM Tris-HCl buffer, pH 7.8, respectively. The broken vertical lines indicate the position of 420 nm.

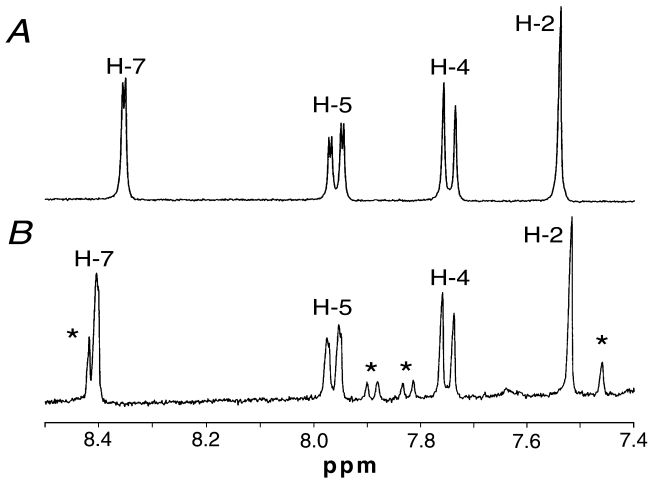

Fig. 8. **^{1}H-NMR spectra of the aromatic region for (A) authentic 6-nitrotryptophan (pH 12.5) and (B) pronase-digested products (pH 11.1) in D_2O.** The peaks in (A) and the major ones in (B) are assigned to 6-nitrotryptophan. Asterisks indicate minor peaks.

any significant absorbance over 250 nm around 25.5 min, therefore we could not identify the +16 Da tryptophan derivatives on this HPLC analysis. The reason for the inability to detect oxindole-3-alanine on HPLC analysis is not clear at present, but the relatively low absorption over 250 nm of this product and the limitation of the sensitivity of the photodiode-array system could be reasons.

Estimation of the Yields of 6-Nitrotryptophan with the Two Modification Systems—Since 6-nitrotryptophan is a specific major product of the reaction between the tryptophan residue and reactive nitrogen species formed with both of the modification systems, it is important to know the yields of 6-nitrotryptophan with the two modification systems. In order to calculate the yields of 6-nitrotryptophan formation under these reaction conditions, we used the absorbance at 420 nm in the difference spectra between the modified and control enzymes with each reaction system for estimation of the amounts of 6-nitrotryptophan, since dihydroxytryptophan, kynurenine, oxindole-3-alanine, and 5-nitrotryptophan exhibit no or negligible absorption at 420 nm. Figure 7 shows the difference spectrum for the MPO system–modified enzyme and control [1] enzyme (A), with that of the peroxynitrite-modified Cu,Zn-SOD (B) (*19*) for comparison. The MPO system–modified SOD showed a similar difference spectrum to that of the peroxynitrite-modified enzyme. We obtained almost the same yields of 6-nitrotryptophan, *i.e.*, 29.6 ± 2.3 mol% (5 preparations) and about 28.4 ± 1.7 mol% (3 preparations) of the total modified tryptophan residue in Cu,Zn-SOD for the peroxynitrite-CO_2 and MPO-H_2O_2-NO_2^- systems, respectively, using 3,320 M^{-1} cm^{-1} as the extinction coefficient of 6-nitrotryptophan at 420 nm, which was calculated with authentic 6-nitrotryptophan. We also calculated the proportion of 6-nitrotryptophan–containing peptide among the total modified peptides by using the intensity of each of the modified peptides on microLC-ESI-Q-TOF mass analyses (Figs. 2 and 4), obtaining 22 ± 7% for the peroxynitrite-CO_2 system and 31 ± 8% for the MPO-H_2O_2-NO_2^- one. Although the accuracy of these values is limited since the ionization-efficiency for each of the peptides could not be the same, these values are fairly similar to the values obtained from the different absorbance at 420 nm.

HPLC Separation of Pronase-Digested Modified-SOD and NMR Measurement of the Fraction Containing Nitrated Tryptophan—In order to confirm the nitrotryptophan and the other oxidized products further, we digested the peroxynitrite-CO_2–modified SOD with pronase extensively. The digested mixture was subjected to reverse-phase HPLC with a photodiode-array as a detector. We found fractions eluted at the same retention times (10.7 min and 21.4 min) as standard kynurenine and 6-nitrotryptophan, respectively, which had the same absorption spectra as the standards (data not shown). We also found fractions having similar spectra to oxindole-3-alanine among the tryptic peptides (Fig. 5B) at the elution times of 11.6 and 9.95 min, respectively (data not shown). We selected the peak material 21.4 min for ^{1}H NMR analysis, in order to further confirm this product to be 6-nitrotryptophan. Figure 8 shows ^{1}H NMR spectra of the aromatic region. The chemical shifts and coupling constants were as follows: Those of authentic 6-nitrotryptophan (D_2O, pH = 12.51) were H-7 (8.35 ppm, 1H, d, J_{5-7} = 2.0 Hz), H-5 (7.96 ppm, 1H, dd, J_{4-5} = 9.0 Hz, J_{5-7} = 2.0 Hz), H-4 (7.75 ppm, 1H, d, J_{4-5} = 9.0 Hz), and H-2 (7.55 ppm, 1H, s) (Fig. 8A). The major peaks in the ^{1}H-NMR spectrum of the pronase-digested modified-SOD (6-nitrotryptophan) (D_2O, pH = 11.09) were H-7 (8.40 ppm, 1H, d, J_{5-7} = 1.8 Hz), H-5 (7.96 ppm, 1H, dd, J_{4-5} = 8.8 Hz, J_{5-7} =

1.8 Hz), H-4 (7.75 ppm, 1H, d, J_{4-5} = 8.8 Hz), and H-2 (7.52 ppm, 1H, s) (Fig. 8B). The major peaks of the pronase-digested product coincided with the peaks of authentic 6-nitrotryptophan in the aromatic region. Since the sample showed no single triplet-peak, we can rule out the possibility of 4-nitrotryptophan and 7-nitrotryptophan. The coupling constants among the data also suggest a 5- or 6-mono substituted tryptophan as a candidate for the product. From these analyses of the absorption spectra of trypsin and pronase-digested samples, we concluded that the major nitrated tryptophan is 6-nitrotryptophan. The pronase-digested fraction also contained minor components (ratio of intensity of major to minor components, 6:1) having signals similar to those of 6-nitrotryptophan. Although we cannot determine the origin of these minor signals, a peptide containing 5- or 6-nitrotryptophan, which is formed through the incomplete digestion by pronase, could be a possible product, based on the same procedure of assignment as described above.

DISCUSSION

Products of Trp32 Modification with the Two Systems—The pH optimum we observed for the tryptophan-modification with the MPO system was the same as the reported value for nitration of tyrosine by eosinoperoxidase (*13*). Sodium bicarbonate had no effect on the modification of the tryptophan residue, except for the acidic region (Fig. 1). No significant effects of sodium bicarbonate on the nitration of tyrosine and related compounds with the myeloperoxidase system have been reported either (*15*, *22*). Although a clear explanation of the enhanced modification of the tryptophan residue in the acidic region is difficult at present, a possible explanation is that an increase in carbon dioxide, which is in equilibrium with the bicarbonate ion, at acidic pH, and an increase in the formation of peroxynitrite from myeloperoxidase at acidic pH (*15*) could have resulted in enhanced nitration of the tryptophan residue.

The copper and zinc contents were not significantly different between the control enzymes and the modified enzyme (Table 1). On amino acid analysis of the MPO-modified enzyme, we observed a little decrease in serine residues, which corresponds to one residue out of ten residues, in addition to disappearance of the tryptophan residue (Table 2). However, we could rule out an influence of this apparent decrease in serine residues on the enzyme activity of the MPO-modified SOD, because a similar decrease in the enzyme activity was observed for the peroxynitrite-CO_2 modified SOD, which showed no decrease in serine residues and disappearance of the tryptophan residue in the modified enzyme (*19*).

We identified the major nitrated product (+45 Da [1]) with both the modification systems as 6-nitrotryptophan and the minor nitrated product (+45 Da [2]) as 5-nitrotryptophan. In contrast to 6-nitrotryptophan, 5-nitrotryptophan was found consistently with the MPO system (3 preparations), but was only found in two preparations in five modification experiments with the peroxynitrite-CO_2 system (data not shown). In addition to these nitrated products, we found kynurenine, oxindole-3-alanine and its modified product, and a dihydroxytryptophan as oxidized products of the tryptophan residue with each of the modification methods. Recently, Herold *et al.* (*21*) reported that the formation of 4-, 5-, and 6-nitrotryptophan in apo-, oxy-, and met myoglobin, and 5- and 6-nitrotryptophan in oxy- and met hemoglobin occurred upon the addition of peroxynitrite-CO_2. They also pointed out that 6-nitrotryptophan seems to be generated to the greatest extent among all the proteins. Although they did not find any oxidized products of tryptophan residues among the reaction products of hemoglobin or myoglobin with peroxynitrite-CO_2, they did not exclude the possible formation of these products. They observed products that absorb at 350 nm at the faster elution position than those of nitrotryptophan in their chromatography (*21*). These products could be oxidized products of the tryptophan residue, such as kynurenine. Suzuki e*t al.* (*22*) reported that they found the formation of 6-nitrotryptophan in bovine serum albumin reacted with various sources of reactive nitrogen species, including peroxynitrite-CO_2 and MPO-H_2O_2-NO_2^- systems, on HPLC with electrochemical detection. With this detection system, other possible reaction products of tryptophan residues could not be detected. Therefore, this is the first report of total products, *i.e.*, nitrated and oxidized products, of the tryptophan residue modified with the peroxynitrite-CO_2 and MPO-H_2O_2-NO_2^- systems. Recently, Sala *et al.* (*31*) reported the formation of the N^1-NO_2 and N^1-NO products, and Suzuki *et al.* (*22*) reported the formation of the N^1-NO_2 product, in addition to 6-nitrotryptophan, as a major nitrated product of the reaction of *N*-acetyl-L-tryptophan with the peroxynitrite-CO_2 and MPO-H_2O_2-NO_2^- systems, respectively. However, we did not observe these products in this study. This discrepancy can be attributed to the difference in reactivity between free tryptophan and tryptophan residues and/or the short half-lives of the N^1-NO_2 and N^1-NO products (*22*), which may have disappeared during the dialysis and protease digestion in our study.

Proposed Reaction Mechanism—In the case of peroxynitrite-CO_2, the carbonate radical ($^\bullet CO_3^-$) and $^\bullet NO_2$ are produced from nitrosoperoxycarbonate (*32–34*). Trp32 in the enzyme may react with $^\bullet CO_3^-$ preferentially to form a tryptophan radical, because of its high reactivity with $^\bullet CO_3^-$, rather than with $^\bullet NO_2$ (Reaction 4) (*35*). Reaction with $^\bullet NO_2$ to form a tryptophan radical may also occur (Reaction 5) to a lower extent.

$$\text{Trp32-SOD} + {}^\bullet CO_3^- \rightarrow {}^\bullet\text{Trp32-SOD} + HCO_3 \quad (4)$$

$$\text{Trp32-SOD} + {}^\bullet NO_2 \rightarrow .\text{Trp32-SOD} + HNO_2 \quad (5)$$

$$^\bullet\text{Trp32-SOD} + {}^\bullet NO_2 \rightarrow NO_2\text{Trp32-SOD} \quad (6)$$

On the other hand, MPO reacts with H_2O_2 and NO_2^- to form $^\bullet NO_2$ and compound II (Reactions 1 and 2). Compound II also oxidizes nitrite to form nitrogen dioxide ($^\bullet NO_2$) (Reaction 3). In contrast to free tryptophan, it may rarely occur that the tryptophan residue in Cu,Zn-SOD reacts directly with compounds I and II, and the tryptophan residue radical being formed (*36*). In this case, $^\bullet NO_2$ may react with the tryptophan residue to form its radical (Reaction 5). The tryptophan radical then recombines with $^\bullet NO_2$ to form nitrotryptophan with both the modification systems (Reaction 6).

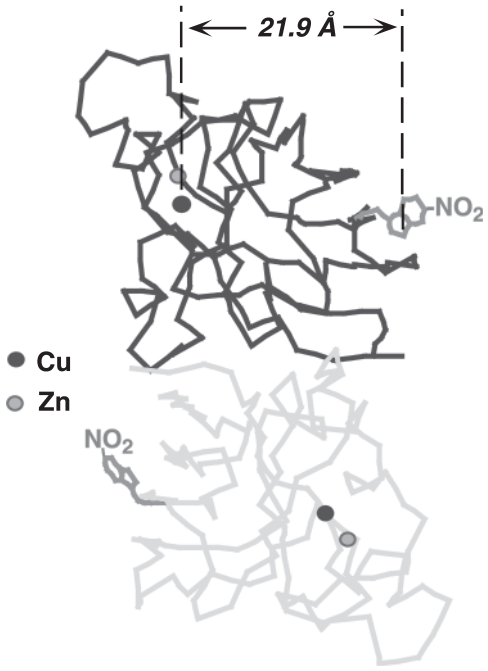

Fig. 9. **Position of Trp32 in the dimer structure of human Cu,Zn-SOD.** The distance between the copper atom and ε1N of Trp32 within the same molecule is 21.9 Å. Trp32 is shown as 6-nitrotryptophan.

The tryptophan (residue) radical is known to react readily with an oxygen molecule to form the tryptophan peroxy radical (Reaction 7). Although the detailed mechanism is not clear at present, this radical may become kynurenine, through the formation of *N*-formylkynurenine. Recently, Zhang *et al.* (*37*) suggested that $^{\bullet}CO_3^-$ produced kynurenine-type products of Trp32 in human Cu,Zn-SOD in the presence of oxygen and then caused covalent aggregation of the enzyme. However, we did not find an aggregated form of Cu,Zn-SOD with the H_2O_2 concentration used in our system (data not shown). This apparent discrepancy may be due to the low concentration of H_2O_2 we used in our study (0.3 mM) and the presence of $^{\bullet}NO_2$, which may compete with oxygen for the tryptophan radical. Nevertheless, the evidence reported by Zhang *et al.* supports the formation of kynurenine through the tryptophan radical in the presence of oxygen.

$$^{\bullet}\text{Trp32-SOD} + O_2 \rightarrow \text{Trp32-OO}^{\bullet}\text{-SOD} \rightarrow \textit{N}\text{-formylkynurenine} \rightarrow \text{Kynurenine} \quad (7)$$

For the formation of oxindole-3-alanine, $^{\bullet}OH$ is the most probable cause of hydroxylation of the tryptophan residue. Lemercier *et al.* (*9*) suggested that a substantial level of hydroxylation of phenol occurred with peroxynitrite in the presence of CO_2 through isomerization of peroxynitrous acid to nitrate and $^{\bullet}OH$. This $^{\bullet}OH$ may be the cause of the hydroxylation reaction of the tryptophan residue in the peroxynitrirte-CO_2 system. We observed no increase in the yield of 6-nitrotryptophan with the MPO system (28.4%) compared with that with the peroxynitrite-CO_2 system (29.6%). This finding is not consistent with the results for tyrosine nitration reported by Brennan *et al.* (*15*), who showed the absence of a hydroxylated product and the presence of a nitrated product in the reaction of MPO-H_2O_2-NO_2^- at pH 7.0. However, they suggested that the MPO system still retained the ability of hydroxylation at neutral pH for salicylate (*15*). Therefore, the ratio of the hydroxylation and nitration reactions with the MPO-H_2O_2-NO_2^- system could differ according to the substrate. Although the mechanism underlying the hydroxylation reaction of the tryptophan residue is not clear at present, Brennan *et al.* suggested the participation of a nascent oxidant liberated from the distal heme of MPO for the hydroxylation of salicylate (*15*).

Physiological Significance—The properties of the modified Cu,Zn-SODs with both the methods showed a little less enzymatic activity [Table 1 (*19*)]. Since Trp32 is located 22 Å away from the active site Cu as shown in Fig. 9, it is not probable that this site is directly perturbed by this modification (Fig. 9). However, if some of the six modified products could affect the local main-chain conformation, it might alter the fine structure of the active environment. Studies on FALS-associated SOD mutants, which show decreased zinc affinity, suggest that diverse mutation sites far from the zinc-binding site can result in similar effect on the metal-binding properties of the zinc site, although the mutated amino acids in these mutants were far from the zinc-binding site (*38*, *39*). We also observed a little less stability of the enzymatic activity of both the modified Cu,Zn-SODs against 6 mM EDTA at pH 5.3 (data not shown). This evidence also supports the possibility mentioned above.

Although the formation of 6-nitrotryptophan comprised about 30% of the total modified tryptophan residue in human Cu,Zn-SOD, 6-nitrotryptophan in proteins could be used as a new tool for revealing the novel functional sites of reactive nitrogen species *in vivo*, since this modification is specific for reactive nitrogen species. Since kynurenine and the other hydroxylated products of the tryptophan residue could also be produced by reactive oxygen species, such as the hydroxyl radical and hydrogen peroxide, these products are not suitable as reactive nitrogen species–specific biomarkers. 6-Nitrotryptophan was also found to be a major product of the reaction between reactive nitrogen species and hemoglobin (*21*), myoglobin (*21*), bovine serum albumin (*22*), and lysozyme (unpublished data). Therefore, 6-nitrotryptophan might be produced widely in the regions where the concentration of peroxynitrite and/or myeloperoxidase-H_2O_2-NO_2^- is elevated. In spite of the possible formation of 3-hydroxytyrosine along with the formation of 3-nitrotyrosine by reactive nitrogen species *in vivo* (*40*), 3-nitrotyrosine in proteins is widely used as a biomarker for the reaction sites of reactive nitrogen species (*1*, *2*).

Although some tryptophan residues are components of the active sites of enzymes (*41*, *42*), many other tryptophan residues participate in the recognition of molecules interactive with enzymes or proteins. Trp286 in human vitamin D receptor (*43*), and Trp158 and 172 in human G protein–coupled receptors (*44*) are examples that show binding sites for interactive molecules, respectively. Therefore, through modification of tryptophan residues to nitrated or oxidized products with reactive nitrogen species, there is the possibility to modulate the interac-

tion of proteins with these interactive molecules. Furthermore, Craig *et al.* (*45*) have reported the presence of a 6-bromotryptophan in a 33–amino acid peptide from *Conus radiatus*, which induces a sleep-like state in mice. They suggested that there was a relation between the pharmacological activity and the bromination at the 6-position of the tryptophan. As shown in this case, modification at this position of tryptophan residues could play a role in the regulation of the recognition of a protein by other molecules. The introduction of a nitro group to the 6 position of the tryptophan residue may affect the structural role of the tryptophan residue in a protein by increasing the bulk at this position. It may also affect the π-electron state of the indole ring through the attractive effect of the nitro group. Therefore, acting as a biomarker for nitrative stress in cells may not be the sole function of 6-nitrotryptophan in proteins. In order to examine this possibility, immunological detection of 6-nitrotryptophan with a specific antibody is underway in our laboratory.

The recommended IUPAC nomenclature for the peroxynitrite anion and peroxynitrous acid is oxoperoxonitrate (1-) and hydrogen oxoperoxonitrate, respectively. Peroxynitrite is used in the text to refer generically to both oxoperoxonitrate (1-) ($ONOO^-$) and its conjugate acid, hydrogen oxoperoxonitrate (ONOOH). We are grateful to Nippon Kayaku Ltd., Tokyo, Japan, for providing the human recombinant Cu,Zn-SOD. We also thank Professor Bruce E. Allen for critical reviewing of the manuscript. This work was supported by grants (E1301) from The Institute for Environmental and Gender-Specific Medicine, Juntendo University Graduate School of Medicine.

REFERENCES

1. Ischiropoulos, H. (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* **35**, 61–11
2. Greenace, S.A.B. and Ischiropoulos, H. (2001) Tyrosine nitration: localization, quantification, consequences for protein function and signal transduction. *Free Rad. Res.* **34**, 541–581
3. Radi, R. (2003) Peroxynitrite reactivity with amino acids and proteins. *Amino Acids* **25**, 295–311
4. Alvarez, B., Rubbo, H., Kirk, M., Barnes, S., Freeman, B.A., and Radi, R. (1996) Peroxynitrite-dependent tryptophan nitration. *Chem. Res. Toxicol.* **9**, 390–396
5. Padmaja, S., Ramazenian, M.S., Bounds, P.L., and Koppenol, W.H. (1996) Reaction of peroxynitrite with L-tryptophan. *Redox Rep.* **2**, 173–177
6. Kato, Y., Kawakishi, S., Aoki, T., Itakura, K., and Osawa, T. (1997) Oxidative modification of tryptophan residues exposed to peroxynitrite. *Biochem. Biophys. Res. Commun.* **234**, 82–84
7. Squadrito, G.L. and Pryor, W. (1998) Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. *Free Radic. Biol. Med.* **25**, 392–403
8. Yermilov, V., Yoshie, Y., Rubio, J., and Ohshima, H. (1996) Effects of carbon dioxide/bicarbonate on induction of DNA single-strand breaks and formation of 8-nitroguanine, 8-oxoguanine and base-propenal mediated by peroxynitrite. *FEBS Lett.* **399**, 67–70
9. Lemercier, J.-N., Padmaja, P., Cueto, R., Squandrito, G.L., Uppu, R.O., and W.A. Pryor, (1997) Carbon dioxide modulation of hydroxylation and nitration of phenol by peroxynitrite. *Arch. Biochem. Biophys.* **345**, 160–170
10. Burner, U., Furtmuller, P.G., Kettle, A.J., Koppenol, W.H., and Obinger, C. (2000) Mechanism of reaction of myeloperoxidase with nitrite. *J. Biol. Chem.* **275**, 20597–20601
11. Pfeiffer, S., Mayer, B., and Hemmens, B., (1999) Nitric oxide: Chemical puzzles posed by a biological messenger. *Angew. Chem. Int. Ed.* **38**, 1714–1731
12. van der Vliet, A., Eiserich, J.P., Halliwell, B., and Cross, C.E. (1997) Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J. Biol. Chem.* **272**, 7617–7625
13. Wu, W., Chen, Y., and Hazen, S.L. (1999) Eosinophil peroxidase nitrates protein tyrosyl residues. Implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. *J. Biol. Chem.* **274**, 25933–25944
14. Sampson, J.B., Ye, Y.Z., Rosen, H., and Beckman, J.S. (1998) Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Arch. Biochem. Biophys.* **356**, 207–213
15. Brennan, M.L., Wu, W., Shen, Z.F., Song, W., Frost, H., Vadseth, C., Narine, L., Lenkiewcz, E., Borchers, M.T., Lusis, A.J., Lee, J.J., Lee, N.A., Abu-Soud, H.M., Ischiropoulos, H., and Hazen, S.L. (2002) A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J. Biol. Chem.* **277**, 17415–17427
16. Ichimori, K., Fukuyama, N., Nakazawa, H., Aratani, Y., Koyama, H., Takizawa, S., Kameoka, Y., Ishida-Okawara, A., Kohi, F., and Suzuki, K. (2003) Myeloperoxidase has directly-opposed effects on nitration reaction-study on myeloperoxidase-deficient patient and myeloperoxidase-knockout mice. *Free Rad. Res.* **37**, 481–489
17. Yamakura, F., Taka, H., Fujimura, T., and Murayama, K. (1998) Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J. Biol. Chem.* **273**, 14085–14089
18. Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J.C., Smith, C.D., and Beckman, J.S. (1992) Peroxynitriter-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch. Biochem. Biophys.* **298**, 431–437
19. Yamakura, F., Matsumoto, T., Fujimura, T., Taka, H., Murayama, K., Imai, T., and Uchida, K. (2001) Modification of a single tryptophan residue in human Cu,Zn-superoxide dismutase by peroxynitrite in the presence of bicarbonate. *Biochim. Biophys. Acta* **1548**, 38–46
20. Alvarez, B., Demichelli, V., Duran, R., Trujillo, M., Cervenansky, C., Freeman, B.A., and Radi, R. (2004) Inactivation of human Cu, Zn-superoxide dismutase by peroxynitrite and formation of histidinyl radical. *Free Radic. Biol. Med.* **37**, 813–822
21. Herold, S., Shivashankar, K., and Mehl, M. (2002) Myoglobin scavenges peroxynitrite without being significantly nitrated. *Biochemistry* **41**, 13460–13472
22. Suzuki, T., Mower, H.W., Friesen, M.D., Gilibert, I., Sawa, T., and Ohshima, H., (2004) Nitration and nitrosation of N-acetyl-L-tryptophan and tryptophan residues in proteins by various reactive nitrogen species. *Free Radic. Biol. Med.* **37**, 671–681
23. Morino, T., Morita, M., Seya, K., Sukenaga, Y., Katoh, K., and Nakamura, T. (1988) Construction of a runaway vector and its use for a high-expression of a cloned human superoxide dismutase gene. *Appl. Microbiol. Biotechnol.* **28**, 170–175
24. Moriya, T., Hagio, K., and Yoneda, N. (1975) A facile synthesis of 6-chloro-D-tryptophan. *Bull. Chem. Soc. Jpn.* **48**, 2217–2218
25. Yamakura, F., Rardin, R.L., Petsko, G.A., Ringe, D., Hiraoka, B.Y., Nakayama, K., Fujimura, T., Taka, H., and Murayama, K. (1998) Inactivation and destruction of conserved Trp159 of Fe-superoxide dismutase from Porphyromonas gingivalis by hydrogen peroxide. *Eur. J. Biochem.* **253**, 49–56
26. Smith, P., Krohn, R.I., Hermanson, G.T., Mallia, A.K. Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85
27. Kuroda, M., Sakiyama, F., and Narita, K. (1975) Oxidation of tryptophan in lysozyme by ozone in aqueous solution. *J. Biochem.* **78**, 641–651

28. Maskos, Z., Rush, J.D., and Koppenol, W.H. (1992) The hydroxylation of tryptophan. *Arch. Biochem. Biophys.* **296**, 514–520
29. Teshima, K., Kuramitsu, S., Hamaguchi, K., Sakiyama, F., Mizuno, K., and Yamasaki, N. (1980) Binding of substrate analogs to hen lysozyme in which Trp 62 is modified to kynurenine. *J. Biochem.* **87**, 1015–1027
30. Berti, G., Da Settimo, A., and Segnini, D. (1960) Studio spettrofotometorico dell'acidità di alcuni nitroderivati indolici. *Gazz.Chim. Ital.* **90**, 539–558
31. Sala, A., Nicolis, S., Roncone, R., Casella, L., and Monzani, E., (2004) Peroxidase catalyzed nitration of tryptophan derivatives. Mechanism, products and comparison with chemical nitrating agents. *Eur. J. Biochem.* **271**, 2841–2852
32. Goldstein, S. and Czapski, G. (1997) The effect of bicarbonate on oxidation by peroxynitrite: implication for its biological activity. *Inorg. Chem.* **36**, 5113–5117
33. Lymar, S.V., Jiang, Q., and Hurst, J.K. (1996) Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. *Biochemistry* **35**, 7855–7861
34. Uppu, R.M., Asquadrito, G.L., and Pryor, W.A. (1996) Acceleration of peroxynitrite oxidations by carbon dioxide. *Arch. Biochem. Biophys.* **327**, 335–343
35. Uppu, R.M., Squadrito, G.L., Bolzan, R.M., and Pryor, W.A. (2000) Nitration and nitrosation by peroxynitrite: role of CO_2 and evidence for common intermediates. *J. Am. Chem. Soc.* **122**, 6911–6916
36. Tien, M. (1999) Myeloperoxidase-catalyzed oxidation of tyrosine. *Arch. Biochem. Biophys.* **367**, 61–66.M
37. Zhang, H., Andrekopoulos, C., Joseph, J., Chandran, K., Karoui, H., Crow, J.P., and Kalyanaraman, B. (2003) Bicarbonate-dependent peroxidase activity of human Cu,Zn-superoxide dismutase induces covalent aggregation of protein: intermediacy of tryptophan-derived oxidation products. *J. Biol. Chem.* **278**, 24078–24089
38. Crow, J.P., Sampson, J.B., Zhuang, X., Tompson, J.A., and Beckman, J.S. (1997) Decreased zinc affinity of amyotrophic lateral sclerosis-associated superoxide dismutase mutants leads to enhanced catalysis of tyrosine nitration by peroxynitrite. *J. Neurochem.* **69**, 1936–1944
39. Lyons, T.J., Liu, H., Goto, J.J., Nersissian, A., Roe, J.A., Graden, J.A., Café, C., Ellerby, L.M., Bredesen, D.E., Gralla, E.B., and Valentine, J.S. (1996) Mutations in copper-zinc superoxide dismutase that cause amyotrophic lateral sclerosis alter the zinc binding site and the redox behavior of the protein. *Proc. Natl Acad. Sci. USA* **93**, 12240–12244
40. Linares, E., Giorgio, S., Mortara, R.A., Santos, C.X., Yamada, A.T., and Augusto, O. (2001) Role of peroxynitrite in macrophage microbicidal mechanisms in vivo revealed by protein nitration and hydroxylation. *Free Radic. Biol. Med.* **30**, 1234–1242
41. Zhang, Z., Ostanin, K., and Van Etten, R.L. (1997) Covalent modification and site-directed mutagenesis of an active site tryptophan of human prostatic acid phosphatase. *Acta Biochim. Pol.* **44**, 659–672
42. Ali, M.S., Shenoy, B.C., Eswaran D., Andersson, L.A., Roche, T.E., and Patel, M.S. (1995) Identification of the tryptophan residue in the thiamin pyrophosphate binding site of mammalian pyruvate dehydrogenase. *J. Biol. Chem.* **270**, 4570–4574
43. Solomon, C., Macoritto, M., Gao, X.L., White, J.H., and Kremer, R. (2001) The unique tryptophan residue of the vitamin D receptor is critical for ligand binding and transcriptional activation. *J. Bone. Miner. Res.* **16**, 39–45
44. Rhee, M.-H., Nevo, I., Bayewitch, M.L., Zagoory, O., and Vogel, Z. (2000) Functional role of tryptophan residues in the fourth transmembrane domain of the CB(2) cannabinoid receptor. *J. Neurochem.* **75**, 2485–2491
45. Craig, A.G., Jimenez, E.C., Dykert, J., Nielsen, D.B., Gulyas, J., Abogadie, F.C., Porter, J., Rivier, J.E., Cruz, L.J., Olivera, B.M., and McIntosh, J.M. (1997) A novel post-translational modification involving bromination of tryptophan. Identification of the residue, L-6-bromotryptophan, in peptides from *Conus imperialis* and *Conus radiatus* venom. *J. Biol. Chem.* **272**, 4689–4698
46. Biemann, K. (1990) Sequencing of peptides by tandem mass spectrometry and high-energy collision-induced dissociation. *Methods Enzymol.* **193**, 455–479