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Oxidative Modification of Tryptophan Residues Exposed to Peroxynitrite

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The aim of this study was to clarify the mechanism of loss of Trp residues in proteins exposed to peroxynitrite. The Trp residues in bovine serum albumin and collagen IV were decreased by peroxynitrite treatment. To identify the degradation products of the Trp residue by peroxynitrite, *tert*-butoxycarbonyl-L-tryptophan (Boc-Trp) was used as a model of the Trp residue in proteins, and the products formed from peroxynitrite-treated Boc-Trp were then isolated. Boc-Trp decreased with an increase in peroxynitrite concentration. N-Formylkynurenine, oxindole, and hydropyrroloindole were identified as major products. The formation of these products may account for the losses of Trp residues in proteins by peroxynitrite.

Nitric oxide (NO) performs many functions when produced *in vivo,* e.g., by neurons and endothelial cells (1). Peroxynitrite ($ONOO^-$), formed by a diffusion-limited reaction of nitric oxide (NO) and superoxide (O_2^-), is capable of the nitration of the tyrosine residue in proteins (2). The 3-nitrotyrosine residue has been detected in atherosclerotic lesion (3, 4), endotoxin shock (5, 6), ischemic injury (7, 8), and lung sections of patients with acute lung injury (9), using specific monoclonal and polyclonal antibodies that recognize nitrotyrosine in proteins (3) or HPLC-ECD (10).

Thus, protein modification by peroxynitrite may contribute to some diseases. Ischiropoulos and Al-Mehdi have investigated the reaction between bovine serum albumin and peroxynitrite *in vitro* (11). The reaction causes protein fragmentation, carbonyl formation, oxidation of Trp and Cys, nitration of Tyr, and the formation of dityrosine (11). However, the mechanism of the degradation of Trp residue remains obscure. The study of protein modification by peroxynitrite concentrates on Tyr modification, whereas Trp residues are also decomposed by peroxynitrite. In this study, we have examined the modification of Trp residues in collagen IV and BSA by peroxynitrite *in vitro.* The loss of Trp residue in proteins was observed by treatment with peroxynitrite. The modification of the Trp residue by peroxynitrite was examined using a model compound of Trp residue in detail. We report on the identification of peroxynitrite-modified Trp.

MATERIALS AND METHODS

Materials. Collagen IV (from human placenta) and bovine serum albumin (BSA) were purchased from Sigma. Hydrogen peroxide was obtained from Mitsubishi Gas Co. $NaNO_2$ was purchased from Wako Pure Chemical Industries.

Synthesis of peroxynitrite. Peroxynitrite was made by the procedure described by Hughes and Nicklin (12). Briefly, an ice cold solution of 0.6 M HCl, 0.7 M H_2O_2 (10 ml) was simultaneously added to a well-stirred, cooled (4°C) solution of 0.6 M $NaNO_2$ (10 ml), immediately followed by the addition of 1.5 M NaOH (20 ml). The synthetic solution in a 50-ml capped centrifuge tube was frozen at −20°C. Due to freeze fractionation, peroxynitrite formed a yellow top layer, which was retained for further studies. The top layer typically contained 150–200 mM peroxynitrite as determined by UV-absorbance spectroscopy at 302 nm in 1.2 M NaOH (e302nm = 1670 M^{-1} cm^{-1}) (13).

Reaction conditions. Protein (0.5 mg/ml) or Boc-Trp (0.1 mM or 1.0 mM) were dissolved in 0.1 M phosphate buffer (pH 7.4) and reacted with peroxynitrite at room temperature for 15 min. To exclude the effects of contaminated sodium chloride, sodium hydroxide, nitrite, nitrate, and hydrogen peroxide on protein modification, as a control, decomposed peroxynitrite was also used. The treatment of decomposed peroxynitrite was as follows: Peroxynitrite was added to 0.1 M phosphate buffer (pH 7.4) and kept at room temperature for 15 min prior to the addition of protein or Boc-Trp.

Determination of Trp loss. Reaction mixtures of the native and peroxynitrite-treated protein were directly used for fluorescence measurement (excitation wavelength, 295 nm; emission wavelength, 340 nm) using a Hitachi F-2000 fluorescence spectrometer.

Isolation and characterization of peroxynitrite-modified Boc-Trp. Boc-Trp was dissolved in 0.1 M phosphate buffer (pH 7.4) at a concentration of 1 mM (for isolation) or 0.1 mM (for analysis) and exposed to peroxynitrite (0–1 mM). Reversed-phase HPLC was used for the analysis of the Trp modification and the isolation of the modified Boc-Trp. Chromatography was performed on a JASCO Galiver using

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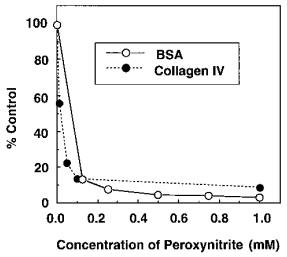

FIG. 1. Changes in fluorescence of Trp residue in BSA and collagen IV exposed to peroxynitrite. Reaction mixtures of the native and peroxynitrite-treated proteins were directly used for fluorescence measurement (ex. 295 nm; em. 340 nm) using Hitachi F-2000 fluorescence spectrometer.

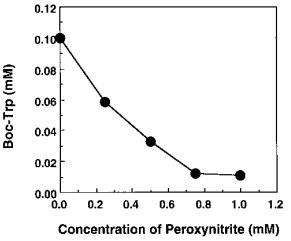

FIG. 2. Loss of Boc-Trp exposed to peroxynitrite. Boc-Trp (0.1 mM) was reacted with peroxynitrite and immediately subjected to HPLC as described in Materials and Methods. The elution was monitored by absorbance at 254 nm. The residual substance was determined from the peak height.

an ODS-HG-5 column (Nomura Chemical Co., 4.6 × 250 mm) for analytical separation and an ODS-HG-5 (8 × 250 mm) for preparative isolations; flow rates were 0.8 and 1.5 ml/min, respectively. A gradient system was used for analysis. Solvent A contained 0.045 M acetate buffer (pH 4.0)/CH_3CN (4/1), and solvent B was CH_3CN. The solvent gradient was 0 min = 100% A, 30 min = 100% A, 60 min = 20% A. For isolations, isocratic elution was performed using a solution of 1% acetic acid/methanol (9/11). The elution was monitored by UV absorbance at 254 nm. For isolation, a 500-ml volume of reaction mixture (containing 1 mM Boc-Trp treated with 1 mM peroxynitrite) was prepared and applied to an XAD-2 column (15 × 100 mm) previously equilibrated with 1% acetic acid. The XAD-2 column was prepared as follows: XAD-2 was loaded with methanol into a glass column and then washed with 1% acetic acid. After the application of the reaction mixture, each 100 ml of 1% acetic acid, 1% acetic acid/methanol (8/2), 1% acetic acid/methanol (6/4), 1% acetic acid/methanol (4/6), 1% acetic acid/methanol (2/8), and methanol was applied to the column. Each fraction was analyzed by HPLC as previously described (for analysis condition). Fractions of 1% acetic acid/methanol (4/6) and 1% acetic acid/methanol (2/8) mainly contained the modified products. These fractions were combined and concentrated and then applied to reversed-phase preparative HPLC. The purification was repeated, and the purified products were used for ^{1}H-NMR and FAB-MS. Three major peaks were detected by HPLC. Two of the three peaks (products) were isomers. Because the other peak was a mixture of two compounds, the isolated products were methylated with diazomethane. The methylation was performed as follows: Diazomethane solution was added to the isolated products in methanol until the yellow color derived from diazomethane disappeared. The methylated derivatives were separated by thin layer chromatography (TLC), which was performed with Silica Gel 60 F_{254} (Merck) using a chloroform/methanol mixture, and used for further analysis. Diazomethane was prepared as follows: KOH (1.2 g) was dissolved in 2 ml of water and added to 4 ml of ethanol. To the solution, N-methyl-N-nitroso-*p*-toluene sulfonamide (4.3 g/25 ml ether) was added under ice cool condition and then distilled. The distilled solution was used for the methylation of the isolated products.

RESULTS

First, collagen IV and BSA were exposed to peroxynitrite and the loss of Trp residue was investigated. The intensity of fluorescence derived from the Trp residue in both type IV collagen and BSA was decreased with increasing peroxynitrite concentration (Fig. 1). The addition of decomposed peroxynitrite to proteins had no effect on Trp fluorescence in proteins. This suggests that contaminated hydrogen peroxide, nitrate, or nitrite in a synthetic solution of peroxynitrite did not contribute to the modification of Trp residues.

To clarify the mechanism of Trp modification, Boc-Trp was used as a model of the Trp residue in protein, and the modification was investigated. The reason why we did not use the acid amide derivative of Boc-Trp is that the acid amide is reactive (14, 15). First, the loss of Boc-Trp by peroxynitrite was determined by HPLC. As shown in Fig. 2, the loss of Boc-Trp by peroxynitrite was observed, accompanied by the formation of products from Boc-Trp (Fig. 3). The products, named A, B, and C, were isolated by HPLC as described in Materials & Methods, and the purified products were identified by ^{1}H-NMR, FAB-MS, and UV scan (14–16). The structures of the identified products are summarized in Fig. 4. When product A (C) was isolated, the isolated product showed two divided peaks (both A and C) on further HPLC purification. In addition, the two peaks have the same UV properties. Products A and C were considered to be isomers. This compound was identified as an oxindole derivative by ^{1}H-NMR, FAB-MS, and its UV properties by comparison with authentic oxin-

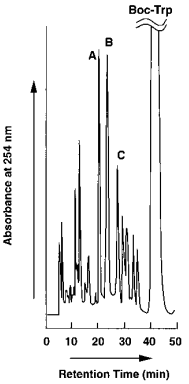

FIG. 3. HPLC profile of peroxynitrite-modified Boc-Trp. Concentrated reaction mixture was applied to HPLC on a Develosil ODS-HG-5 (8 × 250 mm) at a flow rate of 1.5 ml/min with a solution of 1% acetic acid/methanol (45/55). Peaks A, B, and C were collected and characterized.

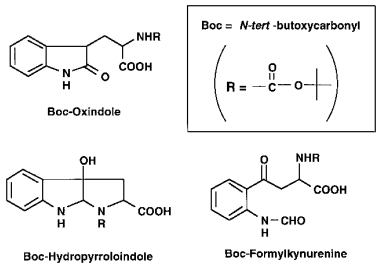

FIG. 4. Summary of products isolated from peroxynitrite-modified Boc-Trp.

dole (14–16). Product B was comprised of two products. Because the peaks were closely eluted, the products could not be separated from each other by HPLC. The mixture of the two peaks was then methylated, and the derivatives were separated by TLC as described in Materials & Methods. The two isolated products were identified as hydropyrroloindole and N-formylkynurenine, respectively, by ^{1}H-NMR, FAB-MS, and UV spectra. The loss of Boc-Trp (1 mM) by peroxynitrite (1 mM) is 29.4%. The yields of oxindole, hydropyrroloindole, and N-formylkynurenine from loss of Boc-Trp are 5.9%, 12.1%, and 2.8%, respectively.

DISCUSSION

It has been reported that the Trp as well as Tyr residues are modified by peroxynitrite (11). We found that Trp residues in both collagen IV and BSA were decreased by incubation with peroxynitrite. The modification mechanism has not been reported in detail. To clarify the mechanism of Trp degradation, the Boc-Trp used for a model of the Trp residue in protein was treated with peroxynitrite, and the modified products were isolated and identified. Hydropyrroloindole, oxindole, and N-formylkynurenine were then identified as the major products formed (Fig. 4). Alvarez *et al.* (17) has been reported the formation of nitrated Trp, hydroxytryptophan, and N-formylkynurenine (or dihydrotryptophan). However, the identification was only performed by spectral characteristics and LC-MS. We identified N-formylkynurenine, oxindole, and hydropyrroloindole from peroxynitrite-modified Trp on the basis of spectral characteristics, MS, and NMR spectrum. As far as we know, oxindole and hydropyrroloindole derived from peroxynitrite-treated Trp were identified for the first time. These products including N-formylkynurenine are probably formed in peroxynitrite-treated proteins. The presence of these peroxynitrite-modified products in proteins has to be proven. It is thought that the treatment of Trp with peroxynitrite may cause the nitration of Trp (11, 17). However, we could not isolate the nitrated molecule, at least, from the three major products obtained. In this experiment, we isolated the products using reversed-phase HPLC from only the fractions before the elution of Boc-Trp. The nitrated Trp possibly elutes after the Boc-Trp.

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