Denaturation of Protein by Chlorine Dioxide: Oxidative Modification of Tryptophan and Tyrosine Residues†

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ABSTRACT: Oxychlorine compounds, such as hypochlorous acid (HOCl) and chlorine dioxide (ClO_2), have potent antimicrobial activity. Although the biochemical mechanism of the antimicrobial activity of HOCl has been extensively investigated, little is known about that of ClO_2. Using bovine serum albumin and glucose-6-phosphate dehydrogenase of *Saccharomyces cerevisiae* as model proteins, here I demonstrate that the antimicrobial activity of ClO_2 is attributable primarily to its protein-denaturing activity. By solubility analysis, circular dichroism spectroscopy, differential scanning calorimetry, and measurement of enzymatic activity, I demonstrate that protein is rapidly denatured by ClO_2 with a concomitant decrease in the concentration of ClO_2 in the reaction mixture. Circular dichroism spectra of the ClO_2-treated proteins show a change in ellipticity at 220 nm, indicating a decrease in α-helical content. Differential scanning calorimetry shows that transition temperature and endothermic transition enthalpy of heat-induced unfolding decrease in the ClO_2-treated protein. The enzymatic activity of glucose-6-phosphate dehydrogenase decreases to 10% within 15 s of treatment with 10 μM ClO_2. Elemental analyses show that oxygen, but not chlorine, atoms are incorporated in the ClO_2-treated protein, providing direct evidence that protein is oxidized by ClO_2. Furthermore, mass spectrometry and nuclear magnetic resonance spectroscopy show that tryptophan residues become *N*-formylkynurenine and tyrosine residues become 3,4-dihydroxyphenylalanine (DOPA) or 2,4,5-trihydroxyphenylalanine (TOPA) in the ClO_2-treated proteins. Taking these results together, I conclude that microbes are inactivated by ClO_2 owing to denaturation of constituent proteins critical to their integrity and/or function, and that this denaturation is caused primarily by covalent oxidative modification of their tryptophan and tyrosine residues.

Proteins are oxidized by many kinds of reactive species, such as superoxide anions ($O_2^{-\bullet}$), hydroxyl radicals ($^\bullet$OH), singlet oxygen ($^1\Delta_g$, $^1O_2$), ozone (O_3), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) (*1*−*7*). Some of these oxidants are generated in the biological systems. For instance, $O_2^{-\bullet}$ is generated during the NADPH oxidase-dependent "respiratory burst" in polymorphonuclear neutrophilic leukocytes (neutrophils) (*8*). The oxidative activities of these species toward microbial proteins are believed to be a protective measure against invading microbes in humans and other animals, but they are also a major cause of disease (*9*). For instance, excessive or misplaced production of these biological oxidants causes host tissue damage, which has been implicated in human disorders (*10*) such as atherosclerosis, cataract, and inflammatory disease (*9*).

HOCl is a highly reactive oxychlorine compound generated in vivo from H_2O_2 and chloride ions by the action of myeloperoxidase (*11*). It is a major oxidant that is generated when neutrophils and macrophages are activated in inflammatory sites. It is released from activated neutrophils during the respiratory burst (*12*, *13*) and is implicated in many bactericidal and cytotoxic responses (*14*). Opsonized microorganisms bind to the surface of phagocytes (neutrophils) and are incorporated into phagosomes that eventually fuse with lysosomes, followed by a burst of oxygen consumption (*15*). The oxygen is then converted to highly reactive oxygen species such as $O_2^{-\bullet}$ and H_2O_2. Myeloperoxidase, together with H_2O_2 from lysosomes, is released into the phagosome. The initial product of the myeloperoxidase−H_2O_2−chloride system in the resulting phagolysosome is HOCl; subsequently chlorine (Cl_2), hydroxyl radical, and singlet oxygen are formed (*1*, *16*−*19*). Cl_2 chlorinates protein and is suggested to play an important role in host defenses and inflammation (*16*). Singlet oxygen is known to kill bacteria in a phagosome of neutrophil (*17*, *19*).

HOCl plays a critical role in destroying microbial pathogens (*20*). In this context, HOCl is considered to be a "natural innate disinfectant". Proteins are major targets of HOCl, and the reaction of proteins with HOCl results in modifications to their side chains, backbone fragmentation, and cross-linking (*21*). Because proteins are major targets of HOCl, oxidative reactions of HOCl with amino acids and peptides have been widely studied (*22*). HOCl readily oxidizes sulfur-containing amino acids, namely, cysteine and methionine (*23*). HOCl also oxidizes tryptophan, tyrosine, and histidine residues of proteins, resulting in protein unfolding (*10*).

Among the oxychlorine compounds with oxidative ability, chlorine dioxide (ClO_2) is known to be extremely potent. Owing to the presence of one unpaired electron in its

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molecular orbital (*24*), it is a free radical. While the presence of ClO_2 in higher organisms, such as human, is not reported in the literature, it is generated in bacteria by a chloroperoxidase-catalyzed dismutation reaction of chlorite (ClO_2^-), which forms chloride (Cl^-), chlorate (ClO_3^-), oxygen, and ClO_2 (*25*). Chlorite is generated by the reduction of either chlorate by chlorate reductase or perchlorate by perchlorate reductase (*26*−*28*). Chlorite is dismutated to chloride and oxygen by chlorite dismutase (*29*). ClO_2 is known to have potent antimicrobial activity against bacteria, viruses, and protozoae (*30*−*38*). Despite a wealth of experimental data on the antimicrobial activity of ClO_2, however, little is known about its biochemical mechanism.

The aim of this work was to elucidate the biochemical mechanism of the antimicrobial activity of ClO_2, on the basis of the working hypothesis that ClO_2 denatures constituent proteins of microbes that are critical for their integrity and/or function, and thereby destroys their viability. To test this hypothesis, I used bovine serum albumin (BSA)[1] and glucose-6-phosphate dehydrogenase (G6PD) of baker's yeast (*Saccharomyces cerevisiae*) as model proteins. I clearly demonstrate here that ClO_2 indeed denatures proteins as assessed by changes in various physicochemical parameters after treatment with ClO_2. I further demonstrate that the denaturation of the proteins is caused by oxidative modification of their tryptophan and tyrosine residues. More specifically, I show that tryptophan becomes *N*-formylkynurenine, and that tyrosine becomes 3,4-dihydroxyphenylalanine (DOPA) or 2,4,5-trihydroxyphenylalanine (TOPA) owing to oxidative covalent modifications. To the best of my knowledge, this is the first report to demonstrate that proteins are oxidatively modified and denatured by ClO_2.

MATERIALS AND METHODS

Materials. Sodium chlorite ($NaClO_2$), trifluoroacetic acid, 3,4-dihydroxy-L-phenylalanine (DOPA), sodium thiosulfate ($Na_2S_2O_3$), sodium azide (NaN_3), high-performance liquid chromatography (HPLC) grade acetonitrile, and a reverse-phase HPLC column (Cosmosil $5C_{18}$-AR-300, 4.6 × 250 mm) were obtained from Nacalai Tesque (Kyoto, Japan). L-Tryptophan, L-tyrosine, BSA (Cohn fraction V, code A 3059, >83% pure), G6PD (code G 6378, >85% pure), aminopeptidase M (porcine kidney microsomal, code L 0632), proteinase K, subtilisin, superoxide dismutase (from *Escherichia coli*), Bradford reagent, and L-kynurenine were obtained from Sigma. Trypsin (code V5111) was obtained from Promega (Madison, WI). $^{18}O_2$ gas (95% isotopic purity) and $H_2{}^{18}O$ (99% isotopic purity) were purchased from Taiyo Nissan (Tokyo, Japan). All synthetic peptides (>95% pure) were obtained from Global Peptide Services (Fort Collins, CO).

Preparation of ClO_2. ClO_2 was prepared by mixing 50 mL of warmed (~48 °C) 550 mM $NaClO_2$ with 5 mL of 2 N HCl for 3 min in a 300 mL flask covered by aluminum foil. The ClO_2 (volatile and soluble in water) generated in the reaction mixture was flushed (bubbled) with a flow of air at 2 L/min for 1.5 min into 50 mL of water chilled on ice. The ClO_2 thus recovered in water (~20 mM) was kept at room temperature in small aliquots in fully filled, tightly capped amber bottles until use.

Iodometric Titration of ClO_2. A 25 μL aliquot of the ClO_2 solution to be assayed was mixed with 475 μL of 105 mM KI. The mixture was titrated with 10 mM $Na_2S_2O_3$ using a micropipet until the brownish yellow color of iodine disappeared. One mole of ClO_2 corresponds to 1 mol of $Na_2S_2O_3$. The experimental error of this assay was ±2%. The validity and accuracy of this assay were confirmed by the absorption of ClO_2 in water, using an absorption maximum, λ_{max}, of 359 nm, and a molar extinction coefficient, ϵ_{max}, of 1230 $M^{-1}\cdot cm^{-1}$ (*25*).

Reactions of Protein, Peptide, and Amino Acid with ClO_2. Unless otherwise specified, standard reactions of protein, peptide, and amino acid with ClO_2 were set up as follows in phosphate-buffered saline (PBS) (20 mM sodium phosphate buffer, pH 7.0, 130 mM NaCl). A ClO_2 stock solution was added (final concentration 1 mM for protein and 0.3 mM for peptide or amino acid) to a solution of protein (final concentration 0.5 mg/mL), peptide or amino acid (each final concentration 0.2 mM), and the reaction was carried out at 25 °C for 2 min. The reaction was terminated by adding a 2-fold molar excess of 200 mM $Na_2S_2O_3$. As a "nontreated" control, ClO_2 that had been premixed with a 2-fold molar excess of $Na_2S_2O_3$ was added to the protein, peptide, or amino acid solution; ClO_2 is reduced to a chlorite anion (ClO_2^-) by an equimolar amount of $Na_2S_2O_3$.

Measurement of Protein Solubility. The solubility of the protein was measured as described (*39*) with some modifications. In brief, the BSA and G6PD solutions (each 0.5 mg/mL in PBS, 200 μL) were treated with varying concentrations of ClO_2 at 25 °C for 2 min, and the reaction was terminated by the addition of an excess amount of $Na_2S_2O_3$ as above. The mixture was then dialyzed for 72 h against 2000 volumes of 10 mM sodium phosphate buffer (pH 7.0) at 4 °C. The dialyzed solution was centrifuged at 15 000 rpm (22000*g*) for 30 min at 4 °C in a 1.5 mL conical plastic centrifuge tube, and the protein concentration of the supernatant was quantified by a Bradford dye-binding assay (*40*) using BSA as a standard. Protein concentrations were corrected for volume changes caused by the dialysis. As a control, ClO_2 premixed with excess $Na_2S_2O_3$ was added to the protein solution, and the mixture was dialyzed and centrifuged as above. Oxidation of protein will not affect the assay of protein concentration, because the assay employed does not rely on reductive capacity of protein.

Calorimetric Measurements of Thermal Denaturation of Protein. Differential scanning calorimetry (DSC) measurements of thermal denaturation (unfolding) of protein were made on a model Q1000 differential scanning calorimeter (TA Instruments, New Castle, DE) using a 15 μL cell for BSA and G6PD solutions (each 10 mg/mL) treated with or without 15 mM ClO_2 in PBS at 25 °C for 2 min. The reaction with ClO_2 was terminated by 30 mM $Na_2S_2O_3$. ClO_2 that had been premixed with $Na_2S_2O_3$ was added to the protein solution as a "nontreated" control. The samples were degassed before being placed in the calorimeter cells. The

[1] Abbreviations: BSA, bovine serum albumin; G6PD, glucose-6-phosphate dehydrogenase; rpm, revolution per minute; DSC, differential scanning calorimetry; CD, circular dichroism; HPLC, high-performance liquid chromatography; MS, mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight MS; ESI, electrospray ionization; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; NOESY, nuclear Overhouser effect spectroscopy; NFK, *N*-formylkynurenine; DOPA, 3,4-dihydroxy-L-phenylalanine; TOPA, 2,4,5-trihydroxy-L-phenylalanine (3,4,6-trihydroxy-L-phenylalanine).

calorimeter was calibrated using indium (melting point 156.61 °C, heat of melting 28.7 J/g). The scans were performed under a flow (50 mL/min) of nitrogen gas at a constant rate of 5.0 °C/min from 25 °C to 95 °C. The DSC curves obtained were corrected for the instrument baseline, which was obtained by heating the solvent alone. The thermal transition temperature of unfolding of protein (T_m, the temperature at which half of the protein molecules are denatured) was determined for each protein sample by curve fitting, assuming a two-state transition of unfolding. The thermal transition enthalpy (ΔH) at T_m was obtained from the area of the unfolding curve below the baseline. The repeated DSC scans for each sample showed an estimated error for T_m and ΔH of ±0.5 °C and ±100 kJ/mol, respectively.

Circular Dichroism (CD) Spectroscopy. CD spectra of ClO_2 (1 mM)-treated (25 °C, 2 min) and nontreated BSA and G6PD proteins (each 3 mg/mL) were acquired in PBS on a spectropolarimeter (model J-715, Jasco, Tokyo, Japan). Before measurement, the ClO_2-treated and nontreated proteins were dialyzed against PBS at 4 °C for 72 h, and centrifuged at 22000*g* for 30 min at 4 °C to remove insoluble materials. The protein concentration of the supernatant was determined by Bradford reagent (*40*). The CD spectrum of the supernatant was acquired in a quartz cell with a 0.1 cm path length, and the result was normalized to a protein concentration of 1.0 mg/mL.

Assay of Enzymatic Activity. The enzymatic activity of G6PD was assayed spectrophotometrically as suggested by the manufacturer (Sigma) using NADP and glucose-6-phosphate as substrates. The reaction of G6PD (80 μg/mL) with 10 μM ClO_2 was done at 25 °C in PBS, and was terminated by adding $Na_2S_2O_3$ to a final concentration of 20 μM. A control G6PD reaction was set up, in which 10 μM ClO_2 was premixed with 20 μM $Na_2S_2O_3$.

Consumption of ClO_2 by Protein. BSA and G6PD (each 0.5 mg/mL in PBS, 0.5 mL) were mixed with ClO_2 (final concentration 1.05 mM for BSA and 0.86 mM for G6PD) for varying time intervals at 25 °C. The reaction was terminated by adding 2 M KI (final concentration 100 mM). The ClO_2 remaining in the mixture was titrated with 10 mM $Na_2S_2O_3$ as described above for the iodometric titration. The time course curve obtained was fitted to a one-phase exponential decay curve using a computer program (Graphpad Prism 3.02, Hallogram Publishing, Aurora, CO).

Absorption and Fluorescence Spectra. Absorption spectra were acquired by a UVIDEC-505 spectrophotometer (Jasco), and fluorescence spectra (excitation at 280 nm) were acquired by a spectrofluorophotometer (model RF-5300PC, Shimadzu, Kyoto, Japan).

Elemental Analysis of Protein. BSA (20 mg) dissolved in 1 mL of PBS was treated with 1 mM ClO_2 at 25 °C for 2 min; the reaction was then terminated by 2 mM $Na_2S_2O_3$. Next, the mixture was dialyzed extensively against 1000 volumes of distilled water for a week, changing the water three times a day. The dialyzed protein was lyophilized, and its weight was carefully measured under a stream of argon gas. For measurement of chlorine content, the protein was combusted in an oxygen flask, the combustion gas was absorbed by a hydrazine solution, and the solution was subjected to potentiometric titration using a potentiometric titrator (model AT-118, Kyoto Denshi Kogyo, Kyoto, Japan). The detection limit of the titrator was 0.01% (w/w). Oxygen content was determined by a CHN-O-Rapid oxygen analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). The accuracy of this analysis was ±0.15%.

Amino Acid Analysis of Protein. BSA and G6PD (each 200 μg) were first treated with 1 mM ClO_2 at 25 °C for 2 min in 50 μL of PBS. The reaction was terminated with 2 mM $Na_2S_2O_3$, and the mixtures were lyophilized. The lyophilized materials were treated with 1 mL of 30 mM 2-mercaptoethanol containing 0.6 M guanidine hydrochloride at 25 °C for 2 h, and then with 30 mM 4-vinylpyridine at 25 °C for 30 min to derivatize cysteine residues to *S*-β-(4-pyridylethyl)cysteine. The derivatized materials were dialyzed against water and lyophilized. The lyophilized materials were next hydrolyzed in 100 μL of 6 N HCl with 1% (w/v) phenol at 110 °C for 24 and 72 h for common amino acids, and in 4.2 N NaOH with 0.5% (w/v) starch at 110 °C for 16 h for tryptophan. The hydrolysates were analyzed by an amino acid analyzer (model L-8500, Hitachi, Tokyo, Japan). The amount of serine was determined by extrapolating the values obtained at 24 and 72 h of hydrolysis to 0 h.

High-Performance Liquid Chromatography (HPLC). Peptide or amino acid was loaded on a reverse-phase column mounted on an HPLC system (model 880, Jasco). The sample was eluted at a flow rate of 1.0 mL/min at 25 °C, first using 0.1% (v/v) trifluoroacetic acid for 6 min, and then using a linear gradient of 0 to 36% (v/v) acetonitrile in 0.1% trifluoroacetic acid over 54 min. The eluant was monitored by absorption at 215 or 270 nm. When a large amount of a peak material was needed for a subsequent analysis, this HPLC separation was repeated many times on the same scale and the relevant peak fractions were pooled. The pooled material was lyophilized for subsequent analyses. Care was exercised to protect tyrosine-containing peptide derivatives from exposure to light.

HPLC−Mass Spectrometry (MS). An aliquot (about 10 nmol) of an amino acid derivative obtained as a peak fraction from HPLC was lyophilized, and then dissolved in 20 μL of solvent A (0.05% (v/v) formic acid, 50% (v/v) acetonitrile). Next, a 1 μL sample was injected into an LCMS-IT-TOF mass spectrometer using an LC-20AD pump (both Shimadzu) at a flow rate of 100 μL/min and solvent A as the mobile phase. The sample was ionized by electrospray ionization (ESI), and positive ions (primarily $[M + H]^+$ and $[M + 2H]^{2+}$) were acquired at a detector voltage of 1550 V. Tyrosine (molecular weight 181.19) was used to calibrate the apparatus. The amino acid sequence of the peptide was determined by *de novo* peptide sequencing by HPLC−ESI-MS/MS using a Bruker Esquire 3000plus mass spectrometer, and the fragment ions obtained were classified according to Biemann's nomenclature for *b*-series and *y*-series ions (*41*).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). Some peptides were analyzed by a Voyager-DE mass spectrometer (PerSeptive Biosystems, Foster City, CA) in linear mode at an acceleration voltage of 20 kV, using α-cyano-4-hydroxycinnamic acid (10 mg/mL in 0.05% trifuoroacetic acid, 50% acetonitrile) as the matrix. Samples were ionized at a laser repetition frequency of 20 Hz.

Nuclear Magnetic Resonance (NMR) Spectroscopy. A derivatized peptide or amino acid (each 2 mg), obtained as

a peak fraction from HPLC and lyophilized, was dissolved in 600 μL of D_2O, and its ^1H NMR signal was acquired in either a one- or two-dimensional mode using an NMR spectrometer (model Unity Inova 600, Varian, Palo Alto, CA) at 25 °C at 599.855 MHz for ^1H. Partial deuterium water (HDO, 4.700 ppm) was used as a standard. Spectra were acquired under the following conditions: preacquisition delay time = 0.000 s, acquisition time = 3.277 s, pulse width = 100.000 μs, spectral width = 6000.6 Hz. Two-dimensional ($^1H-^1H$) total correlation spectroscopy (COSY) of amino acid derivative or derivatized peptide was performed using a mixing sequence of 100 ms flanked by two 2 ms trim pulses with $256t_1$ and $2028t_2$ data points with a pulse repetition time of 6.0 s. After two-dimensional Fourier transformation, the spectra resulted in 2048 × 2048 data points that were phase- and baseline-corrected in both dimensions. A two-dimensional NOESY spectrum was acquired for a spectrum width of 6 kHz × 6 kHz and data points of 2000 × 2000. The pulse repetition time was 3.5 s.

Aminopeptidase M Digestion of Peptide. ClO_2-treated peptide (~100 nmol) was first separated by HPLC as described above, and a fraction corresponding to a major modified peptide peak was collected. This fraction was lyophilized and digested with 2 units of aminoptidase M in 200 μL of 10 mM sodium phosphate buffer (pH 7.0) at 25 °C for 4 h to release amino acids from the modified peptide. The amino acids released were then separated by HPLC, and a fraction corresponding to a peak of modified amino acid was collected. This fraction was subjected to further analyses after lyophilization.

Proteinase K and Aminopeptidase M Digestion of Protein. BSA and G6PD (each 1 mg) were treated with 1 mM ClO_2 in 2 mL of PBS at 25 °C for 2 min, and the reaction was terminated by 2 mM $Na_2S_2O_3$. Next, proteinase K powder was added to a final concentration of 0.5 mg/mL and the mixture was incubated at 37 °C for 2 h. The mixture was then subjected to ultrafiltration using a membrane with a molecular weight cutoff of 10 000. The filtrate was collected and lyophilized. The lyophilized material was dissolved in 170 μL of distilled water containing 5 units of aminopeptidase M. The mixture was incubated at 25 °C for 4 h, and separated by HPLC to isolate modified amino acids.

Synthesis of $Cl^{18}O_2$. NaCl (0.64 g) and $Na_2Cr_2O_7$ (12 mg) were dissolved in 1.7 mL of $H_2{}^{18}O$ containing 4.3 μL of 2 N HCl. The solution was subjected to electrolysis at 60 °C for 3 h at 0.6 A using platinum electrodes. The solution was next slowly and gently mixed with 187 μL of 10.25 M H_2SO_4, 400 μL of 11.4 N HCl, 850 μL of 10 N NaOH, and 510 μL of 30% (w/v) H_2O_2 in this order at 25 °C. The mixed solution was next boiled for 2 min to break down excess H_2O_2 into oxygen and water. The solution was finally mixed with 1.2 mL of 2 N HCl and bubbled gently with air to recover $Cl^{18}O_2$ into 5 mL of distilled water chilled on ice.

^{18}O-Labeling Experiment. Peptide (0.2 mM) was treated with 0.3 mM ClO_2 at 25 °C for 2 min in PBS. In this experiment, all waters used for the reaction were either $H_2{}^{18}O$ in place of common distilled water ($H_2{}^{16}O$) or common distilled water that had been bubbled beforehand with $^{18}O_2$ gas. The $^{18}O_2$-bubbled water was made first by degassing distilled common water under reduced pressure, and then it was bubbled using N_2 gas until dissolved oxygen gas concentration decreased to 2 mg/L (measured by an oxygen meter). This water was next bubbled extensively using $^{18}O_2$ gas until dissolved oxygen gas concentration became 14 mg/L. The peptide treated with ClO_2 was then mixed with excess $Na_2S_2O_3$, and loaded on HPLC to isolate modified peptide as described above. The isolated peptide was analyzed by HPLC−ESI-MS/MS to measure molecular weight of modified amino acid residue in the peptide from fragment ions. In another experiment, peptide was treated with $Cl^{18}O_2$ as above using common distilled water.

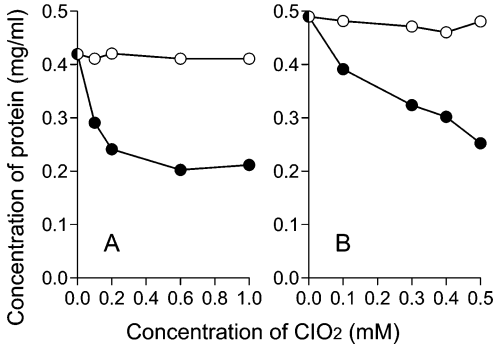

FIGURE 1: Solubility of protein treated with or without ClO_2. BSA (A) and G6PD (B) (each 0.5 mg/mL) were treated in PBS with varying concentrations of ClO_2 at 25 °C for 2 min, and the mixtures were dialyzed after the addition of $Na_2S_2O_3$ to a final concentration of 2 mM. The dialyzed solutions were centrifuged, and the protein concentrations in the supernatant were quantified (filled circles). As a control, ClO_2 that had been premixed with 2 mM $Na_2S_2O_3$ was added to the protein solution, and then treated as above (open circles). Each point represents a mean of two experiments.

Edman Degradation of Peptide. Peptide (~0.5 nmol) was subjected to automated Edman degradation by using a Procise protein sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's recommended protocol.

RESULTS

Denaturation of Protein by ClO_2. When BSA and G6PD were treated with ClO_2, their solubility decreased in a concentration-dependent manner (Figure 1). This result suggests that protein is denatured by ClO_2. Next, the physicochemical parameters of the proteins on thermal denaturation (unfolding) were measured. For BSA, the denaturation temperature (T_m) decreased from 74.5 °C to 70.7 °C upon treatment with ClO_2 (Figure 2A, B); in addition the thermal transition enthalpy (ΔH) decreased from 1390 to 460 kJ/mol after treatment. The T_m of untreated G6PD was 55.6 °C, whereas ClO_2-treated G6PD did not show a clear unfolding temperature, indicating that G6PD had only a marginal amount of higher-order protein structure after treatment with ClO_2. The ΔH of G6PD decreased from 1090 kJ/mol to 0 kJ/mol (actually not detectable) after ClO_2 treatment (Figure 2C,D). Taken together, these results indicate that the higher-order structure of protein becomes less stable after treatment with ClO_2, and is more easily disrupted by an increase in temperature.

This view was further supported by circular dichroism (CD) spectroscopy, in which the ellipticity of BSA and G6PD at ~220 nm (which represents primarily the α-helical content of protein) became closer to zero when the proteins were treated with ClO_2 (Figure 3). This reduction indicates that the α-helical content in BSA and G6PD decreased after treatment with ClO_2. However, the decrease in α-helical

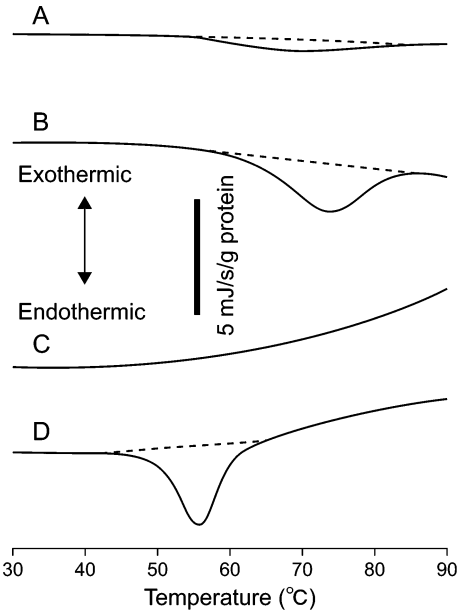

FIGURE 2: Differential scanning calorimetry of heat-induced denaturation of protein. Heat-induced denaturation profiles of BSA (A, B) and G6PD (C, D) treated with (A, C) or without (B, D) ClO_2 were measured by a differential scanning calorimeter. Proteins (each 10 mg/mL) were treated with 15 mM ClO_2 in PBS at 25 °C for 2 min, and the reaction was terminated by 30 mM $Na_2S_2O_3$. ClO_2 that had been premixed with $Na_2S_2O_3$ was added to the protein solution as a "nontreated" control (B, D). The reaction mixtures were directly analyzed by differential scanning calorimetry. Scans were performed from 25 to 95 °C at a constant rate of 5.0 °C/min. Baselines used for calculation of endothermic transition enthalpy ΔH are shown by broken lines. ΔH was calculated from the area of the curve below the broken line. Experiments were done twice independently, and similar results were obtained. The data shown is one of them.

content, as demonstrated by the ellipticity, was relatively small in BSA (Figure 3A).

Inhibition of G6PD Enzymatic Activity by ClO_2. The protein-denaturing activity of ClO_2 was next confirmed by measuring the enzymatic activity of G6PD. When G6PD was treated with ClO_2, its enzymatic activity decreased markedly in both a time- and concentration-dependent manner (Figure 4, filled circles). This inactivation of G6PD enzymatic activity by ClO_2 appeared within 15 s of the addition of 10 μM ClO_2, and then reached a plateau; the enzymatic activity decreased to 10% within 15 s. The addition of a second aliquot of 10 μM ClO_2 at 4 min after the first addition (Figure 4, arrow) inactivated the remaining enzymatic activity. This observation suggests that the first aliquot of ClO_2 added to the protein solution had been totally "consumed" presumably due to its chemical reaction with G6PD molecules, and consequently it was not able to inactivate the remaining enzymatic activity.

Chemical Reaction of Protein with ClO_2. The above idea was further confirmed in an experiment in which the amount of ClO_2 remaining after reaction with BSA or G6PD was quantified. The purpose of this experiment was to determine how much ClO_2 had been consumed by its reaction with protein. As shown in Figure 5, the ClO_2 that had been added to a protein solution decreased exponentially and this decrease was temperature-dependent (Figure 5, insets). This observation suggests that protein is denatured by a chemical reaction involving ClO_2, in which ClO_2 molecules are

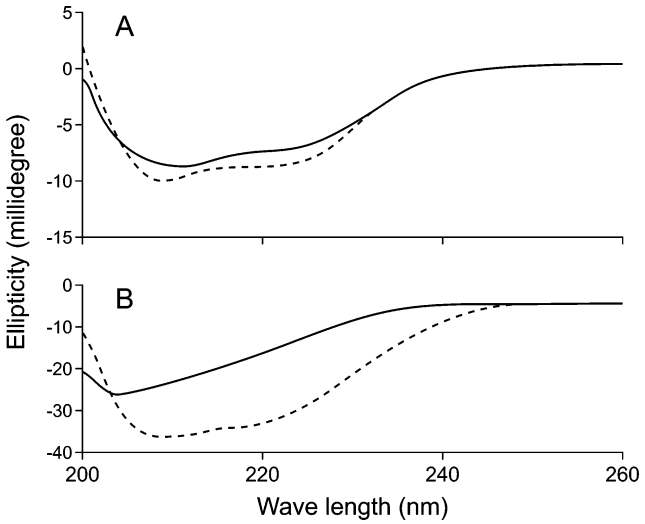

FIGURE 3: CD spectra of protein treated with or without ClO_2. BSA (A) and G6PD (B) (each 3 mg/mL) were treated with (solid line) or without (broken line) 1 mM ClO_2 in PBS at 25 °C for 2 min, and the reaction mixtures were dialyzed after the addition of $Na_2S_2O_3$ to a final concentration of 2 mM. CD spectra of the dialyzed protein solutions were measured after removal of insoluble materials by centrifugation. As a control, 1 mM ClO_2 that had been premixed with 2 mM $Na_2S_2O_3$ was added to the protein solution, and CD spectra were measured as above. The ellipticity was normalized to a protein concentration of 1.0 mg/mL. Experiments were done twice independently, and similar results were obtained. The data shown is one of them.

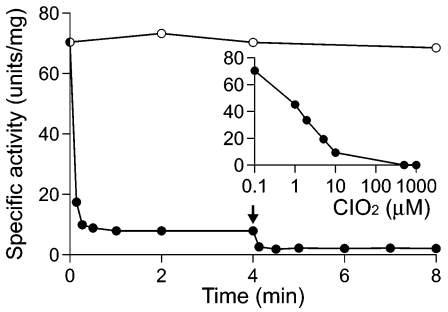

FIGURE 4: Inactivation of the enzymatic activity of G6PD by ClO_2. G6PD (80 μg/mL) was treated with 10 μM ClO_2 in PBS at 25 °C for the times indicated (filled circles). After the reaction of 4 min, a second aliquot of ClO_2 was added to the mixture to a final concentration of 10 μM (arrow). The reaction of ClO_2 was terminated by the addition of a 2-fold molar excess of $Na_2S_2O_3$. The enzymatic activity of G6PD was then assayed. As a control, G6PD was treated with 10 μM ClO_2 that had been premixed with 20 μM $Na_2S_2O_3$, and then its enzymatic activity was assayed (open circles). The inset shows the enzymatic activity of G6PD (80 μg/mL) treated in PBS at 25 °C for 2 min with varying concentrations of ClO_2 indicated. The reaction was terminated by an excess amount of $Na_2S_2O_3$. Each point represents a mean of two experiments.

consumed. It further suggests that protein is covalently modified by ClO_2.

The covalent modification of protein by ClO_2 was examined by elemental analysis of BSA treated with ClO_2 (Table 1). Whereas the amount of chlorine in BSA did not increase after its reaction with ClO_2, the amount of oxygen increased from 26.90% (w/w) to 28.39% (w/w). This increase is significant, with respect to the accuracy of the method of elemental analysis used (±0.15%). This increase in the oxygen content of the ClO_2-treated BSA implies that each molecule of BSA has acquired about 64 oxygen atoms after its reaction with ClO_2. By contrast, not even a single

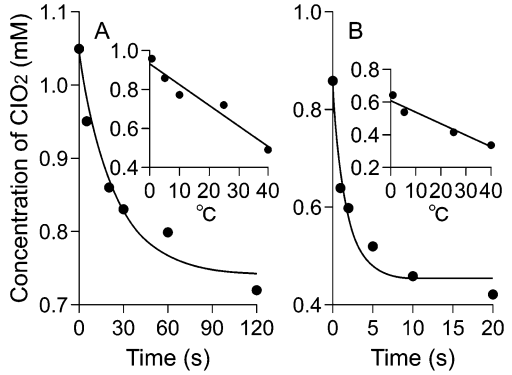

FIGURE 5: Consumption of ClO_2 by the reaction with protein. ClO_2 was mixed at 25 °C with 0.5 mg/mL BSA (A) or G6PD (B) in PBS for the time indicated, and the reaction was terminated by the addition of KI to a final concentration of 100 mM. The amount of ClO_2 remaining in the reaction mixture was measured by iodometric titration. The curve was obtained by fitting the data to a one-phase exponential decay curve. The inset shows ClO_2 concentration remaining after a 2 min reaction as a function of temperature. Each point represents a mean of two experiments.

Table 1: Elemental Analyses of BSA Treated with or without ClO_2[a]

protein	wt % chlorine	wt % oxygen
BSA	0.01	26.90
ClO_2-treated BSA	< 0.01	28.39
BSA mixed with ClO_2^- [b]	0.01	26.86
BSA mixed with 0.1 mg of NaCl[c]	0.29	nd[d]

[a] Each value represents a mean of two experiments. BSA (20 mg) was treated with 1 mM ClO_2 in 1 mL of PBS at 25 °C for 2 min. The reaction was terminated by 2 mM $Na_2S_2O_3$. The reaction mixture was then dialyzed extensively against distilled water and lyophilized. The lyophilized protein was subjected to elemental analyses for chlorine and oxygen. [b] Treated with 1 mM ClO_2 that had been premixed with 2 mM $Na_2S_2O_3$. ClO_2 becomes ClO_2^- anion in this reaction. [c] Added after the dialysis. The theoretical amount of chlorine in this mixture is 0.302% (w/w). [d] Not determined.

molecule of chlorine was incorporated into BSA, as assessed by the detection level of chlorine atoms in the assay (0.01%). Theoretically, if one chlorine atom had been incorporated into one molecule of BSA, the chlorine content of BSA should have become 0.053%, which is well above the detection level of the method used.

To confirm that proteins are covalently modified by ClO_2, the amino acid composition of ClO_2-treated BSA and G6PD was determined. The numbers of tyrosine and tryptophan residues decreased significantly in both BSA and G6PD after ClO_2 treatment (Table 2), suggesting that tyrosine and tryptophan residues are covalently modified by ClO_2. The numbers of other amino acids did not decrease significantly in this analysis.

Absorption and Fluorescence Spectra of ClO_2-Treated Protein. The absorption spectra of ClO_2-treated BSA and G6PD proteins showed that the 280 nm absorption (peak or shoulder) characteristic of the proteins had disappeared (Figure 6, solid line). The spectra showed monotonically decreasing absorption that extended up to ~400 nm. The complete disappearance of the 280 nm absorption peak or shoulder in the ClO_2-treated proteins (Figure 6) strongly suggested that the tryptophan residues were destroyed by ClO_2. This interpretation was further supported by the fluorescence spectra of ClO_2-treated BSA and G6PD proteins

Table 2: Amino Acid Composition of ClO_2-Treated Protein[a]

amino acid	BSA expected	BSA found	G6PD expected	G6PD found
Asp	54	54.7	58	58.5
Thr	33	32.8	22	21.2
Ser	28	29.8	30	29.5
Glu	79	82.1	53	54.7
Gly	16	18.4	32	33.8
Ala	47	46.7	29	29.4
1/2 Cys	35	37.0	1	1.3
Val	36	35.1	37	36.5
Met	4	2.1	11	9.6
Ile	14	13.5	27	28.3
Leu	61	61.9	45	47.8
Tyr	20	13.7	21	14.6
Phe	27	26.7	27	26.7
Lys	59	56.2	43	41.8
His	17	16.1	10	10.1
Trp	2	0.7	5	2.5
Arg	23	22.8	25	24.5
Pro	28	30.4	29	29.0

[a] Values indicate moles of amino acid per mole of protein. Each value, except for tryptophan, represents a mean of two experiments. The value of tryptophan is a mean of three experiments.

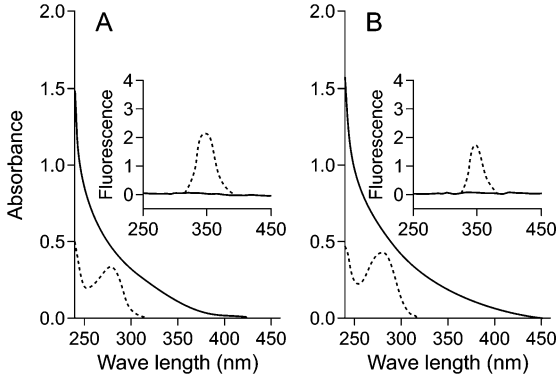

FIGURE 6: Absorption and fluorescence spectra of protein treated with or without ClO_2. The absorption and fluorescence (inset, excitation at 280 nm) spectra of BSA (A) and G6PD (B) (each 0.5 mg/mL) treated with 1 mM ClO_2 in PBS at 25 °C for 2 min are shown (solid lines). The ClO_2 reaction was terminated by 2 mM $Na_2S_2O_3$. As controls, spectra of protein treated with 1 mM ClO_2 that had been premixed with 2 mM $Na_2S_2O_3$ are shown (dotted lines). Each absorption spectrum was obtained after subtracting that of a mixture of 1 mM ClO_2 and 2 mM $Na_2S_2O_3$ as a background. The fluorescence intensity is shown in an arbitrary unit. Experiments were done twice independently, and similar results were obtained. The data shown is one of them.

excited at 280 nm. When excited at 280 nm, proteins that contain tryptophan commonly show a characteristic fluorescence emission peak at ~350 nm. As expected, BSA and G6PD that were not treated with ClO_2 showed a sharp fluorescence emission peak at 348 nm (Figure 6, inset, dotted line). When these proteins were treated with ClO_2, however, this peak disappeared completely (Figure 6, inset, solid line). This result strongly indicates that tryptophan residues in proteins are destroyed by ClO_2.

Reaction of Model Peptides with ClO_2. To substantiate further the covalent modification of tryptophan and tyrosine residues in proteins by ClO_2, I used two model peptides derived from BSA, namely, ALKAWSVAR (residues 209−217) and GSFLYEYSR (residues 327−335). When these peptides were treated with ClO_2, new peptide peaks appeared on the HPLC chromatogram as compared with the nontreated

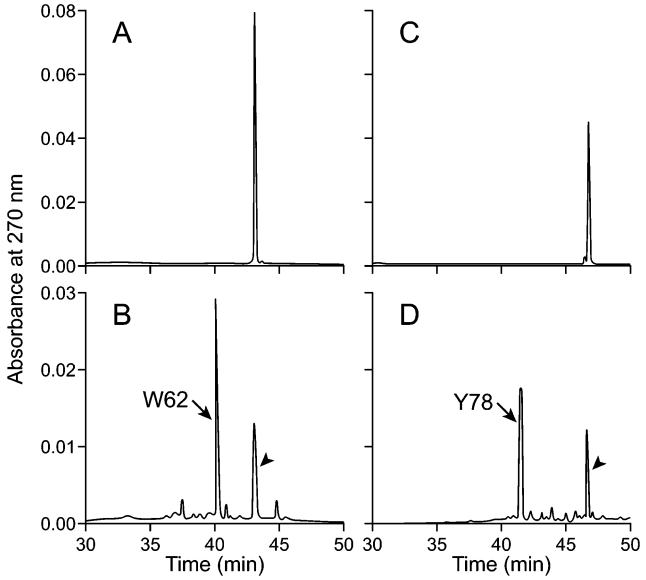

FIGURE 7: HPLC analyses of peptides treated with or without ClO_2. An HPLC profile of 0.2 mM peptide ALKAWSVAR (100 μL) treated with (B) or without (A) 0.3 mM ClO_2 in PBS at 25 °C for 2 min and loaded on a C_{18} reverse-phase column is shown. The reaction was terminated by 0.6 mM $Na_2S_2O_3$. The column was eluted at a flow rate of 1.0 mL/min. Likewise, 0.2 mM peptide GSFLYEYSR treated with (D) or without (C) 0.3 mM ClO_2 is shown. In B and D, major reaction products collected for subsequent analyses (W62 and Y78) are shown by arrows. Intact (unreacted) peptides are shown by arrowheads. Experiments were done five times independently, and similar results were obtained. The data shown is one of them.

peptides (Figure 7B,D, arrows). The major peak observed for the ClO_2-treated peptide ALKAWSVAR was named W62 (Figure 7B, arrow, retention time 40.1 min), and that for the ClO_2-treated peptide GSFLYEYSR was named Y78 (Figure 7D, arrow, retention time 41.6 min). For both peptides, there was also a peak representing unreacted (intact) peptide on the HPLC chromatogram (retention time 43.3 min in Figure 7B, arrowhead, and retention time 46.9 min in Figure 7D, arrowhead). Notably, there were also at least ten small peaks on the HPLC chromatogram of the ClO_2-treated peptides (Figure 7B,D), indicating that the peptides underwent some minor reactions with ClO_2; however, I did not pursue these minor reaction products further. When the tryptophan (W) and tyrosine (Y) residues in these peptides were replaced by glycine (**G**), and the resulting peptides (ALKA**G**SVAR and GSFL**G**E**G**SR, respectively) were treated with ClO_2, no new peptide peaks appeared (Figure 8B,D), providing direct evidence that the new peaks (Figure 7, W62 and Y78) observed after treatment of the peptides ALKAWSVAR and GSFLYEYSR with ClO_2 were due solely to covalent modifications of the tryptophan and tyrosine residues in these peptides.

To characterize further the kind of modifications of the tryptophan and tyrosine residues in these peptides, fractions corresponding to the peptide peaks W62 and Y78 were isolated by HPLC and were analyzed by HPLC−ESI-MS/MS. The fifth residue in the peptide ALKAWSVAR had become 31.8 atomic mass units heavier than tryptophan (molecular weight 204.22) (Figure 9A), indicating that the tryptophan residue had been oxidized by two atoms of oxygen. Both of the fifth and the seventh residues of the peptide GSFLYEYSR had become 15.9 atomic mass units

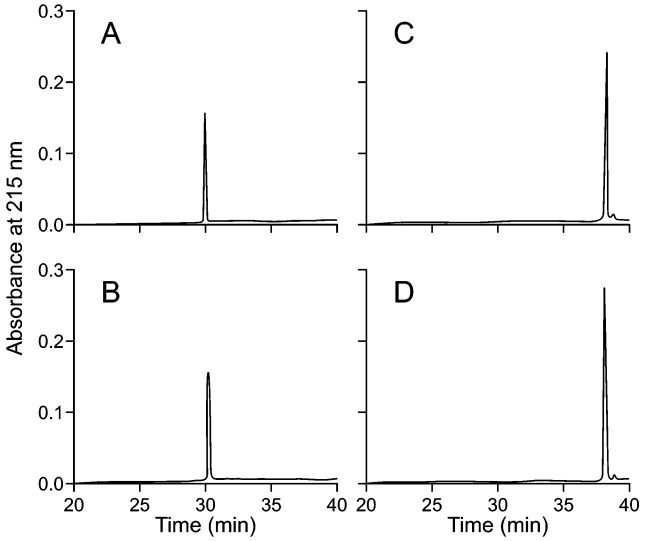

FIGURE 8: HPLC analyses of glycine-substituted peptides treated with or without ClO_2. An HPLC profile of 0.2 mM peptide ALKA**G**SVAR (100 μL) treated with (B) or without (A) 0.3 mM ClO_2 in PBS at 25 °C for 2 min and loaded on a C_{18} reverse-phase column is shown. The reaction was terminated by 0.6 mM $Na_2S_2O_3$. The column was eluted at a flow rate of 1.0 mL/min. Likewise, 0.2 mM peptide GSFL**G**E**G**SR treated with (D) or without (C) 0.3 mM ClO_2 is shown. Experiments were done twice independently, and similar results were obtained. The data shown is one of them.

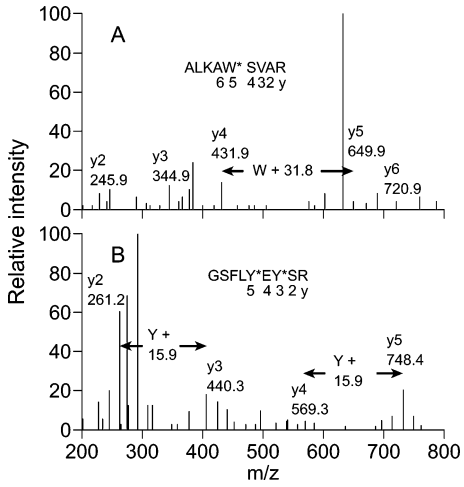

FIGURE 9: Mass spectrometric analyses of ClO_2-treated peptides. Peptide ALKAWSVAR or GSFLYEYSR (each 0.2 mM) was treated with 0.3 mM ClO_2 in PBS at 25 °C for 2 min. The reaction was terminated by 0.6 mM $Na_2S_2O_3$. The reaction mixture was then loaded on HPLC, and peak compounds that were modified by ClO_2 (peak W62 in Figure 7B and peak Y78 in Figure 7D) were collected. They were next analyzed by HPLC−ESI-MS/MS. The *y*-ion series derived from W62 (A) and from Y78 (B) are shown. Modified tryptophan and tyrosine residues are shown by W* and Y*, respectively. The difference of *m*/*z* between *y*5 and *y*4 ions is shown ($W + 31.8$), where W denotes molecular weight of tryptophan − 18.0, namely, 204.2 − 18.0 (A). The differences of *m*/*z* between *y*5 and *y*4 ions, and between *y*3 and *y*2 ions are shown (each $Y + 15.9$), where Y denotes molecular weight of tyrosine − 18.0, namely, 181.2 − 18.0 (B). Experiments were done twice independently, and similar results were obtained. The data shown is one of them.

heavier than tyrosine (molecular weight 181.19) (Figure 9B), indicating that each of the two tyrosine residues in this peptide had been oxidized by one atom of oxygen.

Reaction Stoichiometry of ClO_2. To elucidate the reaction stoichiometry between ClO_2 and protein, peptide, or amino

Table 3: Consumption of ClO_2 by a Reaction with Amino Acid, Peptide, or Protein[a]

	amino acid, peptide, or protein (mM)			ClO_2 (mM)			
	added	recovered[b]	consumed[c]	added	recovered[b]	consumed[c]	stoichiometry[d]
tryptophan	0.50	0.00	0.50	3.4	2.0	1.4	2.8
tyrosine	0.50	0.00	0.50	3.4	1.8	1.6	3.2
ALKAWSVAR	0.50	0.00	0.50	3.4	1.8	1.6	3.2
GSFLYEYSR	0.50	0.00	0.50	3.4	1.3	2.1	4.2
ALKA**G**SVAR	0.50	0.49	0.01	3.4	2.9	0.51	nd[e]
GSFL**G**E**G**SR	0.50	0.52	0.00	3.4	2.9	0.47	nd[e]
BSA	0.0075	nd[e]	nd[e]	3.4	2.5	0.88	nd[e]
G6PD	0.0075	nd[e]	nd[e]	3.4	2.4	1.0	nd[e]

[a] Amino acid, peptide, or protein of indicated concentration was mixed with ClO_2 in PBS at 25 °C for 2 min. It was then mixed with 2 M KI to a final concentration of 100 mM, and ClO_2 concentration remaining in the mixture was quantified by an iodometric titration. [b] Measured after the reaction with amino acid, peptide, or protein. ClO_2 was measured by the iodometric titration. Amino acid and peptide were measured by HPLC. Each data is a mean of five experiments, where the error of the assay was within 4%. [c] Calculated by subtracting the amount recovered from the amount added. [d] A molar ratio of ClO_2 consumed to amino acid, peptide, or protein consumed. [e] Not determined.

acid, I quantified the amount of ClO_2 remaining in the reaction mixture of each type of compound with ClO_2. Each molecule of tryptophan reacted with about three molecules of ClO_2 regardless of whether it was free or bound in peptide (Table 3). Each molecule of tyrosine bound in peptide reacted with two molecules of ClO_2. By contrast, each molecule of free tyrosine reacted with about three molecules of ClO_2, which strongly indicates that the reaction mechanism of free tyrosine with ClO_2 differs from that of tyrosine bound in peptide. Notably, the peptides with glycine replacement, namely, ALKA**G**SVAR and GSFL**G**E**G**SR (Figure 8), reacted with only a small amount of ClO_2 (Table 3). This result is consistent with the data in Figure 8, showing that amino acids other than tryptophan and tyrosine in these peptides do not react with ClO_2.

Amino Acid Residues Modified by ClO_2. Fractions corresponding to the peptide peaks W62 and Y78 (Figure 7B,D) were isolated by HPLC, and then treated with aminopeptidase M to release the modified amino acids for further characterization. The amino acids released by aminopeptidase M were separated by a second round of HPLC (data not shown). The amino acid derivatives released from peptides W62 and Y78 were designated W* and Y*, and showed retention times of 23.8 min and 12.6 min, respectively, on HPLC. From the MS analyses shown in Figure 9, it was already evident that W* was modified tryptophan and Y* was modified tyrosine.

Structures of the Modified Amino Acids. Compound W*, the tryptophan derivative released from peptide W62 by aminopeptidase M and isolated by HPLC (above), was further analyzed by HPLC−MS. From W*, an $[M + H]^+$ ion of $m/z = 237.1$ was generated, which is consistent with *N*-formylkynurenine (NFK), whose theoretical value of an $[M + H]^+$ ion is $m/z = 237.2$ (Figure 10A). A peak at $m/z = 209.1$ was also observed, which is consistent with NFK lacking a formyl residue, i.e., kynurenine, whose theoretical value of an $[M + H]^+$ ion is $m/z = 209.2$. The identification of W* was further checked by its absorption spectrum (data not shown); the spectrum of W* showed λ_{max} values of 240, 260, and 321 nm, and λ_{min} values of 248 and 283 nm, which are exactly the same as those reported for NFK (*42*). The identification of W* as NFK was next confirmed by one-dimensional ^1H NMR and two-dimensional $^1H-^1H$ COSY NMR (Figures 11 and 12). In the ^1H NMR spectrum, a signal at 8.334 ppm was consistent with that of aldehyde. The other signals were also consistent with the identification of W* as NFK. From these results, I conclude that W* obtained from the reaction of a tryptophan-containing peptide with ClO_2 is NFK. In addition, I found that NFK was a major reaction product obtained from the reaction of ClO_2 with free tryptophan (data not shown).

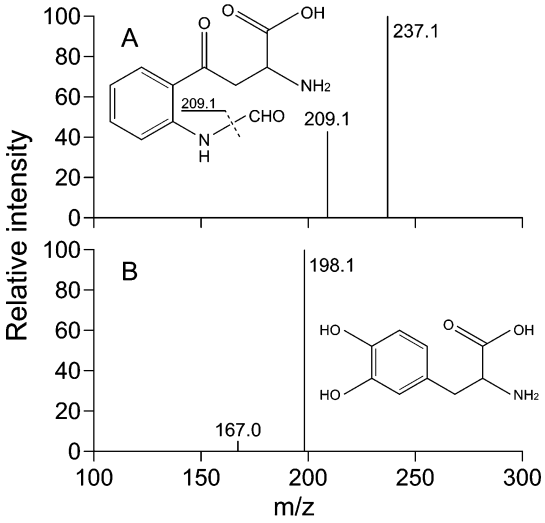

FIGURE 10: Mass spectrometric analyses of modified amino acids obtained from ClO_2-treated peptides. Major reaction products of peptides ALKAWSVAR and GSFLYEYSR (each 0.2 mM) treated with 0.3 mM ClO_2 (peak W62 in Figure 7B and peak Y78 in Figure 7D) were isolated by HPLC and lyophilized. They were then treated with aminopeptidase M to enzymatically release modified amino acids (W* and Y* in Figure 9) from the peptides. The aminopeptidase M-treated peptides were next loaded on HPLC to isolate the modified amino acids. The modified amino acids collected as single major peaks were next analyzed by HPLC−MS. The mass spectra derived from modified tryptophan (A) and tyrosine (B) are shown. Structures of *N*-formylkynurenine (NFK) (A) and 3,4-dihydroxyphenylalanine (DOPA) (B) are shown. Experiments were done twice independently, and similar results were obtained. The data shown is one of them.

Next, I tried to determine the structure of the modified tyrosine residue using peptide Y78 (Figure 7D). I first isolated peptide Y78 by HPLC, and then treated it with aminopeptidase M to release modified tyrosine (Y*). As shown by MS (Figure 9B), it was already evident that the only amino acid modified in peptide Y78 was tyrosine. When peptide Y78 treated with aminopeptidase M was separated by HPLC, a peak at a retention time of 12.6 min was obtained (data not shown). The retention time of Y* was exactly same as that of authentic 3,4-dihydroxyphenylalanine (DOPA) (12.6 min). In addition, the compound Y* showed a λ_{max}

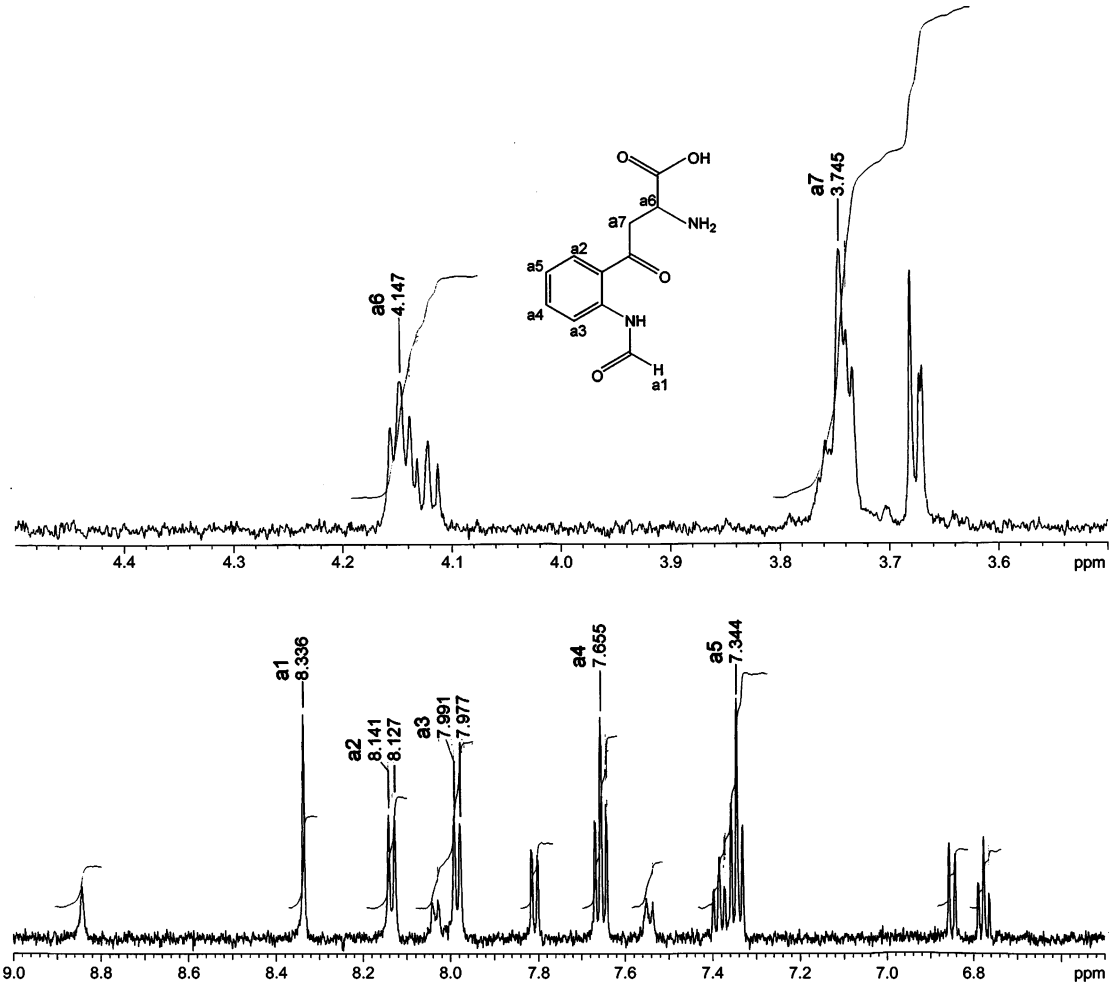

FIGURE 11: A one-dimensional ^{1}H NMR spectrum of modified amino acid isolated from ClO_2-treated peptide. Peptide ALKAWSVAR (0.2 mM) was treated with 0.3 mM ClO_2 in PBS at 25 °C for 2 min, and the reaction was terminated by 0.6 mM $Na_2S_2O_3$. The reaction mixture was then loaded on HPLC, and modified peptide (peak W62 in Figure 7B) was isolated. The isolated peptide was next treated with aminopeptidase M to release modified amino acid (W* in Figure 9A). The modified amino acid (tryptophan derivative) was next isolated by HPLC. Its 600 MHz ^{1}H NMR spectrum is shown. The structure of *N*-formylkynurenine is also shown. Experiments were done twice independently, and similar results were obtained. The data shown is one of them.

value of 279 nm, which is also the same as that of DOPA. Judging from its structure, DOPA is derived from tyrosine by the addition of one atom of oxygen. Therefore, DOPA is consistent with observed modification of the tyrosine residues in Y78 (Figure 9B). These results suggest that the two tyrosine residues in Y78 became DOPA residues after reaction with ClO_2; in other words, Y* is DOPA. This idea was further supported by HPLC−MS of Y*; the spectrum showed a peak at $m/z = 198.1$ for an $[M + H]^+$ ion (Figure 10B), which is almost same as that of DOPA, whose theoretical value for an $[M + H]^+$ ion is $m/z = 198.2$. Taken together, I conclude that the modified tyrosine residues (Y*) in Y78 are DOPA.

I next used a shorter model peptide EYSR (residues 332−325 of BSA) as a starting material. When EYSR (HPLC retention time 21.6 min) was treated with ClO_2, a major reaction product (hereafter called Y93) appeared at a retention time of 23.2 min on HPLC (data not shown). Next, I isolated Y93 by HPLC and analyzed it directly by one-dimensional ^{1}H NMR and two-dimensional ^{1}H NOESY (Figures 13 and 14). In the one-dimensional ^{1}H NMR spectrum, aromatic proton signals of 7.204 and 6.847 ppm were found. Because these signals were found as singlets, these protons were interpreted to be in *para* orientation each other. Interpretation of the ^{1}H NOESY spectrum suggested that the 6.847 ppm signal was from the position 2 proton and the 7.204 ppm signal was from the position 5 proton, because a NOESY correlation signal was found between these protons. In summary, only the tyrosine residue in the peptide EYSR was modified by ClO_2, and it became 2,4,5-trihydroxyphenylalanine (TOPA) on the reaction with ClO_2.

To demonstrate directly that tryptophan and tyrosine residues of proteins are indeed modified by ClO_2, G6PD treated with ClO_2 was digested first with proteinase K and then with aminopeptidase M to release the modified amino acids from the proteinase K-digested protein. These modified amino acids showed peaks at retention times 11.5 and 23.3 min on HPLC (data not shown); these retention times are almost same as those of Y* (12.6 min) and W* (23.8 min), respectively, that were recovered from the ClO_2-treated peptides Y78 and W62. Fractions corresponding to the peaks with these retention times were next subjected to HPLC−MS, which showed a peak at $m/z = 198.1$ ($[M + H]^+$ ion) for the compound with a retention time of 11.5 min and a peak at $m/z = 237.1$ ($[M + H]^+$ ion) for the compound with a retention time of 23.3 min. These m/z values are also consistent with those observed for DOPA ($m/z = 198.2$) and NFK ($m/z = 237.2$). On the basis of the

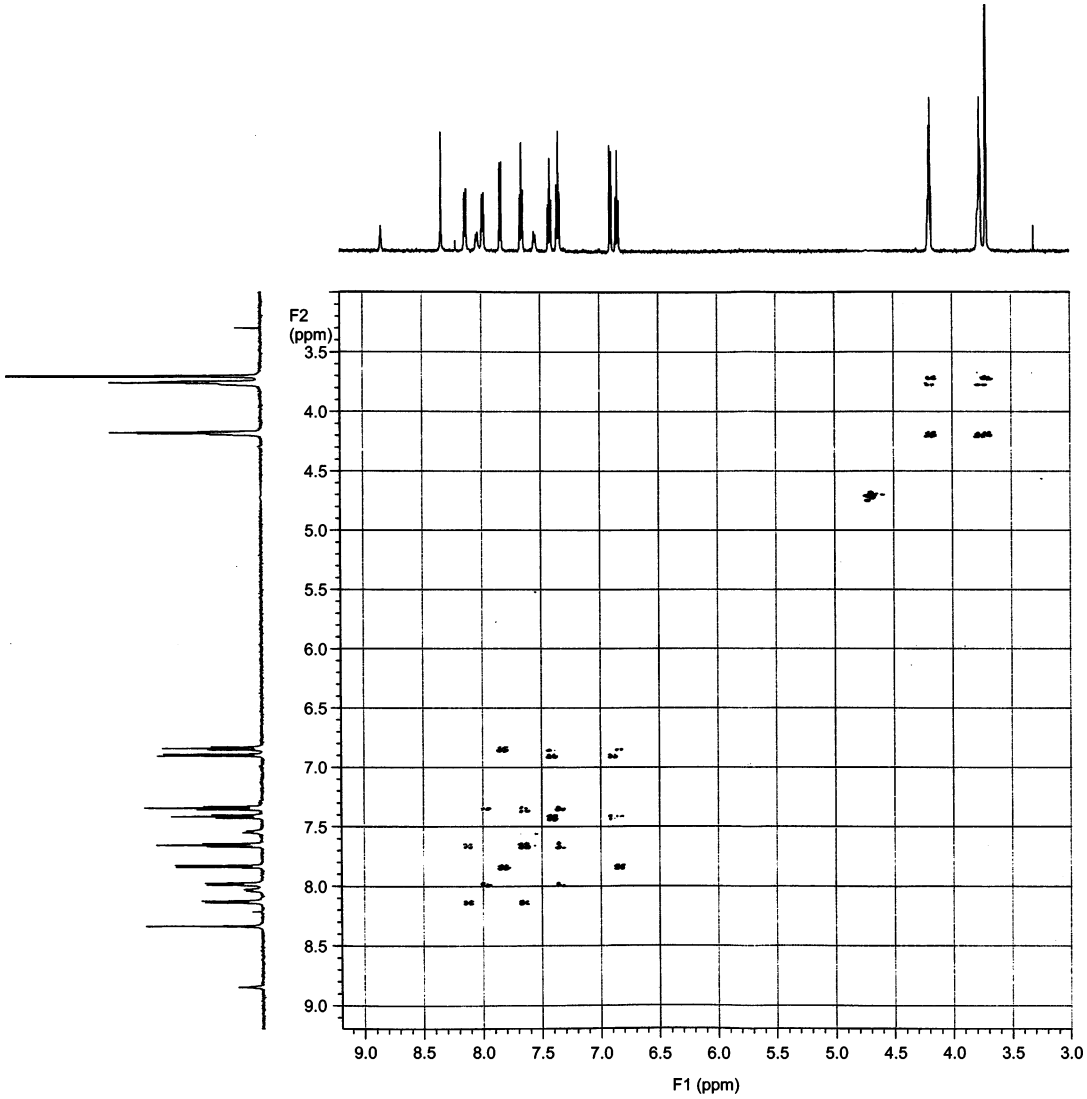

FIGURE 12: A two-dimensional $^1H-^1H$ COSY NMR spectrum of modified amino acid isolated from ClO_2-treated peptide. Peptide ALKAWSVAR (0.2 mM) was treated with 0.3 mM ClO_2 in PBS at 25 °C for 2 min, and the reaction was terminated by 0.6 mM $Na_2S_2O_3$. The reaction mixture was then loaded on HPLC, and modified peptide (peak W62 in Figure 7B) was isolated. The isolated peptide was next treated with aminopeptidase M to release modified amino acid (W* in Figure 9A). The modified amino acid (tryptophan derivative) was next isolated by HPLC. Its $^1H-^1H$ COSY spectrum is shown. Experiments were done twice independently, and similar results were obtained. The data shown is one of them.

HPLC retention times and the *m*/*z* values, I conclude that DOPA and NFK are indeed formed in proteins after treatment with ClO_2.

Origin of the Oxygen Atoms. To elucidate the origin of the oxygen atoms incorporated in the amino acids in the oxidation reaction by ClO_2, an ^{18}O experiment was done. When water or dissolved oxygen gas in the reaction mixture was replaced by $H_2{}^{18}O$ or $^{18}O_2$, respectively, the MS analyses of the reaction product (as in Figure 9) did not show any significant change in *m*/*z* values of product ions despite the high resolution of the *m*/*z* values of the mass spectrometer used, indicating that ^{18}O atoms are not incorporated in the product oxidized by ClO_2 under these conditions (Table 4, lines 6−9). This result strongly suggests that the oxygen atoms in NFK, DOPA, and TOPA do not come from water or dissolved oxygen gas, but rather from ClO_2. Direct evidence in support of this interpretation was obtained in an experiment using $Cl^{18}O_2$. When the peptides were treated with $Cl^{18}O_2$ in common distilled water, tryptophan and tyrosine derivatives having 36.4 and 18.2, respectively, atomic mass units were obtained (Table 4, line 10), indicating that these amino acid residues were oxidized by ^{18}O that came from $Cl^{18}O_2$. The phosphate buffer used for the reaction was not the origin of the oxygen atoms, because the oxidized peptide (Figure 7, W62 and Y78) appeared without the buffer (data not shown).

Sites of Protein Modified by ClO_2. To determine the sites in the proteins modified by ClO_2, BSA and G6PD that had been treated with ClO_2 were digested first with trypsin and then with subtilisin. The digested peptides were separated by HPLC. Edman degradation sequencing of the peptides isolated from BSA identified the peptides EKLGE**X**G (corresponding to residues 395−401), ED**X**LS (residues 449−453), and ET**X**V (residues 494−497). The unknown, and thus presumably modified, residue (**X**) in these peptides was found to be tyrosine upon comparison of the amino acid sequences of these peptides with the sequence of BSA, suggesting that tyrosine residues Tyr^{400}, Tyr^{451}, and Tyr^{496} were the sites modified by ClO_2. Similarly, peptides EG**X**-LDP (residues 38−43), GN**X**DT (residues 93−97), A**X**DD

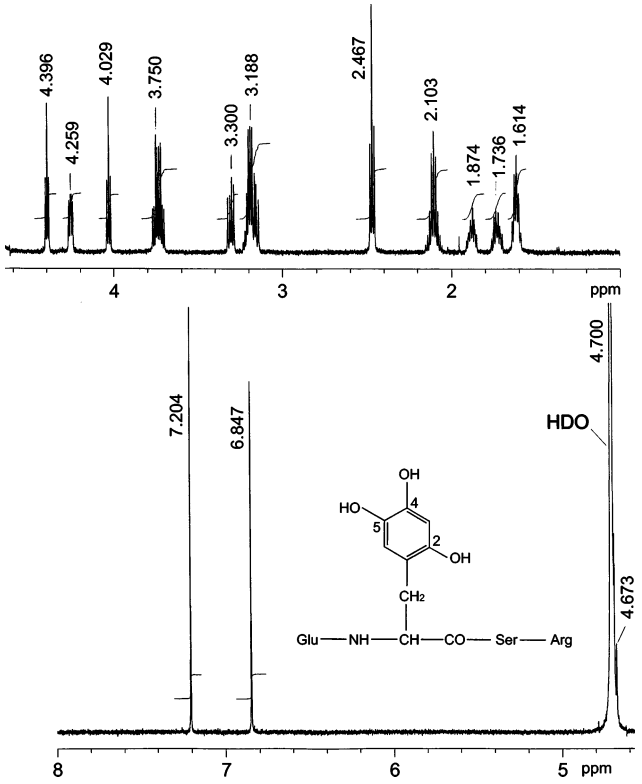

FIGURE 13: A one-dimensional ^{1}H NMR spectrum of peptide treated with ClO_2. Peptide EYSR (4 mM) was treated with 5.1 mM ClO_2 in PBS at 25 °C for 2 min, and the reaction was terminated by 10 mM $Na_2S_2O_3$. The reaction mixture was loaded on HPLC to isolate modified peptide. A ^{1}H NMR spectrum (600 MHz) of the modified peptide is shown. The 4.700 ppm signal is that of partial deuterium water (HDO) used as a standard. The structure of the modified peptide containing a 2,4,5-trihydroxyphenylalanine (TOPA) residue is shown. Experiments were done twice independently, and similar results were obtained. The data shown is one of them.

(residues 304−307), and F**X**IPE (residue 413−417) were obtained from G6PD. By sequence comparison, I found that **X** in F**X**IPE was tryptophan, and the other unknown residue **X** was tyrosine in the G6PD sequence, suggesting that the amino acid residues Tyr^{40}, Tyr^{95}, Tyr^{305}, and Trp^{414} were modified by ClO_2 in G6PD.

DISCUSSION

I have clearly demonstrated that ClO_2-treated tryptophan is oxidized by two atoms of oxygen to form NFK regardless of whether it is free or in a peptide bond. However, I had some difficulty in characterizing the exact reaction products of free tyrosine. Whereas tyrosine is modified to DOPA or TOPA when it is exposed to ClO_2 in a peptide bond, the reaction product formed from free tyrosine seems to be different from them (although complete characterization of ClO_2-treated free tyrosine was unsuccessful.) This difference in reactions might be due to the presence of the free amino and/or carboxyl groups in free tyrosine. I speculate that if amino and carboxyl groups of tyrosine are blocked, as they are in peptide or protein, then no further reaction of the modified tyrosine residue would occur; however, the reaction may proceed further if these groups are free. The peptides treated with ClO_2 showed several minor peaks on HPLC, as well as the major peaks owing to modified tryptophan and tyrosine (Figure 7B,D). By contrast, neither minor nor major HPLC peaks (i.e., reaction products) were seen at all for ClO_2-treated peptides in which the tryptophan and tyrosine residues had been replaced by glycine (Figure 8B,D). These results clearly indicate that minor reactions, other than the formation of NFK, DOPA, and TOPA, occur at the tryptophan and tyrosine residues but not at other amino acid residues in the peptides treated with ClO_2.

Notably, the TOPA formed in peptide EYSR after treatment with ClO_2 might be relatively unstable when it is released from the peptide by aminopeptidase M or it might resist the digestion of aminopeptidase M, because I did not detect an amino acid derivative that appeared to be TOPA on HPLC. It is possible that TOPA is converted to another compound once it is released from the peptide, which would explain why TOPA was not present in the digestion product of protein treated with ClO_2. The lack of availability of authentic TOPA also hindered identification of the compound on HPLC.

I found many sites of tryptophan and tyrosine residues in the proteins treated with and modified by ClO_2, which indicates that there is low regiospecificity in protein in its reaction with ClO_2. It is worth noting, however, that whereas both of the tyrosine residues in the peptide GSFLYEYSR were modified to DOPA (Figures 9B and 10B), the tyrosine residue in the peptide EYSR became TOPA (Figures 13 and 14) after treatment with ClO_2. This observation clearly indicates that the type of reaction that takes place with ClO_2 at a tyrosine side chain in peptide bonds differs depending upon the local fine structure of the peptide.

Whereas the CD spectrum of G6PD changed markedly after ClO_2 treatment, the CD spectrum of BSA changed only a little (Figure 3). This difference might be due to the number of SS (disulfide) linkages in the two proteins: G6PD has no SS linkage, whereas BSA has 17. This large number of SS linkages in BSA may contribute to stabilization of the structure of its α-helices, which might account for the small change in the CD spectrum of BSA.

Elemental analysis showed that about 64 oxygen atoms were found to be incorporated into one molecule of BSA (Table 1), and 48 molecules of ClO_2 reacted with one molecule of BSA (Table 3), whose molecular weight is 66 414. There are 2 tryptophan and 20 tyrosine residues in one molecule of BSA. If all of the tryptophan and tyrosine residues react with ClO_2 and if two oxygen atoms (in tryptophan) and one or two oxygen atoms (in tyrosine) are incorporated in these amino acid residues to form NFK, DOPA, or TOPA, then theoretically the total number of oxygen atoms incorporated in one BSA molecule should be in the range of 24−44, which is 38−69% of the observed value (64 oxygen atoms). This difference of oxygen atom incorporation in protein suggests that there might be some other oxygen-involving reactions of the protein with ClO_2 that were not detected by the analyses used in the present study.

Mudd and his co-workers have shown that ozone reacts rapidly with tryptophan (*43*, *44*). The absorption spectrum of hen egg-white lysozyme exposed to ozone indicates that tryptophan is converted to NFK (*45*). Meiners and his coworkers reported (*46*) that ozone reacts with indole compounds such as tryptophan, 5-hydroxytryptophan, and 5-hydroxytryptamine. The reaction is such that approximately 1 mol of ozone reacts with 1 mol of indole compound when

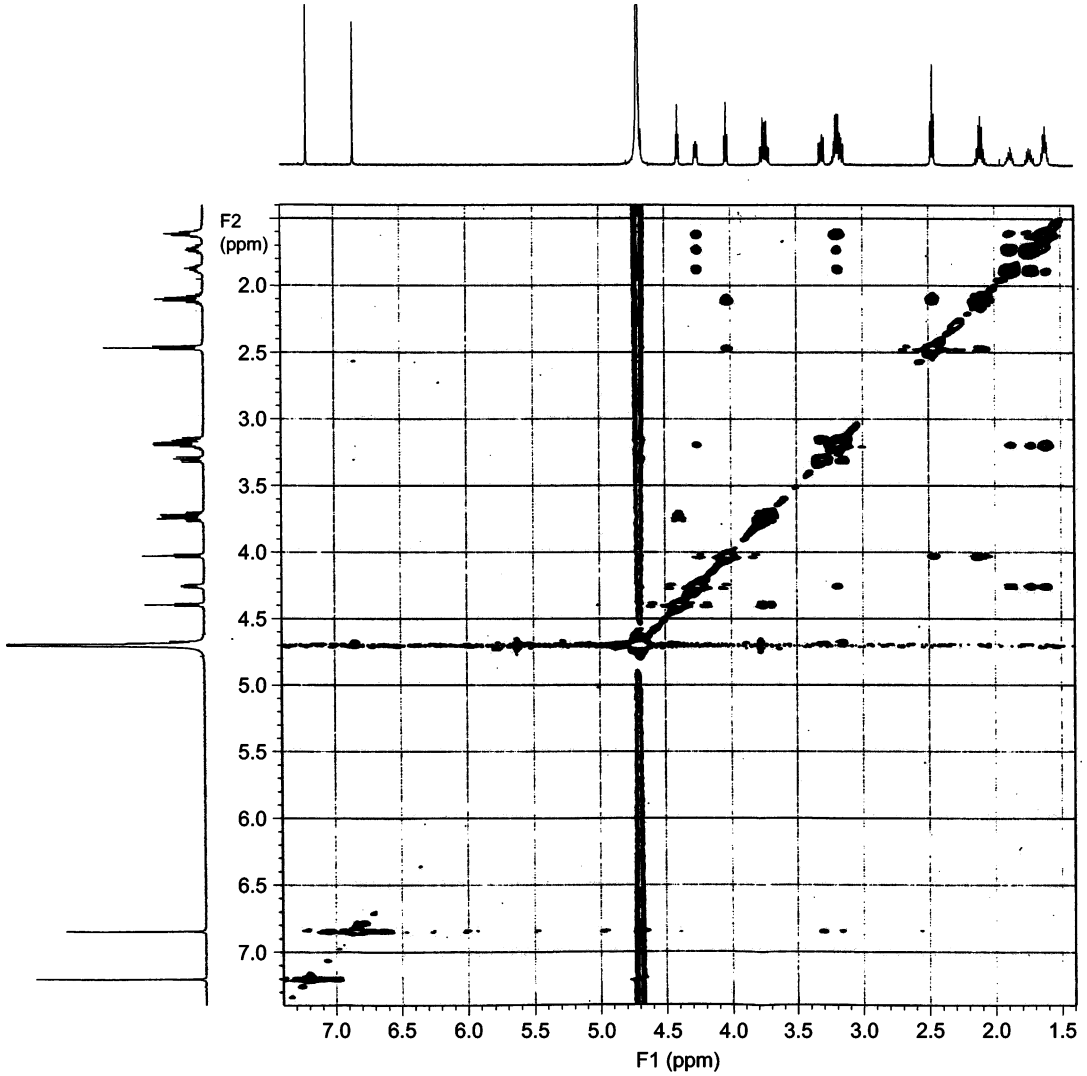

FIGURE 14: A two-dimensional $^1H-^1H$ NOESY NMR spectrum of peptide treated with ClO_2. Peptide EYSR (4 mM) was treated with 5.1 mM ClO_2 in PBS at 25 °C for 2 min, and the reaction was terminated by 10 mM $Na_2S_2O_3$. The reaction mixture was loaded on HPLC to isolate modified peptide. A $^1H-^1H$ NOESY spectrum of the modified peptide is shown. Experiments were done twice independently, and similar results were obtained. The data shown is one of them.

the concentration of the indole compound is 100 μM. Kuroda and his co-workers also demonstrated that a tryptophan residue in hen egg-white lysozyme is modified by ozone in an aqueous solution (*47*), and showed that one of the six tryptophan residues in the enzyme is oxidized to NFK with concomitant loss of enzymatic activity. Taken together, these reports suggest that tryptophan residues in proteins are converted to NFK by chemicals with oxidizing activity. In animals, free tryptophan is enzymatically oxidized to NFK by an iron heme protein tryptophan pyrrolase (tryptophan 2,3-dioxygenase (EC 1.13.11.11)).

Balasubramanian found that tryptophan and tyrosine residues of lens protein crystallin are oxidized by singlet oxygen in a photosensitized oxidation reaction (*48*), in which tryptophan residue becomes NFK. Here I found that the reaction of 0.3 mM ClO_2 with 0.2 mM peptide ALKAWSVAR was markedly inhibited in a concentration-dependent manner by 0.8−20 mM NaN_3, a known singlet oxygen scavenger (Ogata, N., unpublished data). The result suggests that singlet oxygen is involved also in the oxidation of tryptophan by ClO_2. When the reaction of ClO_2 with peptide ALKAWSVAR or GSFLYEYSR was carried out in the presence of superoxide dismutase (final 300 units/mL), the oxidation reaction as assessed by the appearance of Y78 and W62 on HPLC (Figure 7B,D) occurred to the same extent as it did without the enzyme (Ogata, N., unpublished data). This suggests that $O_2^{-\bullet}$ is not involved in the oxidation of protein by ClO_2.

Modifications of amino acids by HOCl are quite different from those of ClO_2. When protein or peptide is treated with HOCl, tyrosine residues become 3-chlorotyrosine or 3,5-dichlorotyrosine (*49*). Methionine residues become methionine sulfoxide (*49*). Cysteine residues become cysteic acid (*49*). In a pentapeptide treated with HOCl, tryptophan residue becomes 2-oxoindolone (*50*) or 3-chloroindolenine (*51*). HOCl is in equilibrium with Cl_2 (*16*). Therefore, it is unclear whether the above-mentioned modifications by "HOCl" are actually by HOCl itself or by Cl_2. The difference of the reactions of amino acid residues in protein with ClO_2 and with HOCl would be primarily due to the difference that ClO_2 is a free radical ($ClO_2^{\bullet}$), but HOCl is not. Since HOCl is in equilibrium with Cl_2 (*16*), it would be possible that chlorination of tyrosine or tryptophan residues occurs directly by a substitution reaction of H and Cl atoms.

Table 4: ^{18}O-Labeling Experiments of Peptide in the Reaction with ClO_2[a]

reaction with	mol wt of modified amino acid: tryptophan in ALKAW*SVAR[c]	mol wt of modified amino acid: tyrosine[b] in GSFLY*EY*SR[c]
$H_2{}^{16}O + Cl^{16}O_2$	204.2 + 32.0	181.2 + 15.9
$H_2{}^{18}O + Cl^{16}O_2$	204.2 + 31.8	181.2 + 16.2
$^{16}O_2$-bubbled $H_2{}^{16}O + Cl^{16}O_2$	204.2 + 32.2	181.2 + 15.9
$^{18}O_2$-bubbled $H_2{}^{16}O + Cl^{16}O_2$	204.2 + 32.2	181.2 + 16.2
$H_2{}^{16}O + Cl^{18}O_2$	204.2 + 36.4	181.2 + 18.2

[a] Experiments were done twice independently, and similar results were obtained. The data shown is one of them. Peptides ALKAWSVAR and GSFLYEYSR (each 0.2 mM) were treated with 0.3 mM ClO_2 at 25 °C for 2 min in PBS, in which either $H_2{}^{18}O$ or $^{18}O_2$-bubbled water was used for the reaction. In another experiment, peptides were treated as above with $Cl^{18}O_2$ in common distilled water. The reaction was terminated by 0.6 mM $Na_2S_2O_3$, and then the reaction mixture was loaded on HPLC to isolate modified peptides (Figure 7, W62 and Y78). The isolated modified peptides were analyzed by HPLC−ESI-MS/MS. Molecular weights of modified amino acids (W* and Y*) in these peptides were determined from the difference of fragment ions found on the MS/MS. Molecular weights of tryptophan and tyrosine were assumed to be 204.2 and 181.2, respectively. [b] The values indicate those of the fifth residue tyrosine in the peptide. The seventh residue tyrosine showed almost the same values. [c] Modified amino acids are shown by asterisks.

As mentioned above, HOCl can inactivate microbes ingested by leukocytes. At very low concentrations ClO_2 can also inactivate microbes in many in vitro experiments (*30*−*38*). However, it is unclear whether in biological systems of higher organisms, such as human, ClO_2 will ever be produced in sufficient amounts to cause inactivation of invading microbes just like HOCl produced intracellularly in leukocytes does (*11*−*15*). In this respect, it would be important to note that ClO_2 is generated from ClO_2^- by the reaction with HOCl (*52*). ClO_2 is made also from ClO_2^- in acidic environments. It is intriguing to speculate that ClO_2 might be made from ClO_2^- in some cells of higher organisms and that it works physiologically to inactivate invading microbes. If ClO_2^-, as a precursor of ClO_2, is indeed found in cells, then it would be possible especially in acidic environments like inside of lysosome. It would also be possible that, similar to NO, ClO_2 has a functional role in cells—for example, in signal transduction or as a local mediator of cellular functions. Most studies of protein oxidation have focused on the reactivity of single amino acid side chains and have ignored gross or higher-order protein structures (*53*). In this context, the present demonstration—namely, that protein side chains are covalently modified by ClO_2 while the protein is simultaneously denatured—will be very important.

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