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Protein Oxidation of Cytochrome *c* by Reactive Halogen Species Enhances Its Peroxidase Activity*

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Yeong-Renn Chen‡§, Leesa J. Deterding¶‖, Bradley E. Sturgeon‡, Kenneth B. Tomer¶, and Ronald P. Mason‡

From the ‡Laboratory of Pharmacology and Chemistry and ¶Laboratory of Structural Biology, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Reactive halogen species (RHS; X_2 and HOX, where X represents Cl, Br, or I) are metabolites mediated by neutrophil activation and its accompanying respiratory burst. We have investigated the interaction between RHS and mitochondrial cytochrome *c* (cyt *c*) by using electrospray mass spectrometry and electron spin resonance (ESR). When the purified cyt *c* was reacted with an excess amount of hypochlorous acid (HOCl) at pH 7.4, the peroxidase activity of cyt *c* was increased by 4.5-, 6.9-, and 8.6-fold at molar ratios (HOCl/cyt *c*) of 2, 4, and 8, respectively. In comparison with native cyt *c*, the mass spectra obtained from the HOCl-treated cyt *c* revealed that oxygen is covalently incorporated into the protein as indicated by molecular ions of *m/z* = 12,360 (cyt *c*), 12,376 (cyt *c* + O), and 12,392 (cyt *c* + 2O). Using tandem mass spectrometry, a peptide (obtained from the tryptic digests of HOCl-treated cyt *c*) corresponding to the amino acid sequence MIFAGIK, which contains the methionine that binds to the heme, was identified to be involved in the oxygen incorporation. The location of the oxygen incorporation was unequivocally determined to be the methionine residue, suggesting that the oxidation of heme ligand (Met-80) by HOCl results in the enhancement of peroxidase activity of cyt *c*. ESR spectroscopy of HOCl-oxidized cyt *c*, when reacted with H_2O_2 in the presence of the nitroso spin trap 2-methyl-2-nitrosopropane (MNP), yielded more immobilized MNP/tyrosyl adduct than native cyt *c*. In the presence of H_2O_2, the peroxidase activity of HOCl-oxidized cyt *c* exhibited an increasing ability to oxidize tyrosine to tyrosyl radical as measured directly by fast flow ESR. Titration of both native cyt *c* and HOCl-oxidized cyt *c* with various amounts of H_2O_2 indicated that the latter has a decreased apparent K_m for H_2O_2, implicating that protein oxidation of cyt *c* increases its accessibility to H_2O_2. HOCl-oxidized cyt *c* also displayed an impaired ability to support oxygen consumption by the purified mitochondrial cytochrome *c* oxidase, suggesting that protein oxidation of cyt *c* may break the electron transport chain and inhibit energy transduction in mitochondria.

Respiratory burst during phagocytosis of bacteria by neutrophils is a physiological response (1). It is well known that during the respiratory burst, a number of enzymatic processes are activated, which then act to produce oxygen metabolites. For example, the hexose monophosphate shunt is activated to increase glucose oxidation and production of CO_2 and NADPH (1). Superoxide anion is generated by activation of NADPH oxidase; superoxide then dismutates to hydrogen peroxide (1).

However, the respiratory burst of neutrophils or monocytes can also mediate the metabolism of halide and generate reactive halogen species (RHS)[1] such as HOX and X_2 (where X represents Cl, Br, or I) (1, 2). In the presence of H_2O_2, a halide anion is a substrate in the peroxidative reaction catalyzed by myeloperoxidase (MPO) released from the primary granule (1, 3, 4). The halide that is commonly most abundant is Cl^- (physiological concentration of 100 mM), and the reactive MPO system is unique in oxidizing chloride ion (Reactions 1 and 2).

$$\text{MPO}(\text{Fe}^{\text{III}}) + \text{H}_2\text{O}_2 \rightarrow \text{MPO-Compound I } [\text{P}^{+\bullet}(\text{Fe}^{\text{IV}})] + \text{H}_2\text{O}$$

$$\text{MPO-Compound I } [\text{P}^{+\bullet}(\text{Fe}^{\text{IV}})] + \text{Cl}^- \rightarrow \text{MPO}(\text{Fe}^{\text{III}}) + \text{HOCl}$$

REACTIONS 1 and 2

The MPO-compound I, which contains a porphyrin cation radical, is responsible for the oxidation of chloride ion to hypochlorous acid (HOCl) (3–5). Other mammalian peroxidases can oxidize bromide and iodide in a similar manner (3, 5).

Destruction of the mitochondrial electron transport chain of oxidative phosphorylation by RHS has been considered to be associated with the antiseptic function of HOCl and to be a causative factor of neutrophil-mediated inflammation (6–8). In support of this idea, mammalian cells and tissue treated with activated neutrophils (8) underwent a drastic loss of ATP and a significant increase of HOCl. Hypochlorous acid is a potent oxidant. It has been shown to deplete energy transduction in bacteria (6, 9) and induce apoptosis in human endothelial cells (10). It may damage host proteins at sites of inflammation *in vivo* (11). In addition, HOCl has been associated with loss of respiration via its oxidation of cytochromes (6).

It has been shown that the protein modifications *in vivo* by HOCl are implicated as an important pathophysiological

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§ To whom correspondence may be addressed: Laboratory of Pharmacology and Chemistry, NIEHS, NIH, P.O. Box 12233, Research Triangle Park, NC 27709. Tel.: 919-541-1501; Fax: 919-541-1043; E-mail: chen6@niehs.nih.gov.

‖ To whom correspondence may be addressed: Laboratory of Structural Biology, NIEHS, NIH, P.O. Box 12233, Research Triangle Park, NC 27709. Tel.: 919-541-3009; Fax: 919-541-0220; E-mail: deterdi2@niehs.nih.gov.

[1] The abbreviations used are: RHS, reactive halogen species; HOCl, the physiological mixture of hypochlorous acid and its anion; cyt *c*, cytochrome *c*; iodo-cyt *c*, native cytochrome *c* modified under iodination conditions; HOCl-cyt *c*, HOCl-treated or HOCl-oxidized cytochrome *c*; ABTS, 2,2′-azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid), diammonium salt; ESI, electrospray ionization; ESR, electron spin resonance; MPO, myeloperoxidase; MNP, 2-methyl-2-nitrosopropane; MS, mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid; GS•, glutathione thiyl radical.

marker of stimulated neutrophils (12). The oxidation of methionine residues in proteins of cells is one of the major protein modifications that can occur both *in vivo* and *in vitro* (13, 14). This oxidation is usually associated with loss of biological activity in a wide range of proteins (13, 14). HOCl-induced methionine oxidation *in vitro* has been demonstrated in some peptides and proteins (*e.g.* bacterial chemotactic inactivation (15), α_1-proteinase inhibitor (13, 14), α-macroglobulin (13), and voltage-dependent potassium channel (16)). In addition, very high amounts of oxidized methionine were detected in the proteins of cells after neutrophil activation as evidenced by *in vivo* studies (17, 18).

Cytochrome *c* (cyt *c*) is a small, globular heme protein localized in the intermembrane space of mitochondria and is present in high concentrations in mitochondria (~200–400 μM). Physiologically, cyt *c* mediates electron shuttling between ubiquinol:cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV) during mitochondrial respiration. In addition to its function in electron transportation, cyt *c* released from mitochodria to the cytosol is a proapoptotic factor (19, 20).

It has been speculated that the location of HOCl-induced oxidation of cyt *c* is a methionine residue (21). A blue shift in the Soret absorption and a loss of the weak 695-nm absorption band were interpreted as an indication of oxidation of the sixth heme ligand at Met-80 (22). Other RHS such as HOBr and HOI also oxidize ferricytochrome *c* in a similar way. However, there is no direct evidence supporting this speculation. Little is known about how oxidation changes protein properties. Prütz *et al.* have shown that the initial reaction of HOCl with ferrocytochrome *c* occurs with a rate constant of $k = 3 \times 10^5\ \mathrm{M^{-1}\ s^{-1}}$ at pH 7.6 (21), but direct evidence for modification of the protein moiety induced by RHS has never been provided. Since RHS have been reported to impair the ability of ferrocytochrome *c* to act as an electron acceptor (21), any modification of the protein by RHS could have a profound influence on its function.

In this investigation, our goal was to elucidate the structure and function of RHS-mediated cyt *c* oxidation products. We used mass spectrometry to study the protein modification and to provide direct evidence that methionine residues are oxidized to methionine sulfoxide during the reaction. We found that the oxidation of cyt *c* increases its peroxidase activity and its ability to scavenge reactive oxygen species. The potential biological relevance of this reaction is discussed.

EXPERIMENTAL PROCEDURES

Reagents—Diethylenetriaminepentaacetic acid, horse heart cytochrome *c*, horseradish peroxidase, glutathione, L-tyrosine, and sodium iodide were purchased from Sigma and used as received. 2,2′-Azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid), diammonium salt (ABTS), 2-methyl-2-nitrosopropane (MNP), and sodium hypochlorite (4% chlorine available in solution) were purchased from Aldrich. Perdeutrated MNP (MNP-d_9) was provided as a gift from Dr. Joy Joseph (Medical College of Wisconsin, Milwaukee, WI). Prepacked Sephadex G-25 (PD-10) size exclusion columns were purchased from Amersham Biosciences. Pronase was obtained from Roche Molecular Biochemicals. Porcine sequencing grade trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated, specific activity >5000 units/mg) was purchased from Promega (Madison, WI).

Analytical Methods—Optical spectra were measured on an SLM Amino DW-2000 UV-visible spectrophotometer. The concentration of cyt *c* was calculated from the difference spectrum after ascorbate reduction using a millimolar extinction coefficient of 18.5 $\mathrm{mM^{-1}\ cm^{-1}}$ at 550 nm. The concentration of HOCl was measured by using the molar extinction coefficient of hypochlorite ($\epsilon_{292\ \mathrm{nm}} = 350\ \mathrm{M^{-1}\ cm^{-1}}$) in 5 mM NaOH solution at pH 11–12 (23). The oxygen consumption by peroxidase or cytochrome *c* oxidase was measured as reported previously (24, 25). Peroxidatic activity was assayed by adding an appropriate amount of protein to 1 ml of the mixture containing 1.3 mM ABTS, 12 mM H_2O_2, and 100 μM diethylenetriaminepentaacetic acid in 100 mM phosphate buffer (pH 7.4) at 23 °C, followed by measuring the increase in absorbance at 420 nm ($\epsilon = 36\ \mathrm{mM^{-1}\ cm^{-1}}$) (26).

Preparation of the HOCl-oxidized Cytochrome c for Mass Spectrometry—Cyt *c* (0.2 mM) was incubated with HOCl (0.4 mM) at room temperature for 7.5 min. The reaction mixture was subjected to gel filtration with Sephadex G-25 to remove excess HOCl. The cyt *c* solution collected from column eluate was concentrated with a Centricon-10 concentrator (~1.76 mM) and immediately dialyzed against 50 mM ammonium bicarbonate buffer, pH 7.9. To avoid any artifact arising from air oxidation, it is imperative to keep experimental conditions free of atmospheric oxygen. Ammonium bicarbonate buffer was prebubbled with argon at room temperature for at least 2 h before it was cooled to 4 °C. All of the sample vials (such as microcentrifuge tubes) and glassware used were flushed with argon for 5 min to ensure that it was free of oxygen. Dialysis was carried out at 4 °C under the argon for 8 h with one change of buffer. The dialysate was then transferred to a sample vial, diluted to a concentration of ~150–325 μM, and flushed with argon for 1 min. Just prior to the electrospray analysis, a small aliquot of the cyt *c* sample or the HOCl-cyt *c* sample was diluted by a factor of 10 with argon-saturated solvent containing 0.05% HCOOH and 50% acetonitrile.

The cyt *c* concentration in mitochondria ranges from 200 to 400 μM (26, 27). However, the concentration of cyt *c* leaking into the cytosol varies, depending on the physiological conditions causing the mitochondrial swelling (20, 28–30). Generally, the plausible concentration is within the range of 1–50 μM. The physiological concentration of Cl^- is 100 mM (31, 32); thus, concentrations of 10–200 μM for HOCl in stimulated cells are plausible. HOCl can also induce the apoptosis, and augment the cyt *c* release in cytosol (10). Therefore, the relative ratios of HOCl to cyt *c* can be expected to fall within the range of 0.05–5 in which our experiments were conducted.

To prepare the tryptic digest, the concentration of cyt *c* (from dialysate) was diluted to 0.32 mM (3.95 mg/ml) with 50 mM argon-saturated NH_4HCO_3 buffer. The endoproteinase, trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated, 20 μg), was dissolved with 10 μl of 50 mM argon-saturated NH_4HCO_3 buffer and then flushed with argon for 1 min prior to mixing with 270 μg of cyt *c*. The reaction of trypsinolysis was carried out at 37 °C for 12 h sealed with parafilm. Approximately 5 μl of the tryptic digest solution was withdrawn under argon, mixed with an equal volume of argon-saturated solvent containing 0.05% HCOOH and 50% acetonitrile, and subjected to electrospray and MS/MS analysis without high pressure liquid chromatography separation.

Iodination of Tyrosine Residues of Cytochrome c—The reaction mixture contained 1-ml aliquots of cyt *c* (200 μM) in 50 mM sodium phosphate buffer, pH 7.4, and NaI (final concentration of 40 mM). Iodination of tyrosine residues of cyt *c* was initiated by the addition of two *N*-chlorobenzenesulfonamide-immobilized beads (IODO-BEADS; Pierce) (33). The reaction was allowed to proceed on a rapid thermomixer at 23 °C for 15 min. To stop the reaction, the solution was removed from the vessel and passed through a prepacked Sephadex G-25 column (under argon-saturated conditions) to remove excess sodium iodide. The fraction containing iodinated cyt *c* was collected and measured with UV-visible spectrophotometry to determine its heme concentration. The protein modification was confirmed using electrospray mass spectrometry. Just prior to analysis, an aliquot of iodo-cyt *c* (0.32 mM) in 50 mM NH_4HCO_3, pH 7.9, was mixed with an equal volume of the argon-saturated solvent containing 0.05% HCOOH and 50% acetonitrile.

Electrospray Mass Spectrometry—Mass spectra were acquired with a Micromass Q-Tof (Altrincham, UK) hybrid tandem mass spectrometer (34) using nanoflow ESI. ESI/MS conditions were as follows: needle voltage, 3050 V; cone voltage, 25 V for the peptide analyses or 40–60 V for the protein analysis; collision energy, 4.0 eV; source temperature, 80 °C; sample infusion rate, ~200 nl/min; acquisition rate, 1.9 s. For the ESI/MS/MS experiments, the parent ion was fragmented by collision with argon atoms at an energy of 25–40 eV. The mass accuracy of this instrument with external calibration is 0.01%; therefore, for molecular masses at 10,000 Da, the accuracy is ±1 Da. Data analysis was accomplished with a MassLynx data system and MaxEnt deconvolution software supplied by the manufacturer.

Electron Spin Resonance Experiments—Electron spin resonance (ESR) experiments were carried out on a Bruker EMX spectrometer operating at 9.8 GHz with 100-kHz modulation frequency at room temperature. The reaction mixture was transferred to a 10-mm quartz ESR flat cell, which was then positioned into the SHQ cavity (Bruker Instrument, Billerica, MA). The sample was scanned using the param-

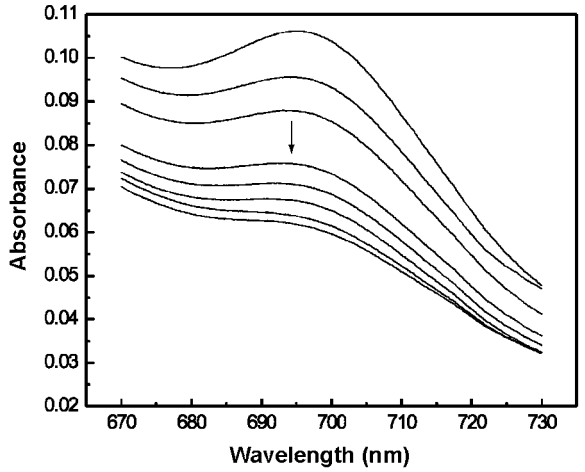

FIG. 1. **Visible absorption spectra of cytochrome *c* following the addition of hypochlorous acid.** The reaction mixture contained cyt *c* (0.17 mM) in 50 mM sodium phosphate, pH 7.4. Visible absorption spectra were recorded from 670 to 730 nm. After the initial scan, 34 μl of HOCl (20 mM stock solution) was added (HOCl/cyt *c* = 4:1). Absorption spectra were recorded every 1.5 min. The spectra show intact cyt *c* (initial scan) and a mixture of cyt *c* and HOCl (second to eighth scan; 3, 4.5, 6, 9, 10.5, 13.5, and 15 min, respectively).

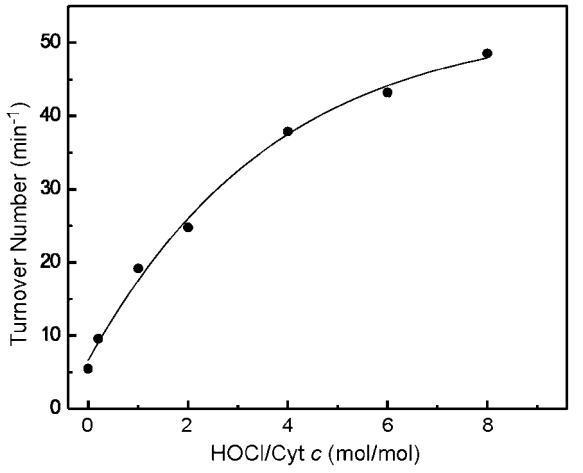

FIG. 2. **The effect of HOCl dosage on the peroxidatic activity of cytochrome *c*.** The reaction mixture containing cyt *c* (200 μM) and various amounts of HOCl in 0.2 ml of 50 mM phosphate buffer (pH 7.4) was allowed to incubate for 15 min at room temperature. The reaction mixture was passed through a Sepahadex G-25 column, and the fractions containing cyt *c* were collected for measuring the enzymatic peroxidase activity by ABTS oxidation. The conditions of peroxidase activity assay are described under "Experimental Procedures."

eters described in the figure legends. The spectral simulations were performed using the WinSim program of the NIEHS public ESR software package available over the Internet (35).

RESULTS

Spectrophotometric Analysis and Peroxidase Activity of HOCl-treated Cytochrome c—When HOCl was added to a cyt *c* solution, the cyt *c* absorbance at 695 nm progressively decreased for up to 15 min (Fig. 1) in agreement with previous results (21). The same result was observed when cyt *c* was treated with chloramine T (36, 37). It has been speculated that this absorbance decrease is due to modification of the sixth ligand (Met-80) of heme c (21, 22). Similar absorbance band bleaching, however, was also detected for the peroxynitrite-induced cyt *c* nitration, in which modification of the sixth heme ligand is not involved (26). HOCl treatment also produced a small blue shift in the Soret γ band of cyt *c*, which is similar to the report in the literature (21).

Purified mitochondrial cyt *c* has peroxidase activity with a turnover number of 5.5 min^{-1} under the assay conditions described under "Experimental Procedures." When cyt *c* was treated with HOCl at room temperature for 15 min, followed by passing the sample through a gel filtration column to remove excess HOCl, its peroxidatic activity increased dramatically (Fig. 2). Maximal activation (8.6-fold enhancement) was observed at a molar ratio of HOCl/cyt *c* of 8:1. It should be noted that a further increase in the amount of HOCl significantly bleached the heme spectrum at the Soret γ band (data not shown), indicating heme degradation as reported by Rosen and Klebanoff (38).

Protein Oxidation of Cytochrome c by HOCl as Characterized by Mass Spectrometry—In order to gain insight into the molecular mechanism of how HOCl interacts with cyt *c*, the HOCl-treated cyt *c* was dialyzed against 50 mM ammonium bicarbonate buffer, pH 7.9, for 8 h with one change of buffer. The dialysate was then subjected to analysis using mass spectrometry. As indicated in Fig. 3, the electrospray mass spectra of HOCl-treated cyt *c* contained a parent molecular ion of average molecular weight (M_r) = 12,360 that corresponds to native cyt *c*, an ion of M_r = 12,376 that corresponds to the addition of one oxygen, and an ion of M_r = 12,392 that corresponds to the addition of two oxygens. These data suggest that oxygen is covalently incorporated into the protein upon treatment with HOCl. The relative abundance of the two-oxygen addition species was affected by the time of the reaction (Fig. 3, *B* and *C*) and the relative concentration of HOCl to cyt *c* (Fig. 3, *C* and *D*). Decreasing the incubation time of the reaction between cyt *c* and HOCl from 15 min (Fig. 3*C*) to 7.5 min (Fig. 3*B*) resulted in the observation of mainly one-oxygen addition product. Likewise, an increase in HOCl concentration from 2:1 (Fig. 3*C*) to 4:1 (Fig. 3*D*) resulted in a higher ratio of native cyt *c* becoming oxidized. It was also observed that the protein was gradually destroyed when the molar ratio of HOCl/cyt *c* was increased to a ratio greater than 10:1 (data not shown). It should also be noted that HOCl-induced oxidation of cyt *c* was also detected in the reaction at low protein concentration (*i.e.* 20 μM cyt *c*) with a molar ratio of HOCl/cyt *c* between 0.2 and 2.

To further provide direct evidence for understanding the molecular mechanism of HOCl-mediated cyt *c* oxidation, it is imperative to determine the location of oxygen binding. To eliminate possible artifacts derived from air oxidation, we prepared HOCl-treated cytochrome *c* under argon saturation. Both native cyt *c* and HOCl-treated cyt *c* (from HOCl/cyt *c* = 2, 7.5-min incubation, Fig. 3*B*) were then digested with trypsin under argon saturation, followed by electrospray mass spectrometry analysis. The resulting mass spectra acquired from the tryptic digest of native cyt *c* and HOCl-treated cyt *c* are shown in Fig. 4, *A* and *B*, respectively. Both spectra contain ions that correspond in mass to tryptic peptides of cyt *c* (ions marked with an *asterisk*) and account for over 90% of the amino acid sequence of cyt *c*.

Because the electrospray spectra of the HOCl-treated cyt *c* samples revealed ions that varied in mass by 16 Da, the mass spectrum from the tryptic digest of HOCl-treated cyt *c* (Fig. 4*B*) was investigated for the addition of 16 Da to the tryptic peptides. This mass difference was observed for two tryptic peptides, T13 (aa 61–72, EETLMEYLENPK) and T16 (aa 80–86, MIFAGIK). The doubly protonated molecule ion $(M + 2H)^{2+}$ of tryptic peptide T13 is observed at *m/z* 748.4, whereas an ion that corresponds in mass to an additional 16 Da is observed at *m/z* 756.5. Likewise, the singly and doubly protonated molecule ions of tryptic peptide T16 are observed at *m/z* 779.4 and *m/z* 390.2, respectively, and the ions corresponding in mass to an increase of 16 Da are observed at *m/z* 795.5 and at *m/z* 398.2,

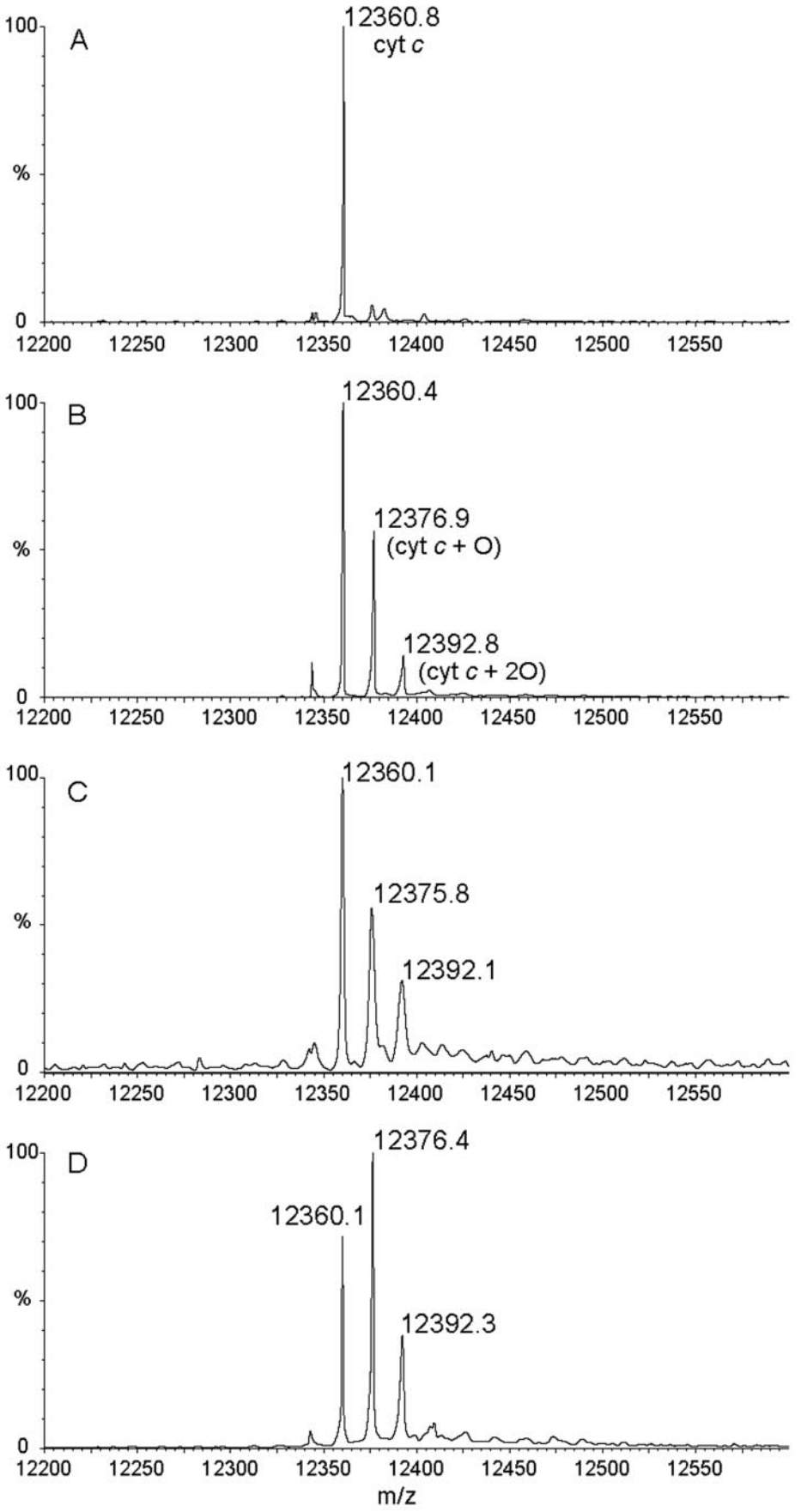

FIG. 3. **Deconvoluted ESI mass spectra of HOCl-oxidized cytochrome *c*.** *A*, native cytochrome *c*. *B*, HOCl-oxidized cytochrome *c* (HOCl:cyt *c* = 2:1, 7.5-min incubation). *C*, HOCl-oxidized cytochrome *c* (HOCl:cyt *c* = 2:1, 15-min incubation). *D*, HOCl-oxidized cytochrome *c* (HOCl:cyt *c* = 4:1, 15-min incubation). Detailed conditions for preparing the HOCl-oxidized cyt *c* are described and discussed under "Experimental Procedures" and "Results."

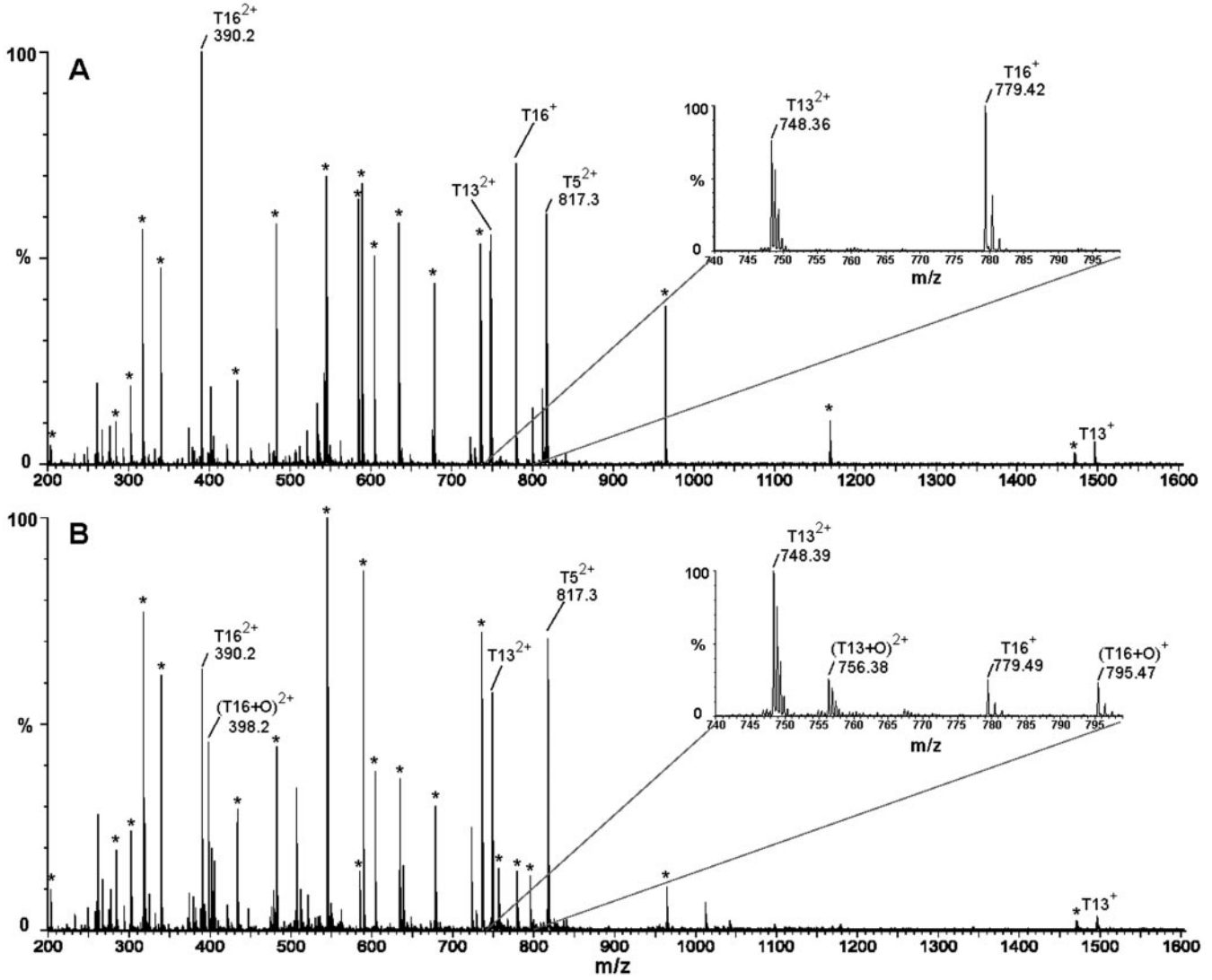

FIG. 4. **ESI mass spectra of tryptic digests of cytochrome *c*.** *A*, native cytochrome *c*. *B*, HOCl-oxidized cytochrome *c* (HOCl:cyt *c* = 2:1, 7.5-min incubation). The protein solution of cyt *c* or HOCl-cyt *c* (1.76 mM) was diluted to a concentration of 0.32 mM with 50 mM ammonium bicarbonate buffer (pH 7.9 and argon-saturated). 68-μl aliquots of cyt *c* or HOCl-cyt *c* were withdrawn and digested with trypsin at a 1:15 ratio (w/w) at 37 °C for 12 h under argon saturation. Ions marked with an *asterisk* correspond in mass to tryptic peptides other than T13 and T16. The *insets* are the ESI spectra with a *m/z* range of 740–800.

respectively. The addition of 16 Da to these ions is not observed in the mass spectrum of the tryptic digest of native cyt *c* (Fig. 4*A*). These data suggest that one oxygen is covalently bound to one of the residues of cyt *c* in T13 (residues 61–72) and T16 (residues 80–86) upon HOCl treatment.

To determine whether T13 or T16 might be the preferred domain for HOCl oxidation, the relative abundance of the oxidized protonated molecules was compared with the relative abundance of the unmodified protonated molecules (Fig. 4*B*, *inset*). The relative abundance of the oxidized T13 ion $(T13 + O)^{2+}$ is ~23% ($n = 3$) of the relative abundance of the corresponding T13 molecular ion ($T13^{2+}$). Conversely, the relative abundance of the oxidized T16 molecular ion $(T16 + O)^{+}$ is 72% ($n = 3$) of the relative abundance of the corresponding T16 molecular ion ($T16^{+}$). These results suggest that the T16 tryptic peptide is more selective for oxygen incorporation during HOCl-induced cyt *c* oxidation.

Because the porphyrin configuration of heme c is covalently bound to Cys-14 and Cys-17, it has been proposed that the thioethers of the heme pocket could be potential sites for RHS oxidation (21). This speculation can be disproven by the observation of the molecular ion $m/z = 817.5$ (doubly protonated, $z = +2$), corresponding to the heme-containing peptide BAQCHTVEK (T5 peptide, where B represents Cys-14 + heme) with no corresponding oxygenated species (Fig. 4).

To determine which amino acid(s) was covalently linked with the oxygen, the MS/MS spectra of the $(T16 + O)^{+}$ ion of *m/z* 795.5 and the $(T13 + O)^{2+}$ ion of *m/z* 756.4 were acquired (Fig. 5). The MS/MS spectrum shown in Fig. 5*B* has been deconvoluted to the single charge state using MaxEnt software. In both spectra, both *y* and *b* ions (39, 40) are observed which correspond to cleavages along the peptide backbone. The *y* series ions result from C-terminal peptide backbone cleavages, and the *b* series ions result from N-terminal backbone cleavages. In both spectra, an ion due to the loss of 64 Da, $SOHCH_3$, from the protonated molecule is observed, indicating the presence of methionine sulfoxide (41, 42). In addition, the loss of 64 Da is observed from some of the structurally informative fragment ions. These data allow the unequivocal assignment of the oxygen adduct to the methionine 80 residue of tryptic peptide T16 and methionine 65 residue of tryptic peptide T13.

Iodination of the Tyrosine Residues of Cytochrome c—A reaction system used to generate iodine from iodide provided an alternative means of studying the interaction between RHS

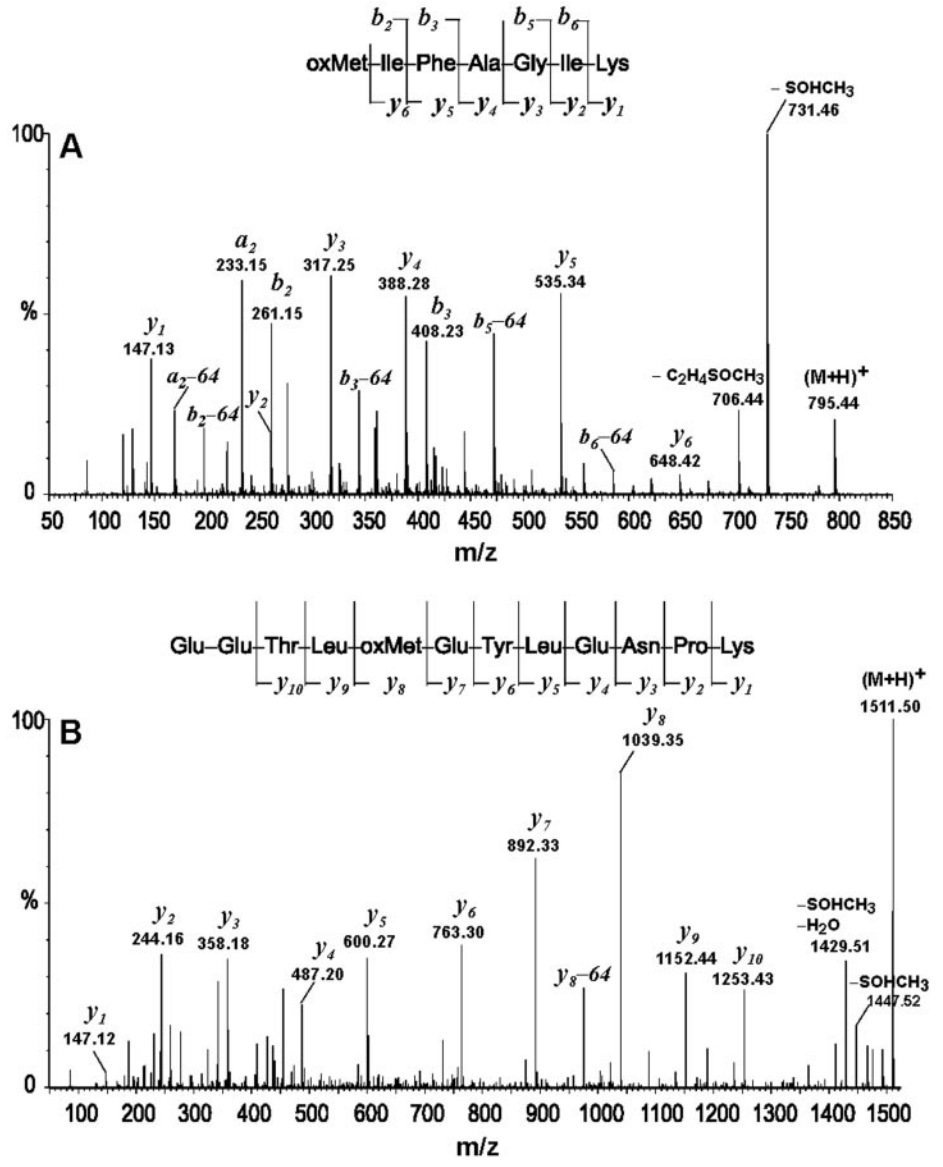

FIG. 5. **Tandem mass spectra of the protonated molecular ions of the oxygenated peptides.** *A*, MIFAGIK; *B*, EETLMEYLENPK. The sequence-specific ions are labeled as *y* and *b* ions on the spectra. *oxMet*, methionine sulfoxide.

and cyt *c*. Oxidation of iodide to iodine can be catalyzed by *N*-chlorobenzenesulfonamide, and the reaction yields a mixture of I_2, HOI, and I_3^{-1} (Reactions 3–5), whereas I_2 and HOI are both potential oxidants that can induce iodination of tyrosine and oxidation of methionine residues on protein moieties.

$$2I^- \rightarrow I_2$$

$$I_2 + H_2O_2 \rightleftarrows HOI + I^- + H^+$$

$$I_2 + I^- \rightarrow I_3^{-1}$$

REACTIONS 3–5

After inclusion of cyt *c* in the reaction system of I_2/HOI, the reaction mixture was analyzed by electrospray mass spectrometry (Fig. 6). Multiple species were observed, which corresponded in mass to iodine plus cyt *c* and iodine/oxygen plus cyt *c*. The modified cyt *c* (iodo-cyt *c*) obtained from this reactive iodine system (under the conditions described under "Experimental Procedures") showed high peroxidative activity (turnover number of ABTS oxidation by iodo-cyt *c* was 110 min^{-1}), absorption loss at 695 nm, and a blue shift in the Soret γ band (data not shown). As indicated by the mass spectrum (Fig. 6), the main products with *m/z* 12,865.60 and 12,881.60 contain four iodine incorporations, consistent with the four tyrosine residues available in mitochondrial cyt *c* from horse heart.

Protein Oxidation of Cytochrome c by HOCl Enhances H_2O_2-induced Protein-derived Tyrosyl Radical—Barr *et al.* have reported that H_2O_2 induces radical(s) derived from cyt *c*, as detected by ESR spin-trapping techniques (43). Studies of several heme proteins imply that protein-centered radicals are formed by the migration of the oxidizing equivalents from the porphyrin cation radical (compound I-like species) (25, 44–46). Presumably, for any globin radical, the intensity of the ESR signal can be affected by the peroxidase activity of the heme protein. To test this hypothesis, we measured the ESR spectrum from the reaction of HOCl-cyt *c* with H_2O_2 in the presence of MNP. The protein MNP radical adduct is seen as an immobilized nitroxide (25, 43, 44); when HOCl-cyt *c* was included in the reaction mixture, the immobilized nitroxide was enhanced compared with the native cyt *c* (Fig. 7, *A* and *B*). The enhanced immobilized nitroxide (Fig. 7, *A* and *B*) was persistent even after gel filtration and increased with increased HOCl concentration (Fig. 7*A*). Formation of the protein radical adduct could be prevented by prior iodination

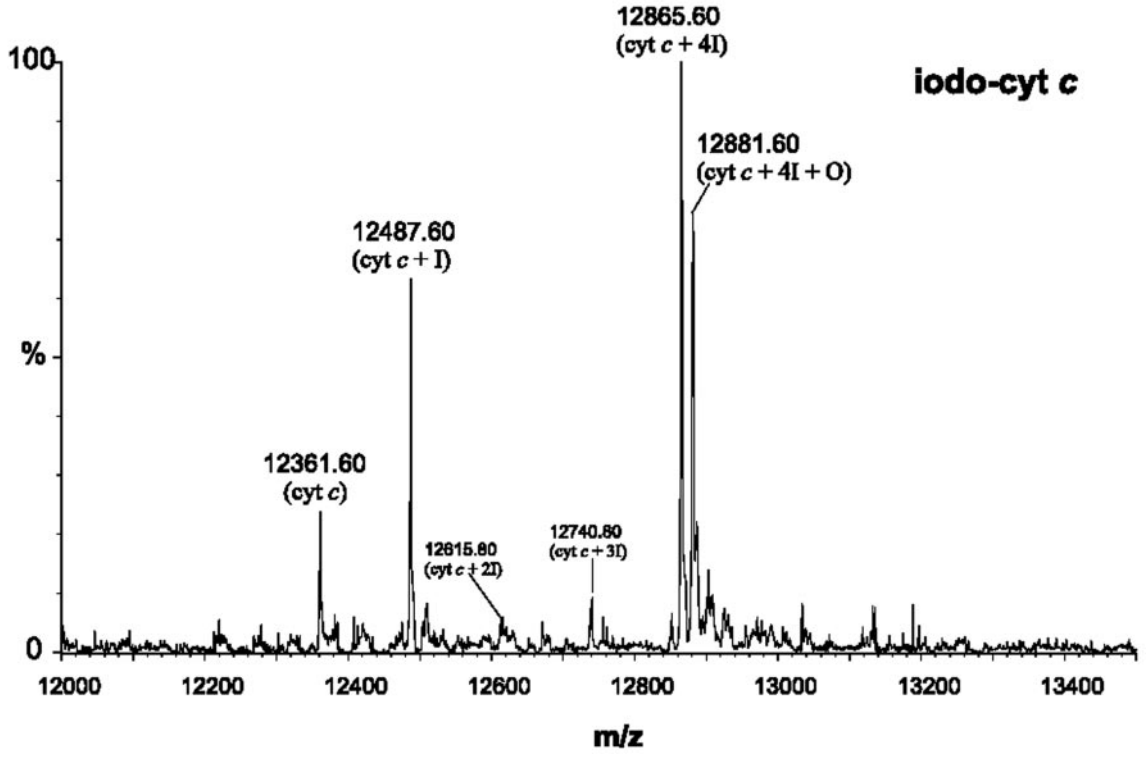

FIG. 6. **Deconvoluted ESI mass spectra of the iodocytochrome *c* (iodo-cyt *c*).** The sample was prepared by iodination of native cyt *c* as described under "Experimental Procedures." Aliquots of iodo-cyt *c* (0.32 mM) in 50 mM NH_4HCO_3, pH 7.9, were mixed with an equal volume of the argon-saturated solvent containing 0.05% HCOOH and 50% acetonitrile and then subjected to electrospray analysis.

of cyt *c* (see above), thereby indicating that tyrosine residues were involved in the radical formation (Fig. 7*C*). Nonspecific proteolytic digestion of the enhanced immobilized nitroxide by Pronase yielded an isotropic three-line spectrum with a^N = 15.6 G, indicative of a tyrosyl radical adduct (Fig. 7*D*). Assignment of this protein radical as the tyrosyl radical was confirmed with perdeuterated MNP (MNP-d_9) (Fig. 7*E*) (43, 44, 47, 48).

Peroxidase Activity of HOCl-oxidized Cytochrome c Exhibited an Increasing Ability to Oxidize Tyrosine to Tyrosyl Radical—In the presence of H_2O_2, horseradish peroxidase can convert tyrosine to the tyrosyl radical as measured directly by fast flow ESR (24). To compare the activities of native cyt *c* and HOCl-cyt *c*, we immobilized small amounts of both on agarose beads (~11 nmol of cyt *c*/ml of agarose beads) and packed the beads into a flat cell for ESR measurement (49). With a solution containing tyrosine (2 mM) and H_2O_2 (1 mM) flowing over immobilized HOCl-cyt *c*, the ESR spectrum of the tyrosyl radical was significantly stronger than that obtained using immobilized native cyt *c* (Fig. 8). The hyperfine coupling constants of the detected tyrosyl radical were the same as the values reported in the literature (24, 49, 50).

A biologically generated radical such as the tyrosyl radical can react with GSH to form the thiyl radical (GS•) and then undergo further reactions to give superoxide (Reactions 6–8) (24).

$$Tyr^{\bullet} + GSH \rightarrow Tyr + GS^{\bullet}$$

$$GS^{\bullet} + GS^{-} \rightleftarrows GSSG^{\bar{\bullet}}$$

$$GSSG^{\bar{\bullet}} + O_2 \rightarrow GSSG + O^{\bar{\bullet}}{}_2$$

REACTIONS 6–8

To test whether the RHS-enhanced peroxidase activity of cyt *c* could initiate the above reactions in the presence of H_2O_2, we measured oxygen consumption in a reaction mixture containing H_2O_2, tyrosine, and GSH. The addition of native cyt *c* did not cause any detectable oxygen consumption (Fig. 9*A*). Replacement of native protein with RHS-treated cyt *c* (HOCl-oxidized cyt *c* and iodo-cyt *c*) in the system resulted in significant oxygen consumption (Fig. 9, *B–D*). The extent of oxygen uptake was proportional to the peroxidative activity of the protein. Postaddition (1 min after cyt *c* initiation) of superoxide dismutase inhibited the rate and extent of oxygen consumption, indicating the generation of superoxide (data not shown).

HOCl-oxidized Cytochrome c Shows Decreased K_m, Increased Binding Affinity for H_2O_2, and Impaired Ability to Support Oxygen Consumption by Cytochrome c Oxidase—Peroxidases such as horseradish peroxidase typically show high turnover numbers and high substrate affinity. It was expected that methionine oxidation of cytochrome *c* would increase its accessibility to H_2O_2. To test this hypothesis, both native and HOCl-oxidized cyt *c* were titrated with various amounts of H_2O_2; then the oxidation of ABTS was measured. Fig. 10 shows the H_2O_2-dependent activity titration curve for native cyt *c* and HOCl-oxidized cyt *c*. Compared with the native protein, the peroxidative activity of HOCl-oxidized cyt *c* was saturated more easily by H_2O_2, implying that cyt *c* oxidation by HOCl increased its binding affinity for H_2O_2. The apparent K_m for H_2O_2 for the HOCl-cyt *c* as determined by Lineweaver-Burk plots was between 18 and 20 mM. In contrast, the peroxidase activity of native cyt *c* could not be saturated by H_2O_2 in the range of concentrations studied (up to 70 mM).

Incubation of mitochondrial cytochrome *c* oxidase, ferricytochrome *c*, and an appropriate amount of an electron donor such as ascorbate resulted in oxygen consumption as indicated (Fig. 11). The electron transport between reduced cyt *c* and molecular oxygen was catalyzed by cytochrome *c* oxidase and was sensitive to the presence of cyanide (Fig. 11). Oxygen consumption by cytochrome *c* oxidase was greatly inhibited when the native cyt *c* was replaced with HOCl-oxidized cyt *c*, and inhi-

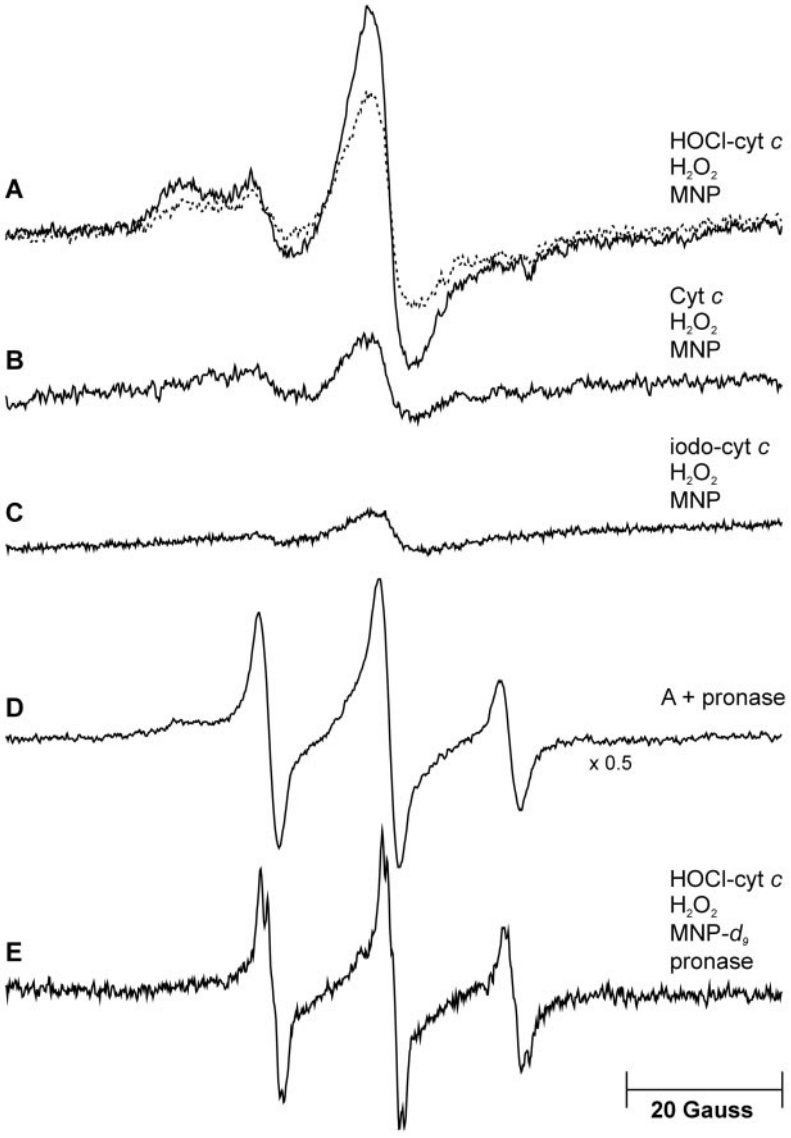

FIG. 7. **ESR spectra of the immobilized nitroxide obtained from the reaction of HOCl-oxidized cytochrome *c* with H_2O_2 in the presence of MNP.** *A*, the reaction mixture containing HOCl-cyt *c* (0.5 mM, *solid line*, HOCl:cyt *c* = 8:1), H_2O_2 (2.5 mM), and 18 mM MNP in 50 mM sodium phosphate (pH 7.4) was subjected to ESR measurement. The *dashed line* represents the spectrum obtained by using the same amount of HOCl-cyt *c* at a molar ratio of HOCl/cyt *c* = 2. *B*, as in *A*, but HOCl-cyt *c* was replaced with native cyt *c*. *C*, as in *A*, but HOCl-cyt *c* was replaced with iodo-cyt *c*. *D*, sample *A* (HOCl-cyt *c* at a molar ratio of HOCl/cyt *c* = 8) was passed through a Sephadex G-25 column. Pronase was then added (5 mg/ml) before the ESR spectrum was rerecorded. *E*, as in *D*, but MNP was replaced with perdeuterated isotope (MNP-d_9). Instrument settings were as follows: center field, 3490 G; sweep width, 100 G; microwave power, 20 milliwatts; time constant, 655.36 ms; modulation amplitude, 2 G for spectra A, B, C, and D and 0.5 G for spectrum E; gain, 1×10^5 for spectra A, B, C, and D and 2.52×10^5 for spectrum E; sweep rate, 0.298 G/s for spectra A, B, C, and D and 0.149 G/s for spectrum E.

bition was proportional to the ratio of HOCl/cyt *c*. More than 50% inhibition was detected at a ratio of 2, and nearly complete inhibitions were observed at a ratio of 8 (Fig. 11). These results suggest that RHS-induced cyt *c* oxidation may interrupt the electron transport chain and, thus, inhibit energy transduction in mitochondria.

DISCUSSION

In the current investigation, we provide the first direct evidence for the molecular mechanism of HOCl-induced protein oxidation for cyt *c*. We have used electrospray mass spectrometry (Fig. 3) to demonstrate that one oxygen is added to the protein at lower HOCl concentrations (HOCl/cyt *c* = 0.2–2; Fig. 3*B*), whereas at increased reaction times or increased HOCl concentrations, two oxygen molecules are added (Fig. 3, *C* and *D*). Mass spectral analysis of the tryptic peptides showed that oxygen is incorporated into T13 (aa 61–72) and T16 (aa 80–86), with T16 favored (72% *versus* 23%; Fig. 4, *inset*). MS/MS analysis of the oxidized T13 and the oxidized T16 showed that oxygen binds covalently to methionine 65 in T13 and methionine 80 in T16 (Fig. 5). These data suggest that methionine 80 (the heme ligand) is the preferred site of HOCl-induced oxidation on cyt *c*.

In this reaction, we observed only oxidation, with none of the competing reactions that have been postulated. Hypochlorous acid did not cause specific sulfoxidation of cyt *c* at Cys-14 or Cys-17, which are covalently linked to porphyrin. However, HOCl-induced oxidation of cysteine has recently been reported for glutathione in endothelial cells and the activation of matrix metalloproteinase (23, 31). Our results did not show this reaction, because cyt *c* does not contain free cys. It has been reported that HOCl chlorinates tyrosine residues of proteins or peptides (11, 51), and this reaction has been proposed as an indicator for the production of hypochlorous acid *in vivo*. However, in our current investigation, we did not observe chlorination in HOCl-treated cyt *c*, although halogenation of cyt *c* was dominant under iodination conditions (Fig. 6). One possible reason for these results is that direct reaction of HOCl with tyrosine (k_{obs} = 44 M^{-1} s^{-1}) is considerably slower than oxidation of methionine (k_{obs} = 3.8×10^7 M^{-1} s^{-1}) (21, 52, 53).

Our results are consistent with the notable disappearance of the weak UV-visible absorbance at 695 nm (21). However, this disappearance could have other causes. For example, the loss of the 695-nm absorption in cyt *c* can also be caused by nitration of tyrosine residues, which does not induce protein oxidation as indicated by mass spectral analysis (26). As proposed by Cassina *et al.* (26), modification of the heme-vicinal tyrosine (Tyr-67) could promote a conformational change, displacing the

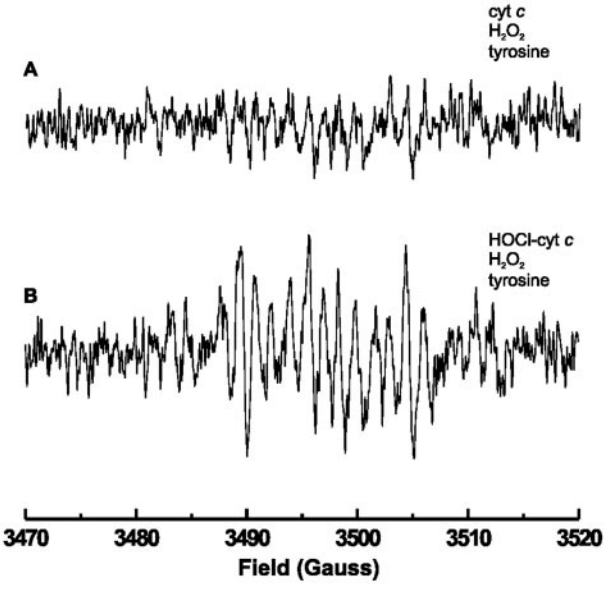

FIG. 8. **Direct ESR spectra of the enhanced tyrosyl radical generated by HOCl-oxidized cytochrome *c*.** Cyt *c* and HOCl-cyt *c* were dialyzed against 0.1 M MOPS buffer (pH 7.5) for 8 h with one change of buffer. Protein was recovered from the dialysis cassette and coupled to an activated agarose gel bead support (Affi-Gel 10, Bio-Rad) via the primary amino group as outlined in the product literature. Immobilized protein (11 nmol/ml of beads, 100 μl) was transferred to a 10-mm flat cell. A solution of tyrosine (2 mM)/H_2O_2 (1 mM) in 50 mM phosphate buffer (pH 7.4, 200 μM diethylenetriaminepentaacetic acid) was passed over the beads at a flow rate of 1 ml/min. All measurements were done at room temperature (49). *A*, complete system with immobilized cyt *c*, tyrosine, and H_2O_2. *B*, as in *A*, but immobilized cyt *c* was replaced with immobilized HOCl-cyt *c*. Instrument conditions were as follows: center field, 3495 G; sweep width, 50 G; microwave power, 20 mW; modulation amplitude, 0.5 G; time constant, 163.84 ms; gain, 6.32×10^4; sweep rate, 0.596 G/s.

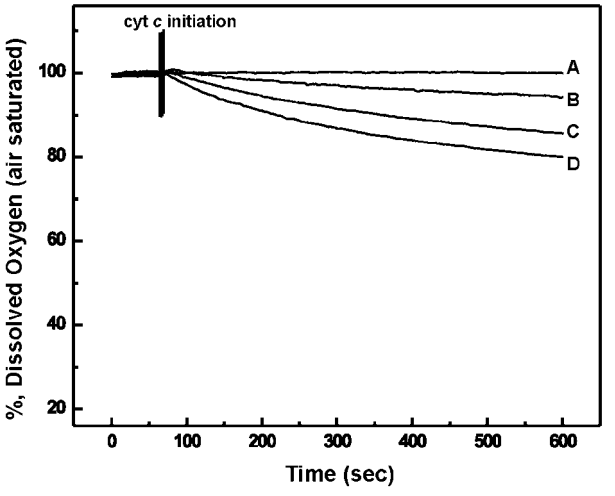

FIG. 9. **Oxygen consumption by the cytochrome *c* system with 8 mM glutathione.** *Trace A*, the system contained cyt *c* (30 μM), tyrosine (1.85 mM), H_2O_2 (0.53 mM), and GSH (8 mM). *Trace B*, as in *trace A*, but cyt *c* was replaced with HOCl-cyt *c* (4:1). *Trace C*, as in *trace A*, but cyt *c* was replaced with HOCl-cyt *c* (8:1). *Trace D*, as in *trace A*, but cyt *c* was replaced with iodo-cyt *c*.

Met-80 heme ligand, followed by a characteristic loss of 695-nm absorbance. Nevertheless, our mass spectrometry data provide direct and unequivocal evidence for HOCl-induced cyt *c* oxidation.

The modification of the heme ligand (Met-80) also accounts for HOCl's enhancement of the peroxidative activity of cyt *c*. A similar enhancement was also observed for the nitration of cyt

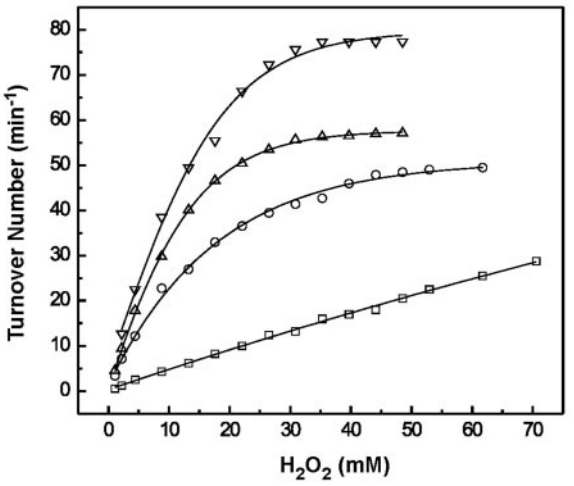

FIG. 10. **Titration curve of native cyt *c* and HOCl-oxidized cytochrome *c* with various amounts of H_2O_2.** 5- or 10-μl aliquots of cyt *c* (□) or HOCl-cyt *c* (2:1, ○; 4:1, △; 8:1, ▽), 95 nmol/ml, in 50 mM ammonium bicarbonate (pH 7.9) were added to a 1-ml assay mixture (see "Experimental Procedures") containing the indicated concentrations of H_2O_2. Each data point was an average value of duplicate assays of two different batches of protein preparations.

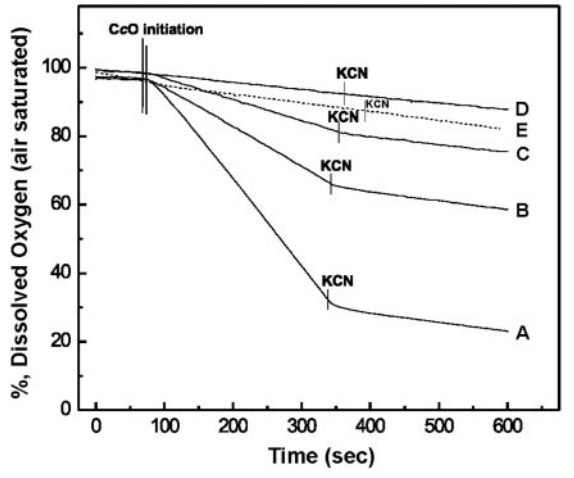

FIG. 11. **Oxygen consumption by the mitochondrial cytochrome *c* oxidase (*CcO*) system with cyt *c*, HOCl-cyt *c*, or iodo-cyt *c* in the presence of ascorbate.** *Trace A*, the system contained cytochrome *c* oxidase (0.6 μM), ascorbate (20 mM), and cyt *c* (20 μM) in 50 mM phosphate buffer (pH 7.4). Potassium cyanide (2 mM) was added at 4.5 min after cytochrome *c* oxidase initiation. *Trace B*, as in *trace A*, but cyt *c* was replaced with HOCl-cyt *c* (2:1). *Trace C*, as in *trace A*, but cyt *c* was replaced with HOCl-cyt *c* (4:1). *Trace D*, as in *trace A*, but cyt *c* was replaced with HOCl-cyt *c* (8:1). *Trace E* (shown with *dashed line*), as in *trace A*, but cyt *c* was replaced with iodo-cyt *c*.

c with peroxynitrite (26), the iodination of cyt *c* (this work), and the pretreatment of cyt *c* with chloramine T (data not shown). All of these modifications can bleach the 695-nm band, although they may not share the same mechanism. It is logical to assume that sulfoxidation of Met-80 would change the protein conformation, leaving the sixth coordinate opened and occupied by a water molecule. This substitution will make the heme environment of cyt *c* resemble that of horseradish peroxidase, which utilizes water as the sixth ligand (54). Such a change in the heme environment would increase the binding affinity of HOCl-cyt *c* for H_2O_2. This prediction is strongly supported by the titration curve with H_2O_2 and the observation that HOCl-oxidized cyt *c* has a decreased K_m for H_2O_2 (Fig. 10).

In view of this enhancement, we employed ESR spectroscopy to evaluate the biochemical consequences of gaining peroxidative activity by protein oxidation of cyt *c*. We found that the cyt

c-derived tyrosyl radical signal could be greatly enhanced when excess H_2O_2 was reacted with HOCl-cyt *c* (Fig. 7). The formation of the protein tyrosyl radical is presumably due to electron transfer from vicinal tyrosine residue(s) to the porphyrin cation radical of a horseradish peroxidase compound I-type intermediate. Since HOCl-oxidized cyt *c* has higher peroxidative activity and higher affinity for H_2O_2, it would be expected to facilitate the formation of compound I-type species upon reaction with hydrogen peroxide.

Additional evidence supporting RHS enhancement of cyt *c* peroxidative activity is provided by direct ESR spectra of increased tyrosyl radical from modified cyt *c* H_2O_2/tyrosine (Fig. 8). This result has physiological significance. Intracellular GSH in mammalian cells has concentrations as high as millimolar (55, 56). The tyrosyl radical generated by HOCl-treated cyt *c* could oxidize GSH to yield $GS^{\bullet}$, which can potentially undergo further reactions to generate superoxide anion (Reactions 6–8). This potential was unequivocally confirmed when HOCl-cyt *c* or iodo-cyt *c* was introduced into the reaction system of H_2O_2/tyrosine/GSH, where oxygen consumption was detected (Fig. 9).

The pathophysiological role of cyt *c*-derived radicals remains to be defined. It has been speculated that they can contribute to lipid peroxidation of the mitochondrial membrane and indirectly cause permeability transition pores to induce proapoptotic events (19, 20). Recently, oxidative stress involving cyt *c* has been linked to a molecular mechanism of protein aggregates associated with Parkinson's disease (57). The two proteins that have been found in Lewy bodies (abnormal protein aggregates found in neuron cells) are cyt *c* and α-synuclein; it has recently been demonstrated that cyt *c* stimulates α-synuclein aggregation in the presence of H_2O_2 (57). It would be logical to assume that H_2O_2-induced cyt *c* protein radical(s) initiates α-synuclein aggregates via radical transfer and radical-radical reaction. In view of this possibility, the enhancement of peroxidase activity of cyt *c* by halogen oxidants could also contribute to Lewy body formation resulting from neuroinflammation.

Finally, the oxygen consumption of mitochondrial cytochrome *c* oxidase demonstrates that when cyt *c* is oxidized at methionine, its ability to act in electron transport is significantly impaired (Fig. 11). Methionine oxidation greatly reduced the ability of cyt *c* to accept an electron from electron partners in mitochondria such as ascorbate or the cytochrome c_1 of complex III. This defect in its electron transfer ability can affect the coupling for ATP formation by complex III and complex IV, thus inhibiting energy transduction in mitochondria. It is also correlated with the destruction of the adenine nucleotide pool (ATP + ADP + AMP) (8, 9). Albrich *et al.* (6) have reported decreased respiration in bacterial cells treated with HOCl or the MPO/H_2O_2/Cl^- system and suggested that oxidation of the electron transport chains is one likely consequence of HOCl-induced loss of respiration. Our results support this hypothesis.

Among the electron transport chain components, cyt *c* may not be the only target for RHS attack. Several sites of the electron transport chain could be susceptible to damage by RHS. For example, the cytochrome c_1 subunit of complex III contains a heme configuration and heme environment similar to those of cyt *c* (58, 59). It could undergo the same reaction mechanism when oxidative damage of complex III is induced by halogen oxidants. The present results clarify the molecular mechanism of protein oxidation caused by halogen oxidants. This mechanism may not be specific only to mitochondrial cyt *c*, but may also apply to many other heme and non-heme proteins.

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