Specific Sequence Motifs Direct the Oxygenation and Chlorination of Tryptophan by Myeloperoxidase†

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Received November 15, 2005; Revised Manuscript Received January 10, 2006

ABSTRACT: Most studies of protein oxidation have typically focused on the reactivity of single amino acid side chains while ignoring the potential importance of adjacent sequences in directing the reaction pathway. We previously showed that hypochlorous acid (HOCl), a specific product of myeloperoxidase, inactivates matrilysin by modifying adjacent tryptophan and glycine (WG) residues in the catalytic domain. Here, we use model peptides that mimic the region of matrilysin involved in this reaction, VVWGTA, VVWATA, and the library VVWXTA, to determine whether specific sequence motifs are targeted for chlorination or oxygenation by myeloperoxidase. Our results demonstrate that HOCl generated by myeloperoxidase or activated neutrophils converts the peptide VVWGTA to a chlorinated product, WG+32-(Cl). Tandem mass spectrometry in concert with high resolution ^{1}H and two-dimensional NMR analysis revealed that the modification required cross-linking of the tryptophan to the amide of glycine followed by chlorination of the indole ring of tryptophan. In contrast, when glycine in the peptide was replaced with alanine, the major products were mono- and dioxygenated tryptophan residues. When the peptide library VVWXTA (where X represents all 20 common amino acids) was exposed to HOCl, only WG produced a high yield of the chloroindolenine derivative. However, when glycine was replaced by other amino acids, oxygenated tryptophan derivatives were the major products. Our observations indicate that WG may represent a specific sequence motif in proteins that is targeted for chlorination by myeloperoxidase.

Activated phagocytic white blood cells, the cellular hallmark of inflammation, use a membrane-associated NADPH oxidase to generate superoxide, which can dismutate to hydrogen peroxide (H_2O_2).[1] Activated neutrophils, monocytes, and some populations of human macrophages also secrete myeloperoxidase, a heme protein that uses H_2O_2 to convert chloride to hypochlorous acid (HOCl) (*1*−*4*). HOCl is a highly reactive cytotoxin that plays a key role in host defense by oxidizing cellular constituents of pathogens (*5*, *6*). However, it can also damage host proteins at sites of inflammation (*7*−*11*). For example, myeloperoxidase has been detected in human atherosclerotic tissue, and levels of 3-chlorotyrosine, a specific product of HOCl, are elevated in low-density lipoprotein and high-density lipoprotein isolated from these chronic inflammatory lesions (*12*, *13*). Indeed, oxidative footprints of myeloperoxidase have been found in human disorders ranging from renal failure (*14*) to an array of pulmonary diseases (*15*−*17*).

It has long been appreciated that thiols and the thioether group of methionine are major targets for oxidation in proteins. More recent studies indicate that amino groups and tyrosine residues are important sites for chlorination by HOCl (*13*, *18*−*22*). In contrast, remarkably little is known about the reactions of the aromatic amino acid tryptophan (Trp) with HOCl (*23*−*25*). Tryptophan is oxygenated by reagent HOCl, and Trp fluorescence significantly decreases when lipoproteins are exposed to the myeloperoxidase−H_2O_2−chloride system (*26*), suggesting that Trp residues are potential biological substrates for the hypochlorous acid.

The indole ring of Trp has a high reduction potential and reacts rapidly with a wide range of oxidants (*27*). Oxidative reactions convert Trp to kynurenine, *N*-formylkynurenine, oxindolylalanine, and hydroxytrptophan, altering protein function as well as structure (*28*−*34*). Indeed, pioneering studies by Kuroda and co-workers demonstrated that oxidation of a single Trp residue inactivates lysozyme in vitro (*30*). Moreover, a carbonate radical anion cross-links the enzyme superoxide dismutase by a reaction pathway involving Trp residues (*31*). High molecular weight aggregates of

† This work was supported by Grants HL075381, P50 HL073996, P30 ES073996, P30 DK035816, PO1 HL030086, and HL078527 from the National Institutes of Health.

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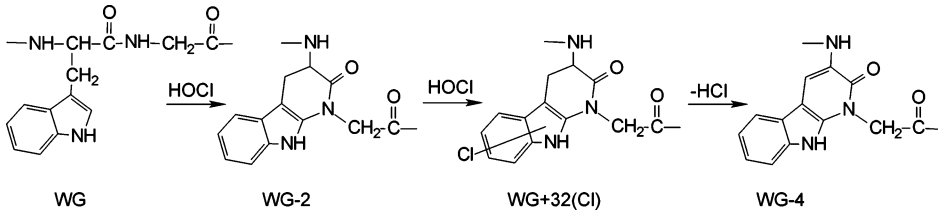
[1] Abbreviations: HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; MPO, myeloperoxidase; WG+32(Cl), chlorinated peptide; VVWXTA, peptide library (where X represents all 20 common amino acids); PMA, phorbol myristate acetate; PBS, phosphate-buffered saline; LC, liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; *m*/*z*, mass-to-charge ratio; amu, atomic mass unit; RIC, reconstructed ion chromatogram; TIC, total ion chromatogram; t_R, retention time; NMR, nuclear magnetic resonance.

10.1021/bi052339+ CCC: $33.50
Published on Web 03/01/2006

Scheme 1: Proposed Reaction Pathway for the Formation of WG-4



superoxide dismutase have been detected in patients suffering from amyotropic lateral sclerosis (*31*), suggesting that cross-linking of Trp residues might be pathogenic. *Pseudomonas fluorescens* produces a halogenating enzyme, PrnA, that catalyzes the regioselective chlorination of the 7 position of tryptophan (*35*). A variety of oxygenated Trp products have been identified in mitochondrial proteins and photosystem II of plants (*32*, *33*). Both protein complexes have been implicated in the generation of reactive intermediates, indicating that Trp is a physiologically relevant target for oxidation.

Most studies of protein oxidation have focused on the reactivity of amino acid side chains and have ignored the potential importance of adjacent sequences. However, we previously showed that HOCl inactivates matrilysin (MMP-7), a matrix metalloproteinase involved in cancer metastasis and inflammation, in concert with cross-linking and aromatization of adjacent Trp and Gly residues (WG) to form WG-4 (*36*, *37*). The reaction pathway (Scheme 1) has been proposed to involve the formation of a chlorinated species, but the structure of this intermediate has not been established, nor has it been determined whether amino acid residues adjacent to the Trp residue have a major impact on the reaction pathway for oxidation.

In the current studies, we use synthetic peptides and a peptide library based on the sequence of matrilysin to identify motifs that might drive chlorination of tryptophan by myeloperoxidase. Our results demonstrate that HOCl generated by myeloperoxidase converts WG to WG+32(Cl), a chloroindolenine derivative. In contrast, when the Gly residue adjacent to Trp is replaced by Ala or any other common amino acid, oxygenated Trp isomers are the major products of the myeloperoxidase reaction. Our observations suggest that WG might play a role in inflammatory diseases by directing site-specific chlorination of proteins.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise indicated, all materials were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hypochlorite (NaOCl), H_2O_2, and HPLC-grade CH_3CN were obtained from Fisher Scientific (Pittsburgh, PA). Peptides VVWGTA and VVWATA were synthesized by the Protein and Nucleic Acid Chemistry Laboratory (Washington University, St. Louis, MO). Myeloperoxidase (A_{430}/A_{280} ratio > 0.8) was purified from HL-60 cells by sequential lectin affinity, ion exchange, and size exclusion chromatographies (*38*, *39*). Myeloperoxidase concentrations were determined spectrophotometrically ($\epsilon_{430} = 0.178\ M^{-1}\ cm^{-1}$) (*40*).

Methods. 1. Peptide Library. Combinatorial libraries of VVWXTA were prepared by combining equal molar amounts of the *N*-(9-fluorenyl)methoxycarbonyl derivatives of all 20 common amino acids in a single cartridge, using the standard single-coupling protocol, and purifying the products on a C18 reverse-phase HPLC column.

2. Reaction Conditions. Peptides were incubated in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 138 mM NaCl, and 2.7 mM KCl, pH 7.4) for the indicated time at 37 °C. Reactions were initiated by adding oxidant and terminated by adding a 10-fold molar excess (relative to oxidant) of L-methionine. Concentrations of OCl^- and H_2O_2 were determined spectrophotometrically ($\epsilon_{292} = 350\ M^{-1}\ cm^{-1}$ and $\epsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$) (*41*, *42*).

3. Isolation of Human Neutrophils. Neutrophils were isolated from EDTA-anticoagulated blood by buoyant density centrifugation at 4 °C using Polymorph-Prep (Robbins Scientific, Sunnyvale, CA) (*43*). Neutrophils were washed twice by centrifugation with buffer A (magnesium-, calcium-, phenol-, and bicarbonate-free Hank's balanced salt solution, pH 7.4; Invitrogen Corporation, Grand Island, NY); 100 μM diethylenetriaminepentaacetic acid was included to inhibit reactions catalyzed by metal ions. Residual red blood cells were removed by hypotonic lysis at 4 °C. Neutrophils were pelleted by centrifugation, resuspended in buffer A, and immediately used for experiments. Peptide VVWGTA (1 μM) was exposed for 1 h to neutrophils (1×10^6 cells/mL) in buffer A at 37 °C. Neutrophils were activated with 200 nM phorbol myristate acetate and maintained in suspension by periodic inversion. Cells were removed by centrifugation, and the supernatant was incubated for 24 h at 37 °C. Reactions were terminated by adding L-methionine (100 μM).

4. Liquid Chromatography (LC)−Electrospray Ionization (ESI)−Mass Spectrometry (MS). LC−ESI−MS analyses were performed in the positive ion mode with a Finnigan Mat LCQ ion trap instrument (San Jose, CA) coupled to a Waters 2690 HPLC system (Milford, MA) as described (*44*). Peptides were separated at a flow rate of 0.2 mL/min on a reverse-phase column (Vydac C18 MS column, 2.1 mm × 250 mm) using solvent A (0.2% formic acid in water) and solvent B (0.2% formic acid in 90% CH_3CN, 10% water). Peptides from the neutrophil reaction mixture were eluted using the following linear gradient: 0−10% B over 5 min, 10−30% B over 50 min, then 30−70% B over 5 min; or 0−15% B over 5 min, 15−35% B over 20 min, then 35−65% B over 5 min. The electrospray needle was held at 4500 V. Nitrogen, the sheath gas, was set at 70 units. The collision gas was helium. The temperature of the heated capillary was 220 °C.

5. HPLC Analysis. Peptides were separated at a flow rate of 1.0 mL/min on a reverse-phase column (Ultra-sphere, C18, 5 μm, 4.6 mm × 250 mm; Beckman, Fullerton, CA) using a Beckman HPLC system equipped with a photodiode array detector. Peptides were eluted using a linear gradient of 20−50% CH_3CN in either 0.2% HCOOH or 0.06% trifluoroacetic acid over 20 min.

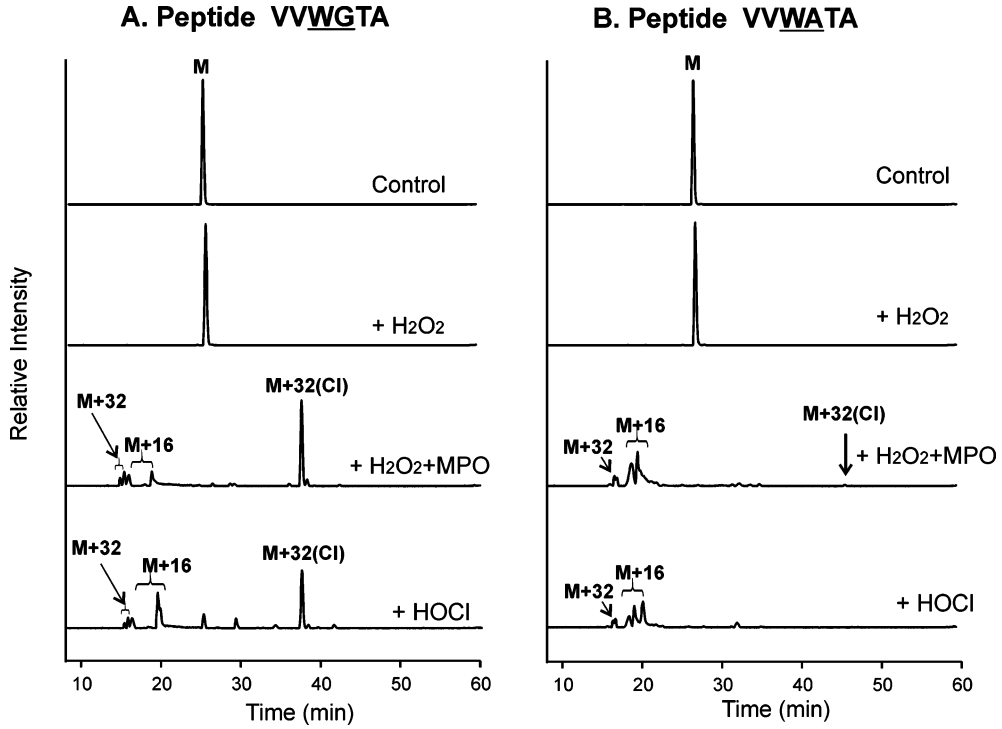


FIGURE 1: Total ion chromatograms of VVWGTA (A) or VVWATA (B) exposed to H_2O_2, the myeloperoxidase−H_2O_2−chloride system, or HOCl. Peptide (20 μM) VVWGTA or VVWATA was incubated for 30 min at 37 °C in buffer (PBS, pH 7.4) alone (Control) or supplemented with 100 μM H_2O_2, 100 μM H_2O_2 and 50 nM myeloperoxidase (MPO), or 40 μM HOCl. Reactions were initiated by adding oxidant and terminated by adding L-methionine (1:10, mol/mol, oxidant/Met). The reaction mixture was analyzed by LC−ESI−MS. Ion chromatograms of precursor and product peptides were normalized to the ion current of VVWGTA or VVWATA incubated in PBS alone. M, peptide.

6. NMR Spectrometry. NMR experiments were performed at 25 °C on Varian Inova 600 spectrometer (Varian, Inc., Palo Alto, CA) with a 5 mm triple-axis gradient probe. The two-dimensional NMR spectra were obtained from a 10 mM peptide solution in a mixture of 90% H_2O and 10% D_2O (*45*). The total correlation spectra were recorded using an MELV-17 mixing sequence of 100 ms flanked by two 2 ms trim pulses. Phase-sensitive 2D spectra were obtained with the hypercomplex method. A total of $2 \times 256 \times 2048$ data matrices with 16 scans per t_1 value were collected. Gaussian and sine-bell apodization functions were used in weighting the t_2 and t_1 dimensions, respectively. After two-dimensional Fourier transformation, the spectra resulted in 2048×2048 data points, which were then phase- and baseline-corrected in both dimensions.

RESULTS

The Myeloperoxidase−H_2O_2−Chloride System Converts Tryptophan in the Peptides VVWGTA and VVWATA to Different Oxidation Products. We previously showed that the loss of protease activity that is seen when HOCl oxidizes MMP-7 occurs in concert with the oxidation of adjacent WG residues. To investigate the potential importance of adjacent sequences in directing the specificity of Trp oxidation, we performed parallel experiments with the peptides VVWGTA, which mimics the region of MMP-7 containing WG, and VVWATA, in which Ala replaces Gly. We exposed the peptides to the complete myeloperoxidase system (enzyme + H_2O_2 + Cl^-) for 30 min at 37 °C in a physiological buffer at neutral pH. The peptide and hydrogen peroxide were present at a 1:5 molar ratio. The reaction was terminated by adding a molar excess (relative to oxidant) of methionine.

HPLC analysis of the VVWGTA reaction mixture revealed a major peak of new material that eluted later than the precursor peptide (Figure 1A). Minor products (t_R = 15−21 min) that eluted before the precursor peptide were also detected. HOCl yielded the same spectrum of oxidized peptides, implicating HOCl generation by myeloperoxidase in the reaction pathway. In contrast to the results obtained with VVWGTA, major early eluting oxidation products were observed when VVWATA was incubated with myeloperoxidase, H_2O_2, and Cl^- (Figure 1B). H_2O_2 alone (5:1, mol/mol, oxidant/peptide) failed to convert either VVWGTA or VVWATA into new products (Figure 1).

To identify the oxidative modifications that occurred when VVWGTA (M, *m*/*z* 632.2) was exposed to the complete myeloperoxidase system, we subjected the reaction mixture to LC−ESI−MS analysis (Figure 2, left panel). Four or more peaks of new material eluting between 15 and 21 min (Figure 1A) had gained 16 or 32 amu (Figure 2A,B; M+16, mass-to-charge ratio (*m*/*z*) 648.2 and M+32, *m*/*z* 664.2), suggesting that the peptides were oxidized to multiple different species by acquisition of one or two oxygen atoms. Indeed, Trp oxidation has been shown to yield at least five different oxygenated isomers (*32*, *33*). Consistent with this proposal, each of the product peptides had a shorter retention time—and therefore were likely to be more hydrophilic—than the precursor peptide. Interestingly, the major peak of new material (Figure 1A; t_R = 37.4 min) also had gained 32 amu (Figure 2C; M+32, *m*/*z* 664.2), but it had a longer retention time than the precursor peptide, suggesting that it was more hydrophobic. Moreover, the peptide exhibited the characteristic isotopic pattern of a chlorinated molecule (3:1 ratio of M and M+2, representing ^{35}Cl and ^{37}Cl). We termed this

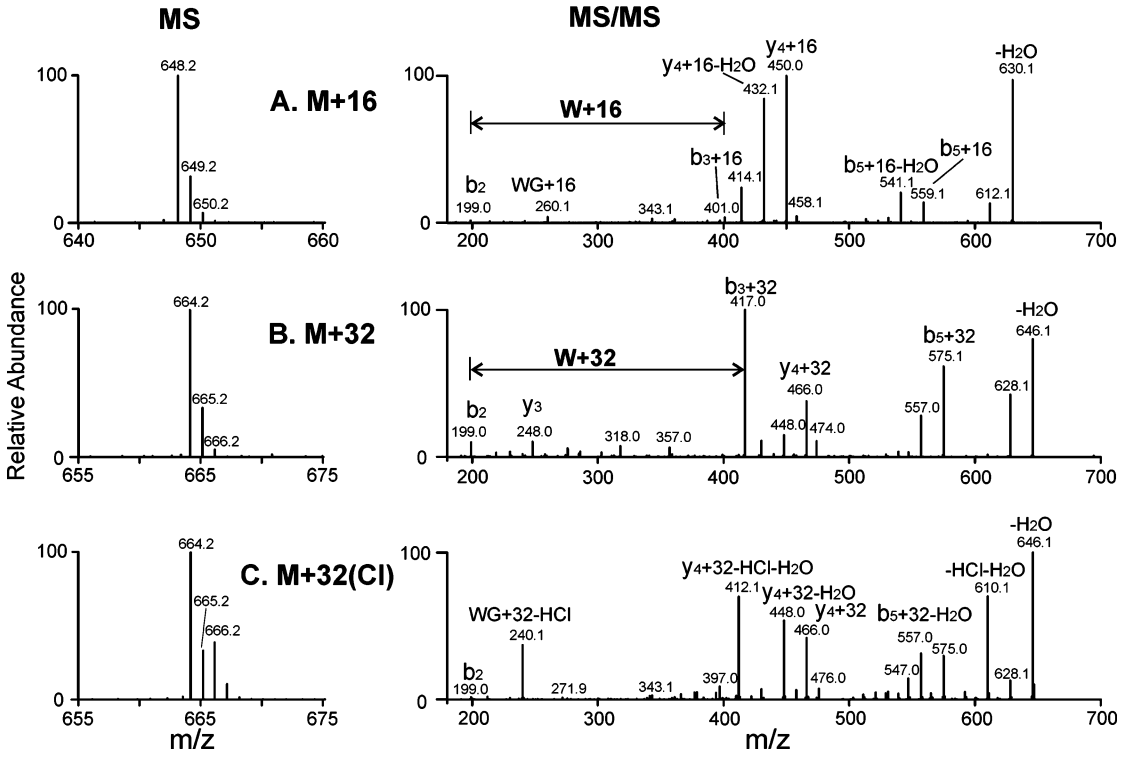


FIGURE 2: MS and MS/MS analysis of VVWGTA exposed to the complete myeloperoxidase−H_2O_2−Cl^- system. Reaction conditions were identical to those described in the legend to Figure 1. (A) MS (left) and MS/MS (right) of M+16 products; (B) MS (left) and MS/MS (right) of M+32 products; (C) MS (left) and MS/MS (right) of M+32(Cl) product. Note that the ions of *m*/*z* 664.2 and 666.21 in the full scan mass spectrum of M+32(Cl) exhibit the isotopic ratio of a chlorinated compound (~3:1, the anticipated relative abundance of ^{35}Cl and ^{37}Cl).

peptide M+32(Cl) to distinguish it from the oxygenated peptides (M+16 and M+32). These observations suggest that the complete myeloperoxidase−H_2O_2−Cl^- system, but not H_2O_2 alone, converts VVWGTA to a major chlorinated product and several minor oxygenated products.

LC−ESI−MS analysis of VVWATA exposed to the complete myeloperoxidase system or HOCl demonstrated multiple peptide peaks eluting between 15 and 21 min (Figure 1B) that had gained 16 or 32 amu (M+16, *m*/*z* 662.2, and M+32, *m*/*z* 678.1; data not shown). The full scan mass spectra of the new peaks were consistent with the addition of one or two oxygen atoms to the precursor peptide. These peaks of material likely represent oxygenated Trp products. In contrast, only a minor peak of material eluted later than the precursor peptide (Figure 1B). This late eluting peak demonstrated the characteristic isotopic pattern of a chlorinated molecule (data not shown).

These observations suggest that the myeloperoxidase system generates a relatively stable chlorinated peptide when it oxidizes VVWGTA but yields multiple oxygenated species as its major products when it oxidizes VVWATA.

Chlorinated Peptide Is the Major Product When the Myeloperoxidase−H_2O_2−Chloride System Oxidizes VVWGTA. To identify the major sites that are oxidized when VVWGTA is exposed to the complete myeloperoxidase system, we analyzed the structure of M+32(Cl) using LC−ESI−MS/MS (Figure 2, right panel). MS/MS (Figure 2C, right panel) revealed prominent ions at *m*/*z* 412.1 and *m*/*z* 610.1, implying the loss of HCl from the precursor ion, M+32(Cl) (y_4+32-HCl−H_2O and M+H−HCl). Importantly, an ion of *m*/*z* 240.1—consistent with the formation of WG+32-HCl—was apparent in the mass spectrum. Detection of this ion strongly implied that both Trp and Gly had been oxidatively modified. These observations support the proposal that M+32(Cl) is chlorinated. The high yield of this product suggests that the reaction pathway might be physiologically relevant.

Oxygenated Tryptophan Residues Are Minor Products When the Myeloperoxidase−H_2O_2−Chloride System Oxidizes VVWGTA. LC−ESI−MS/MS analysis of material from the minor peaks (t_R 15−21 min) that formed when VVWGTA was exposed to the myeloperoxidase system demonstrated that the Trp residue had gained one or two oxygen atoms (Figure 2A,B; 16 or 32 amu). MS/MS analysis demonstrated at least four oxygenated peptides with different retention times. MS/MS analysis indicated that Trp was the site of oxygen addition (Figure 1A). Two peptides gained one oxygen atom (M+16) and two peptides gained two oxygen atoms (M+32). These results are consistent with previous studies demonstrating that HOCl and other reactive intermediates convert the indole ring of Trp to hydroxy or dihydroxy derivatives (*28*, *29*). Thus, oxygenation of Trp appears to be a minor oxidative pathway when VVWGTA is exposed to HOCl generated by myeloperoxidase.

Oxygenated Tryptophans Are Major Products When the Myeloperoxidase−H_2O_2−Chloride System Oxidizes VVWATA. When we replaced the Gly residue of VVWGTA with Ala and oxidized the resulting peptide with either HOCl or the complete myeloperoxidase system (Figure 1B), LC−ESI−MS/MS detected only low yields of the chlorinated Trp product (M+32(Cl)). The major products were derived from the addition of either one or two oxygens to the Trp residue (M+16 or M+32). Importantly, the product yield was much lower when M+32(Cl) formed from VVWATA rather than

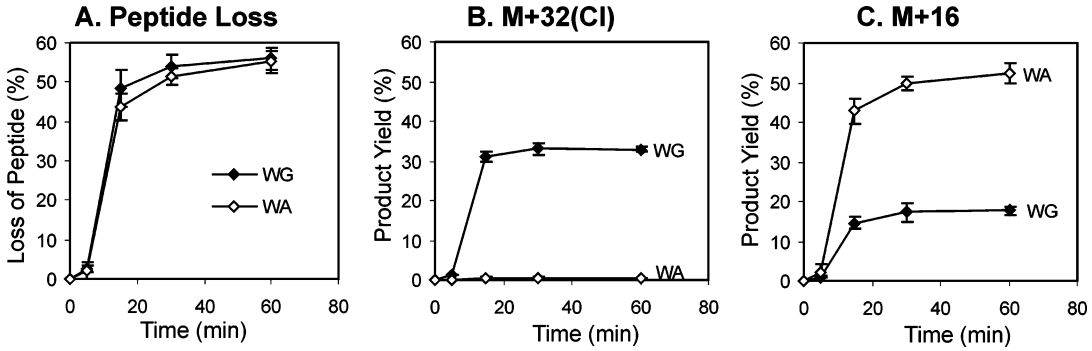


FIGURE 3: Kinetics (A) and product yields (B and C) of VVWGTA and VVWATA oxidized by the complete myeloperoxidase−H_2O_2−Cl^- system. A peptide mixture of 20 μM VVWGTA and 20 μM VVWATA was incubated with 100 μM H_2O_2 and 50 nM myeloperoxidase (MPO) for the indicated time at 37 °C in buffer (PBS, pH 7.4). Reaction mixtures were analyzed by LC−ESI−MS, and peptides were quantified by reconstructed ion chromatograms. The results of M+16 represent the sum of all isomers of M+16. Results represent the average and SD of four independent experiments.

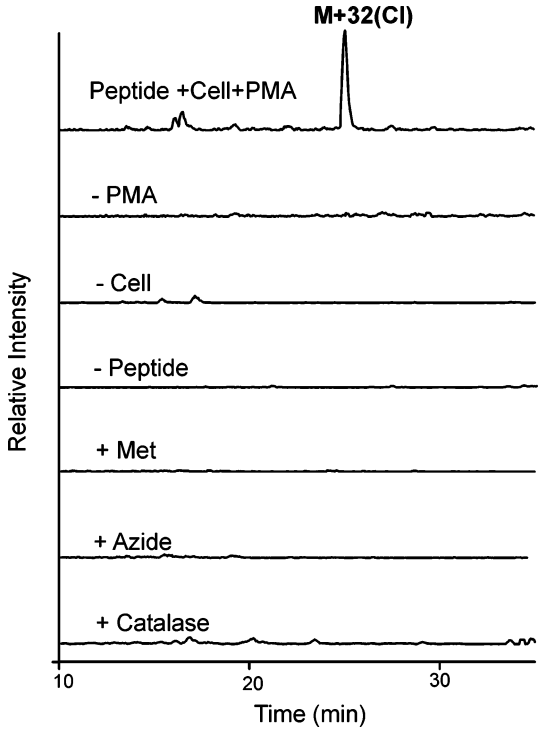


FIGURE 4: Chlorination of VVWGTA by activated human neutrophils. VVWGTA (1 μM) was incubated with neutrophils (10^6/mL) in buffer A (magnesium-, calcium-, phenol-, and bicarbonate-free Hank's balanced salt solution, pH 7.4) for 1 h at 37 °C. Neutrophils were activated with 200 nM phorbol myristate acetate (PMA), and the supernatant of the cells was incubated overnight at 37 °C. Where indicated, PMA or cells were omitted or 1 mM L-methionine (L-met), 1 mM azide, or 100 nM catalase was included in the buffer. Production of the chlorinated peptide M+32(Cl) was monitored by LC−ESI−MS with detection of ions of *m*/*z* 664.2 on reconstructed ion chromatograms.

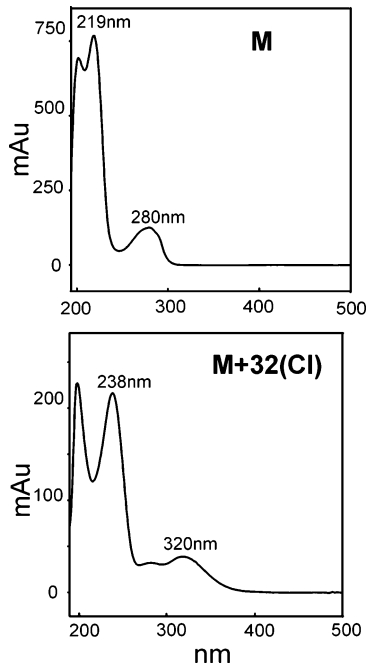


FIGURE 5: UV analysis of chlorinated VVWGTA. Peptide VVWGTA (20 μM) was incubated for 30 min at 37 °C in buffer supplemented with 40 μM HOCl. Reactions were initiated by adding oxidant and terminated by adding L-methionine (1:10, mol/mol, oxidant/Met). The reaction mixture was analyzed by HPLC equipped with an on-line diode array spectrophotometer. Note that M+32(Cl) exhibits an absorption maximum at 320 nm, suggesting that the number of conjugated double bonds had increased relative to the Trp containing precursor peptide.

from VVWGTA (estimated molar ratio of WG+32(Cl) to WA+32(Cl) ~70:1). These observations support the hypothesis that HOCl oxidizes WG and WA via distinct reaction pathways.

Relative Rates of Oxidation of VVWGTA and VVWATA by the Myeloperoxidase−H_2O_2−Chloride System. To directly compare the relative susceptibilities of the two peptides to oxidation, we exposed a reaction mixture containing equal concentrations (20 μM) of VVWGTA and VVWATA to the complete myeloperoxidase system containing H_2O_2 (100 μM). Under these conditions, oxidation of the two peptides produced almost identical progress curves (Figure 3A), indicating similar reaction rates with HOCl. Moreover, the extent of oxidation of both peptides was similar (~55% loss of precursor peptide). However, the relative yields of the oxidation products differed markedly (Figure 3B,C). The major product (~33% product yield) of VVWGTA was the chlorinated species M+32(Cl), with a lower yield (~18% product yield) of M+16 isomers. In contrast, the major product (~50% product yield) of VVWATA oxidation was multiple isomers of the oxygenated species M+16, and the product yield of chlorinated peptide was less than 2%. For both VVWGTA and VVWATA, ~2−4% of the precursor peptide was converted to M+32 isomers that had gained two oxygen atoms (data not shown). These observations provide strong evidence that the intrinsic reactivity of Trp with HOCl

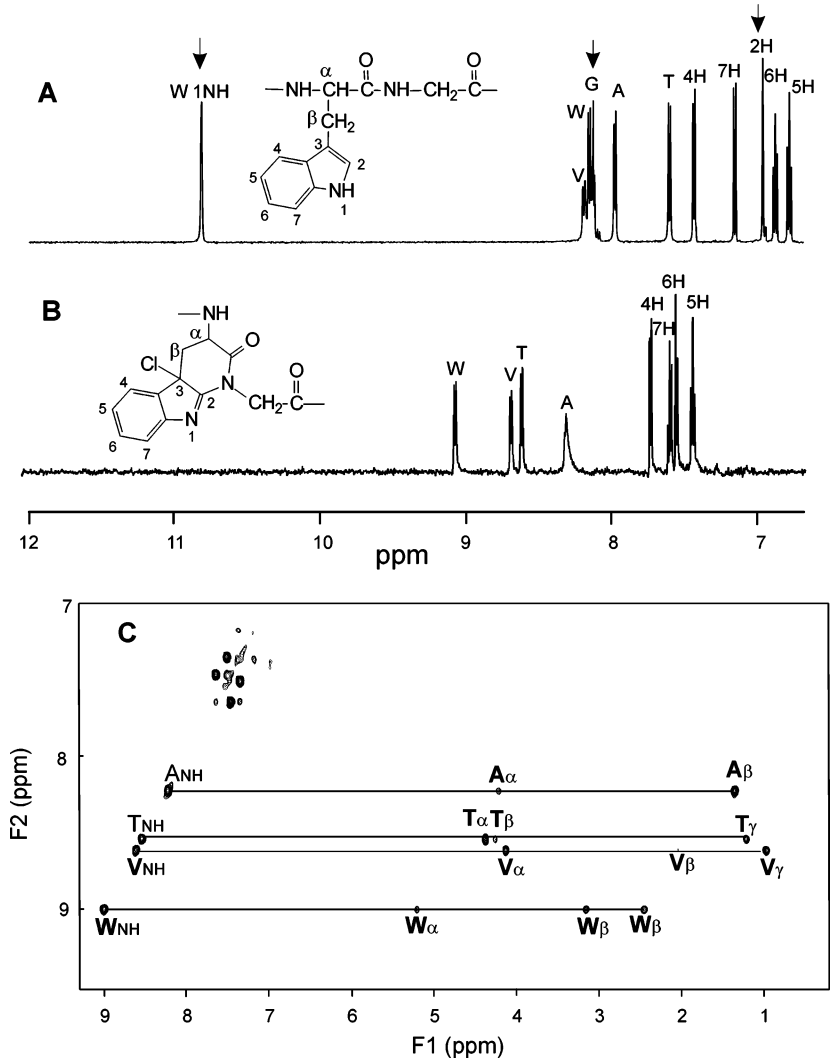


FIGURE 6: High-resolution ^{1}H NMR and total correlated spectroscopy analysis of chlorinated VVWGTA. (A) ^{1}H NMR analysis of the precursor peptide VVWGTA; (B) ^{1}H NMR analysis of chlorinated VVWGTA (M+32(Cl)); (C) total correlated spectroscopy analysis of chlorinated VVWGTA. VVWGTA (20 μM) was incubated for 30 min at 37 °C in buffer supplemented with 40 μM HOCl. Following the addition of methionine, M+32(Cl) was isolated from the reaction mixture by reverse-phase HPLC, dried under vacuum, and subjected to NMR analysis.

is similar in the two peptides but that the amino acid that lies on the C-terminal side of Trp determines the final reaction pathway.

Human Neutrophils Use the Myeloperoxidase−H_2O_2 System To Chlorinate VVWGTA. To investigate the potential physiological relevance of Trp−Gly chlorination, we determined whether oxidants generated by neutrophils might also convert WG to WG+32(Cl). Human neutrophils were activated with phorbol myristate acetate (PMA) at 37 °C in buffer A supplemented with 1 μM VVWGTA. LC−ESI−MS analysis with reconstructed ion chromatograms revealed the formation of M+32(Cl) in the medium of the stimulated cells (Figure 4). MS/MS analysis confirmed that the oxidation product was the chlorinated product WG+32(Cl). Generation of this product required cellular activation and was inhibited by the peroxide scavenger catalase, the heme poison sodium azide, and the HOCl scavenger methionine. These observations indicate that HOCl generated by the myeloperoxidase system of activated human neutrophils can react with the model peptide VVWGTA to form WG+32(Cl). Thus, the WG motif in proteins might be chlorinated by activated phagocytes at sites of inflammation.

UV Analysis of Chlorinated Peptide M+32(Cl). We used HPLC together with a photodiode array detector to determine the UV−visible spectra of peptide VVWGTA and its chlorinated product. The precursor peptide had an absorption maximum at 280 nm, which confirmed that it contained Trp (Figure 5; M). We previously demonstrated that HOCl initially converts WG to M−2 by a reaction pathway that involves the loss of two hydrogen atoms (*37*). M−2 reacts rapidly with a second HOCl molecule to form M+32(Cl). The absorption spectrum of M+32(Cl) was further red-shifted, with maximal absorption at 320 nm (Figure 5; M+32(Cl)). Collectively, these observations suggest M+32-(Cl) might represent an unusual aromatic compound.

NMR Analysis Indicates That M+32(Cl) Is a Cross-Linked Chloroindolenine Derivative. We used NMR spectroscopy to further investigate the structure of M+32(Cl). High-resolution ^{1}H NMR analysis of M+32(Cl) that had been purified by HPLC demonstrated loss of the Trp indole amine NH resonance (W 1NH, arrow), the indole ring 2H resonance (arrow), and the glycine amide NH resonance (G, arrow) from VVWGTA (compare Figure 6, panels A and B). The downfield shift in the peptide amide resonances of W, V, T,

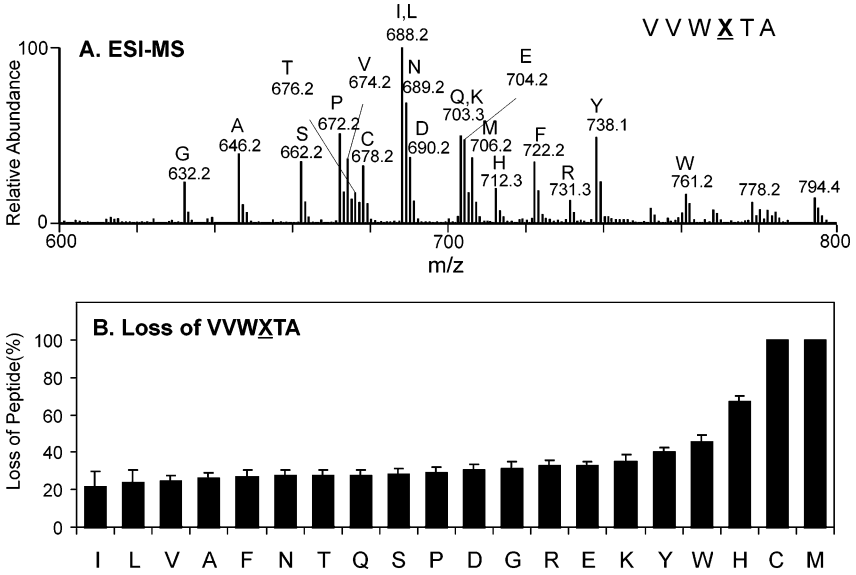


FIGURE 7: Mass spectrometric analysis of the peptide library VVWXTA after exposure to the complete myeloperoxidase−H_2O_2−Cl^- system. (A) Full scan mass spectrum of VVWXTA (X = all 20 common amino acids). Peptide library (20 μM total peptide; ~1 pmol/μL of each peptide) solubilized in 0.2% formic acid, 20% CH_3CN was continuously infused (3 μL/min) into the ion trap mass spectrometer. Single letters indicate the anticipated amino acid at position X in each peptide. Note that peaks of material expected to contain isobaric amino acids (I, L) exhibited ~2-fold more ion current, suggesting that the mixture included peptides containing both amino acids. (B) Oxidation of the peptide library by myeloperoxidase. VVWXTA (100 μM final concentration) was incubated with 100 μM H_2O_2 and 50 nM myeloperoxidase for 1 h at 37 °C in buffer (PBS, pH 7.4). The reaction was terminated by adding L-methionine (1:10, mol/mol, oxidant/Met), and the reaction mixture was analyzed by LC−ESI−MS. Peptide loss was quantified from the ratio of the ion current of each peptide in the oxidized or control reaction mixtures relative to that of the internal standard VVGWVTA, which was added to the reaction mixture after the reaction was terminated. Results represent the average and SD of six independent experiments.

and A as well as the Trp indole ring hydrogens is consistent with the addition of an electron-withdrawing group to the aromatic ring system. In particular, the large downfield shift of the Trp amide resonance indicated that the chlorine substitution may appear at the Trp residue. Total correlated spectroscopy confirmed one α hydrogen and two β hydrogens in the chlorinated product (Figure 6C) and the loss of the Trp indole amine NH resonance. These observations, together with the increase of 32 amu in M+32(Cl), are consistent with the formation of a cross-link between the Gly amide and the C2 of Trp. The loss of NH resonance (Figure 6B, inset) suggests the formation of a chloroindolenine (*46*−*51*).

Oxidation of the Peptide Library VVWXTA Indicates That WG Is the Dominant Motif for Chlorination. To determine whether the nitrogen atom in the indole ring of Trp is chlorinated only when Trp is adjacent to Gly, we used the peptide library VVWXTA, where X represents all 20 common amino acids. We first characterized the library by ESI−MS and MS/MS. MS analysis detected ions with the *m*/*z* anticipated for all 20 peptides in the library (Figure 7A). The sequences of the peptides were confirmed by MS/MS analysis. Moreover, the ion currents of the different peptides were similar, suggesting that the library contained a similar concentration of each peptide. To investigate the role of the amino acid on the C-terminal side of Trp, we exposed the 20 peptides in the library to the complete myeloperoxidase−H_2O_2−chloride system and separated the products by HPLC. At a 1:1 molar ratio of oxidant to total peptides, peptides containing Cys and Met were completely converted to their corresponding oxygenated peptides, which is consistent with the well-established reactivity of sulfur compounds with HOCl ($k_{cys} = 3 \times 10^7$ M^{-1} s^{-1}, $k_{met} = 4 \times 10^7$ M^{-1} s^{-1} (*18*, *52*)). Most of the 20 peptides were converted to a derivative that had gained one or two oxygens, and MS/MS analysis suggested that—with the exception of WG—the predominant products were oxygenated and dioxgenated Trp derivatives. The relative loss of precursor peptide for most of the amino acids was 20−40%, suggesting that the reactivity of Trp was not greatly altered by the identity of its neighbor on the C-terminal side. However, the five peptides that exhibited the least oxidation all contained a hydrophobic residue, suggesting that this class of amino acids lowered the reactivity of an adjacent Trp residue. Moreover, we observed a significantly greater loss of precursor peptide (~67%) when X was His, suggesting that peptide containing WH might be especially reactive with HOCl—presumably because the peptide contains a highly reactive His residues ($k_{his} = 1 \times 10^5$ M^{-1} s^{-1} (*52*)). There was also a trend toward increased loss of the peptide (~46%) containing two adjacent Trp residues ($k_{trp} = 1.1 \times 10^4$ M^{-1} s^{-1} (*52*)).

We used reconstructed ion chromatograms to search for material with the anticipated *m*/*z* of chlorinated peptides after we exposed the library to various molar ratios of HOCl (Figure 8A). This approach detected a single major chlorinated product, WG+32(Cl) (t_R = 36.7 min), which was derived from VVWGTA. Two minor chlorinated products, WA+32(Cl) (t_R = 45.1 min) and WS+32(Cl) (t_R = 45.7 min), were also present. Both the isotope pattern of the oxidized peptides and MS/MS analysis of the ions of *m*/*z* 664.2 and *m*/*z* 678.2 were consistent with chlorination of W in VVWGTA and VVWATA, respectively (data not shown). The full scan mass spectrum of WS+32(Cl) also exhibited the isotope pattern of a chlorinated product, but MS/MS failed to reveal fragmentation ions that suggested loss of HCl (e.g., ions consistent with (WS)+32-HCl and y_4+32-HCl−H_2O; Figures 2C and 3C). These observations suggest that WS might yield a chlorinated species that is structurally

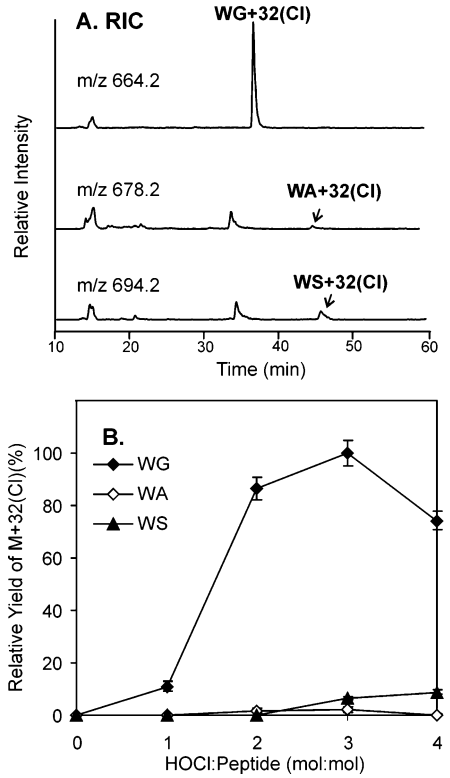


FIGURE 8: Detection of chlorinated peptides in the library VVWXTA exposed to HOCl. Peptide library VVWXTA (100 μM final concentration) was oxidized with 200 μM HOCl as described in the legend to Figure 7B. Chlorinated peptides were detected by LC−ESI−MS with monitoring of reconstructed ion chromatograms (RIC). (A) Material with the anticipated *m*/*z* of a chlorinated peptide was detected for WG (*m*/*z* 664.2), WA (*m*/*z* 678.2), and WS (*m*/*z* 694.2). The identities of the peptides were confirmed by MS/MS analysis. (B) Relative yield of chlorinated peptides WG, WA, and WS. The peptide library was oxidized with the indicated molar ratio of HOCl and analyzed by LC−ESI−MS. Relative yield was assessed by determining the ratio of the ion currents of the chlorinated and precursor peptides and was normalized to WG+32 (Cl). Results represent the average and SD of three independent experiments.

distinct from those derived from WG and WA. However, WG was the major target for oxidation over a wide range of HOCl concentrations (Figure 8B). These observations suggest that WG is likely to be the major source of relatively stable chloroindolenine derivative in proteins, but that WA and WS might yield smaller quantities of chlorinated species.

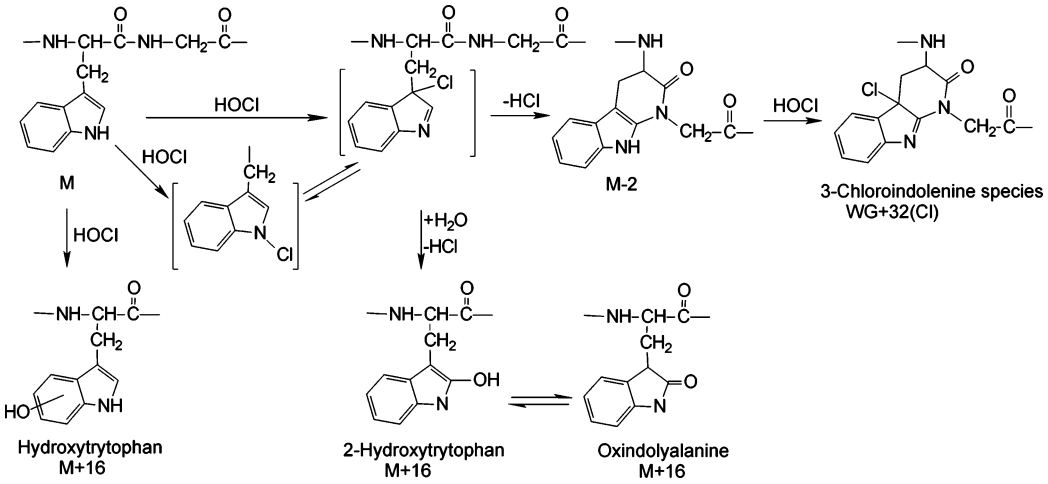
DISCUSSION

In the current studies, we used model peptides to investigate the factors that control the chlorination and oxygenation of Trp by myeloperoxidase. Significantly, we found that the identity of the adjacent residue determined which pathway oxidized Trp. When we exposed VVWGTA to HOCl or the myeloperoxidase−H_2O_2−Cl^- system, the major product was the chlorinated peptide WG+32(Cl), which was produced in high yield. Tandem mass spectrometric and NMR studies provided strong evidence that M+32(Cl) is a chlorinated species derived from the indole ring of Trp. In contrast, when the substrate was VVWATA (created by replacing the peptide's Gly residue with Ala), the major oxidation products were M+16 and M+32, which were mono- and dioxygenated derivatives of Trp. Thus, the sequences flanking Trp have a major impact on the way Trp reacts with HOCl.

On the basis of indole chemistry and the reactions of HOCl, we propose the following pathway for Trp oxygenation and chlorination (Scheme 2). The indole ring of Trp initially reacts with HOCl to yield a chlorinated species, which is likely to be the 3-chloroindolenine or perhaps the *N*-chloroindole species (*46*, *53*−*57*). When Trp is adjacent to most common amino acids, this reactive intermediate undergoes an addition reaction with H_2O and loss of HCl to form oxygenated products such as oxindolylalanine or 2-hydroxytryptophan. Alternatively, HOCl can oxidize directly the benzene ring of the indole to yield a variety of oxygenated isomers (*28*, *55*).

When a Gly residue is adjacent to the 3-chloroindolenine, however, its side chain—a single hydrogen atom—does not exert the steric hindrance seen with other amino acids. Therefore, the nitrogen on the amide group of Gly can cross-link to C2 of the intermediate's indole ring. The cross-linked intermediate, M−2, then reacts with HOCl to form the chlorinated species WG+32(Cl), which may be the 3-chloroindolenine or *N*-chloro derivative (*47*−*51*). However, it is important to note that *N*-chloro derivatives of primary and secondary amines react readily with Met (*20*), the thiol ether containing amino acid we used to terminate all the oxidation

Scheme 2: Proposed Reaction Pathways for the Oxygenation and Chlorination of Tryptophan



reactions. Thus, WG+32(Cl) must be stable in the presence of Met, suggesting that the 3-chloroindolenine derivation is likely to be the major species. Consistent with this proposal, the product yield of WG+32(Cl) was not diminished when 5-thio-2-nitrobenzoic acid was added to the reaction mixture (data not shown), indicating that the chlorinated intermediate was unlikely to contain a N−Cl bond (*18*). Our NMR results are consistent with a 3-chloroindolenine derivative as the intermediate in the reaction pathway. We also detected a small peak of material whose MS and MS/MS spectra were identical to those of WG+32(Cl) but that exhibited a different retention time on HPLC analysis. This peak might represent an additional isomer that forms when Cl^+ attacks one of the two faces of the indole ring. These observations indicate that the amino acid directly adjacent to Trp determines in part which pathway oxidizes the indole ring. They further suggest that long-range interactions within the same or different regions of proteins might have a similar effect if, for example, Trp and Gly residues from different parts of the primary sequence are brought together by protein folding.

Importantly, we also found that activated human neutrophils produced WG+32(Cl). The reaction pathway was dependent upon activation of the cells and was blocked by the peroxide scavenger catalase, the HOCl scavenger methionine, and the heme inhibitor azide, implicating HOCl generated by myeloperoxidase in the reaction pathway. Collectively, these observations raise the possibility that chlorinated species derived from the indole ring of Trp might play a previously unsuspected role in oxidation reactions executed by myeloperoxidase in vivo.

To globally assess the factors that might affect the oxidation of Trp by HOCl, we exposed the peptide library VVWXTA (X = all 20 common amino acids) to the oxidant. MS and MS/MS analysis suggested that the library contained roughly equal molar concentrations of the 20 possible peptides. Because each peptide had to compete with the other 19 peptides to react with HOCl, quantifying the oxidation products should have provided an index of the relative reactivity of Trp in the different sequence contexts.

Previous studies have indicated that amino acid side chains containing thiols and thioethers react the most rapidly with HOCl (*5*, *57*). Therefore, when the library was reacted with an equimolar concentration of HOCl (relative to the total peptide concentration), the peptides containing Cys or Met were consumed quantitatively in concert with the appearance of oxygenated sulfur products. In contrast, most of the other peptides were oxidized to a similar extent (∼20−40%), suggesting that the local amino acid environment affected the intrinsic reactivity of Trp with HOCl only minimally. However, the peptide containing WH was more sensitive to oxidation. His is also highly reactive with HOCl (*52*), suggesting that the presence of an oxidant-sensitive amino acid increased the overall reactivity of the peptides. MS/MS analysis indicated that, with the exception of WG, the major products of peptide oxidation were oxygenated Trp derivatives. Previous studies have shown that oxygenated species are the major oxidation products when Trp is exposed to HOCl or other oxidants (*56*−*59*). Thus, Trp residues react like free Trp in most peptides.

Remarkably, WG+32(Cl) was the major chlorinated product when the library was exposed to HOCl. This observation strongly supports the proposal that Trp can be converted to its chloroindolenine derivative only when it resides next to a residue whose side chain creates no steric hindrance. However, we did detect small amounts of chlorinated intermediates derived from WA and WS, indicating that Ala and Ser might also promote the formation of chlorinated species derived from Trp. MS/MS analysis suggested that WS might yield a chlorinated species that is structurally distinct from that derived from WG and WA. These observations suggest that WG is the preferred sequence for Trp cross-linking and the chlorination reaction but that WA and WS might yield much smaller amounts of chlorinated indole derivatives.

Chlorinated tyrosine and chlorinated nucleobases identical to those formed by myeloperoxidase or HOCl in vitro have been detected in inflamed human tissue (*12*−*17*, *60*), but remarkably little is known about chlorination of Trp residues in proteins. Our model system studies suggest that HOCl generated by myeloperoxidase selectively forms a chloroindolenine species when it oxidizes the sequence motif WG, and that this reaction leads to the formation of WG-4. Our previous work showed that low concentrations of HOCl *activate* pro-matrilysin by oxygenating a specific thiol residue in the enzyme's prodomain, whereas WG-4 production associates strongly with oxidative *inactivation* of MMP-7 (*36*, *37*, *44*). Indeed, we have recently shown that reactive intermediates derived from the NADPH oxidase of phagocytes appear to regulate MMP activity in vivo (*61*). Such transient activation could be important for regulating proteolytic activity during inflammation. Thus, oxidant production by phagocytes would strictly confine proteinase activity in space and time, permitting only bursts of pericellular proteolysis.

ACKNOWLEDGMENT

We thank Dr. Michael Davies for insightful comments on the proposed reaction pathways. Mass spectrometric studies were performed by the Mass Spectrometry Resource, Department of Medicine, University of Washington.

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BI052339+