Oxidative Cross-linking of Tryptophan to Glycine Restrains Matrix Metalloproteinase Activity

SPECIFIC STRUCTURAL MOTIFS CONTROL PROTEIN OXIDATION*

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Matrix metalloproteinases (MMPs) play a central role in the proteolytic regulation of proteins involved in inflammation and repair, in the turnover of extracellular matrix, and in pathological destruction of tissue proteins. Dyregulation of MMPs is implicated in the pathogenesis of destructive diseases, such as aneurysms, emphysema, and arthritis, as well as tumor growth and metastasis (2–4).

MMPs are synthesized as zymogens (1). The domain of latent MMPs interacts with the zinc ion in the catalytic domain. This interaction is critical to maintaining the enzyme in an inactive state. The mechanisms that control the activation and subsequent inactivation of MMPs have generally been attributed to other proteinases and protein inhibitors, respectively (1). However, the physiological mechanisms regulating MMP proteolysis remain largely unknown. Indeed, reactive oxygen and nitrogen intermediates regulate MMP activity in vitro (5–9) raising the possibility that similar reactions control MMP activity in vivo.

We previously demonstrated that hypochlorous acid (HOCI), a potent oxidant generated by neutrophils, monocytes, and macrophages (10–12), can activate pro-MMP-7 (promatrixins) by converting the thiol residue of the prodomain to the corresponding sulfenic acid (7). Recent studies suggest that oxygenation of pro-MMPs may be physiologically relevant (13).

The modification of MMPs provides a relevant paradigm illustrating how HOCI can regulate protein function by targeting specific amino acids within critical domains. Although HOCI can initially activate pro-MMPs, subsequent inhibition of enzyme activity is the predominant effect at physiologic concentrations of the oxidant (6–8, 10). We recently reported that loss of MMP-7 activity occurred in concert with the formation of WG–4, an unusual oxidation product of tryptophan and glycine residues (WG) that has lost 4 atomic mass units (14). In the current studies, we demonstrate that WG–4 forms an aromatic six-membered ring which kinks the peptide backbone. Our observations suggest that sequential oxidative modifications of specific structural motifs provide a single mechanism for controlling proteolytic enzyme activity by phagocytes.

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1 The abbreviations used are: MMP, matrix metalloproteinase; HOCI, hypochlorous acid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NOESY, nuclear Overhauser spectroscopy; TOCSY, two-dimensional total correlated spectroscopy; HPLC, high performance liquid chromatography.
RESULTS

Oxidation of VVWGTA (M) with HOCl Produces M–2, M+32, and M–4—To investigate the reaction pathway that produces WG–4, we exposed the peptide VVWGTA to HOCl. This peptide duplicates the region in the catalytic domain of MMP-7 that is oxidatively modified in concert with enzyme inactivation (14). Analysis of the reaction mixture by reverse-phase HPLC revealed the unmodified peptide (M) and five major oxidized peptides (Fig. 1A, peaks 1–5). MS analysis of the material in peak 5 demonstrated a major ion of mass-to-charge ratio (m/z) 628.2 (M–4), the predicted mass of singly charged peptide minus 4 atomic mass units (peak 5; M–4), gained 32 atomic mass units (peaks 2 and 4; M+32), or lost 2 atomic mass units (peak 3; M–2). MS/MS analysis of peak 5 revealed a series of ions consistent with 4 atomic mass unit losses from WG (Fig. 1B). Importantly, we observed a major ion of m/z 240.1, the predicted mass of adjacent tryptophan and glycine residues that had lost 4 atomic mass units, indicating that WG–4 is produced when HOCl oxidizes VVWGTA.

The presence of multiple HPLC peaks of material in VVWGTA exposed to HOCl suggested that multiple intermediates participated in the reaction pathway. To explore this possibility, we subjected peaks 1–4 to MS and MS/MS analysis. The major product in the reaction mixture, peak 4 (M+32), exhibited a major ion of m/z 664.2 (Fig. 1B, [M+32 + H]+), suggesting that VVWGTA had gained two oxygen atoms. However, the molecular ion exhibited the characteristic isotopic pattern of a chlorinated compound (Fig. 1B, inset; note the peaks derived from 35Cl and 37Cl). Consistent with this interpretation, MS/MS analysis demonstrated a series of ions consistent with loss of HCl from peak 4. Peak 2 also contained one chlorine atom and had the same m/z as peak 4, indicating that peaks 4 and 2 were isomers. MS/MS analysis of peak 4 revealed an ion of m/z 240.1, the m/z of WG–4.

Fig. 1. Reaction pathway for the production of WG–4. Peptide VVWGTA (100 μM) was incubated in buffer A (150 mM NaCl, 5 mM CaCl2, 10 mM HEPES, pH 7.4) for 30 min at 37°C. Reactions were initiated by adding HOCl (5:1, mol/mol, oxidant/peptide) and terminated by adding l-methionine (10:1, mol/mol, Met/oxidant). A, HPLC analysis of VVWGTA (M) exposed to HOCl. B, MS analysis (left panels) and MS/MS analysis (right panels) of peaks 2–5 in oxidized VVWGTA. Note that VVWGTA has lost 4 atomic mass units (peak 5; M–4), gained 32 atomic mass units (peaks 2 and 4; M+32), or lost 2 atomic mass units (peak 3; M–2). C, HPLC analysis of isolated M–2 and M+32 incubated with HOCl and under basic conditions, respectively. D, proposed reaction pathway for the formation of WG–4. Consistent with this proposal, VVWGTA incorporated 11 deuterium atoms (11H-D) whereas M–2, M+32, and M–4 incorporated 10, 9, and 10 deuterium atoms, respectively.
These observations indicate that peak 2 and peak 4 are chlorinated isomers, and they suggest that M+32 can convert to WG–4 in the gas phase and therefore might be an intermediate in the reaction pathway in aqueous solution.

MS analysis of peak 3 (M–2) revealed a major ion of \( m/z \) 630.2, the predicted mass of VVWGTA without 2 hydrogen atoms (Fig. 1B, [M – 2H + H]^+). MS/MS analysis revealed a series of ions consistent with loss of 2 atomic mass units from WG. MS analysis of peak 1 (M+16) demonstrated a major ion of \( m/z \) 648.2 (data not shown), the predicted mass of the peptide plus 1 oxygen ([M + 16 + H]^+). MS/MS analysis indicated that the tryptophan residue in VVWGTA had been oxygenated, likely to oxidodylalaline. Collectively, these observations indicate that HOCl produces WG–4, WG–2, and W+16 in VVWGTA and chlorinates the peptide. The generation of WG–4 from the chlorinated peptide (M+32) during MS/MS analysis suggests that tryptophan is targeted for chlorination.

Formation of WG–4 Involves an Initial Loss of Two Hydrogens followed by Chlorination and Finally Loss of HCl—To explore the reaction pathway, we used reverse-phase HPLC to quantify the influence of time and HOCl concentration on the yields of M–2, M+32, and M–4. M–2 was the major product early in the incubation, suggesting that it was the first stable product when HOCl oxidized VVWGTA. To verify this observation, we isolated peak 2, concentrated the sample, and exposed the compound to HOCl. HPLC analysis demonstrated that M–2 reacted further to generate both isomeric chlorinated peptides (M+32) as well as WG–4 (Fig. 1C).

The kinetics of VVWGTA oxidation by HOCl demonstrated that the initial rapid increase in M–2 concentration was followed by a gradual decline. In contrast, M–4 concentration increased with time, suggesting that M–4 was the final stable product. Consistent with this idea, M+32 was almost quantitatively converted to M–4 after 1 h of incubation in alkaline buffer (Fig. 1C). These observations suggest the following reaction pathway: VVWGTA (M) initially reacts with HOCl to generate M–2, which is then chlorinated by HOCl to form M+32. This intermediate subsequently loses HCl (36 atomic mass units) to yield a stable product, M–4.

Reaction Pathway for the Formation of WG–4—Based on our observations and the known reactivity of HOCl (16–19), we propose the following reaction pathway for the generation of WG–4 (Fig. 1D). Initially, HOCl reacts with the double bond in the five-member ring of tryptophan to yield a 3-chloroindolenine (18, 19). The unstable intermediate is converted to M–2, a cyclic intermediate. In this reaction, the lone pair electrons of the adjacent amide bond attack C-2 of the indolenine. A glycine adjacent to the chlorinated tryptophan intermediate favors this reaction by providing an unhindered local environment for nucleophilic attack by the amide. M–2 then reacts with a second molecule of HOCl to yield two chlorinated isomers, M+32, which subsequently lose HCl to form WG–4.

MS Analysis of Hydrogen-Deuterium Exchange of Oxidized Peptides—To study further the proposed mechanism for oxidation of WG by HOCl, we used hydrogen-deuterium exchange and MS analysis to determine the number of exchangeable hydrogens on each intermediate in the reaction pathway. VVWGTA incorporated 11 deuterium atoms (5 amide bond hydrogens, 3 N-terminal amino hydrogens, the hydrogen on the nitrogen of the indole ring, the hydrogen on the alcohol of threonine, and the hydrogen on the COOH group of the C terminus). In contrast, M–2, M+32, and M–4 incorporated 10, 9, and 10 deuterium atoms, respectively, which suggests that exchangeable hydrogens in WG are involved in the reaction pathway. The proposed mechanism for converting WG to WG–4 (Fig. 1D) is consistent with these observations.

WG–4 Is an Unusual Aromatic Compound—To determine whether WG is oxidized to a more aromatic compound, we isolated M–4 by reverse-phase HPLC and measured its absorption spectrum. The absorption maximum of VVWGTA was 280 nm (\( \epsilon = 5.4 \times 10^3 \)), whereas that of M–4 was 363 nm, and the intensity was greater (\( \epsilon = 1.8 \times 10^5 \)) (Fig. 2A). The oxidized peptide also exhibited a marked increase in fluorescence emission intensity accompanied by a shift in the excitation and emission maxima to longer wavelengths (Fig. 2B). VV(WG–4)TA, \( \lambda_{\text{em}} = 363 \text{ nm}, \lambda_{\text{em}} = 412 \text{ nm} \). The loss of 4 atomic mass units and spectral alterations suggest that WG–4 might be an unusual aromatic compound.

WG–4 Is Produced by Cross-linking the Indole Ring of Tryptophan to the Amide Nitrogen of Glycine—Proton chemical shifts of VVWGTA were assigned by TOCSY and nuclear Overhauser spectroscopy (NOESY) (20). High resolution \(^1\)H NMR spectra of HPLC-purified M+32, when compared with those of VVWGTA, showed loss of the tryptophan ring NH resonance. TOCSY spectra indicated the presence of the tryptophan \( \alpha \) and \( \beta \) protons. These observations suggest that N-chloroindole and 3-chloroindolenine (Fig. 1D, M+32) account for the isomers in peaks 2 and 4 (19).

High resolution \(^1\)H NMR spectra of HPLC-purified WG–4 (M–4) showed a marked downfield shift of the tryptophan ring NH resonance, from 10.9 to 11.95 ppm. TOCSY spectra confirmed the absence of the glycine amide proton and indicated loss of the tryptophan \( \alpha \) proton (Fig. 2, C and D). For WG–4, heteronuclear multiple quantum coherence also revealed loss...
of the tryptophan C-2 proton and confirmed the αβ double bond. NOESY for WG–4 demonstrated a short range interaction between the aromatic tryptophan NH proton and the glycine α proton. Modeling confirmed that WG–4 formed a kink in the backbone of the host peptide (Fig. 2, E and F) and that the oxidized peptide was rigid and markedly constrained in its range of conformations. These observations can be explained by invoking a pathway in which HOCl oxidizes adjacent tryptophan and glycine residues into an unusual cross-linked, aromatic compound (Fig. 1D) that induces a kink in the peptide backbone and restricts local conformational space.

**HOCl Targets the Sequence Motif WG for Cross-linking—**To determine whether tryptophan-glycine represents a specific motif for oxidative modification of proteins, we used a combinatorial approach to search for tryptophan residues that cross-link to other residues during oxidation. We used a peptide library based on the motif present in the peptide of MMP-7 associated with oxidative inactivation of the enzyme, VVWXTA, where X represents all 20 common amino acids.

After the library was exposed to HOCl and separated by HPLC with monitoring of absorbance at 363 nm, we detected a single major peak of new material. The absorbance and fluorescence excitation and emission spectra of this material were similar to those of WG–4. MS and MS/MS analysis confirmed that the product was VV(WG−4)/TTA. Similar reactions involving tryptophan residues may account for the cross-linking of superoxide dismutase by carbonate radical anion (21).

**DISCUSSION**

Chlorinated amino acids and nucleobases identical to those formed by myeloperoxidase or HOCl in vitro have been detected in inflamed human tissue (22, 23), but remarkably little is known about the reaction of HOCl with tryptophan residues in proteins (24–26). Our studies demonstrate that HOCl targets tryptophan residues adjacent to glycine residues in the primary sequence. The end product, WG–4, is an intensely fluorescent aromatic compound with a cross-link between the indole ring and the adjacent amide bond of glycine. NMR studies demonstrated that WG–4 introduces marked conformational restraints and a kinked backbone into its host peptide. Thus, WG–4 may exert long range effects on protein function by altering local peptide structure.

Most studies have focused on the reactivity of protein amino acid side chains while ignoring the potential importance of adjacent sequences. Our results indicate that specific structural motifs are important for controlling protein oxidation. It is noteworthy that similar observations have recently been reported for iron regulatory protein 2, which coordinates cellular reactivity of amino acid side chains.

**H2O2 has been proposed to act as a second messenger by transiently inhibiting protein tyrosine phosphatases. Recent structural studies of protein tyrosine phosphatase 1B exposed to H2O2 have revealed a sulfenyl-amide species formed by cross-linking of the catalytic cysteine residue and an adjacent main chain nitrogen (28, 29). Oxidation of the thiol to the sulfenyl-amide is associated with a conformational change in the active site of the enzyme that may inhibit substrate binding. These observations suggest that cross-linking of amino acid side chains to peptide amides may be a common structural motif in oxidized proteins.**

Our results indicate that HOCl selectively oxidizes the sequence motif WG and that the resulting formation of WG–4 associates strongly with oxidative inactivation of MMP-7 (14). This finding complements our previous work (7), which showed that low concentrations of HOCl activate pro-MMP-7 by oxygenating a specific thiol residue in the predomain of the enzyme. Such transient activation could be important for control of proteolytic activity during inflammation. Sequential MMP activation and inactivation could occur on or near phagocytes, which store MMPs and myeloperoxidase in secretory compartments and produce H2O2 at the cell membrane. Phagocytes therefore could create high local concentrations of oxidant-generating enzymes, substrates, and targets at the cell surface where they degranulate. Initial targeting of the cysteine switch thiol would rapidly activate the MMP, but sustained or high levels of HOCl production would modify other sites (7). Once specific sites in the protein were modified, the MMP would lose its catalytic activity (14). Thus, oxidant production by phagocytes would strictly confine MMP activity in space and time, permitting only bursts of pericellular proteolysis.

Our suggestion that HOCl inactivates MMP-7 by promoting the site-specific oxidation of WG to WG–4 provides an alternative mechanism for MMP inactivation, which is typically ascribed to protein inhibitors such as tissue inhibitors of matrix metalloproteinases (1). Our observations suggest that phagocyte-derived oxidants may restrain proteolytic activity by site-specific modification of MMPs, raising the possibility that oxidative pathways play a physiological role in limiting the degradation of healthy tissue.

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