THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 31, Issue of August 1, pp. 28403–28409, 2003
Printed in U.S.A.

Hypochlorous Acid Generated by Myeloperoxidase Modifies Adjacent Tryptophan and Glycine Residues in the Catalytic Domain of Matrix Metalloproteinase-7 (Matrilysin)

AN OXIDATIVE MECHANISM FOR RESTRAINING PROTEOLYTIC ACTIVITY DURING INFLAMMATION*

Received for publication, May 6, 2003
Published, JBC Papers in Press, May 20, 2003, DOI 10.1074/jbc.M304739200

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Dysregulation of matrix metalloproteinase (MMP) activity is implicated in tissue destruction under inflammatory conditions. An important mechanism controlling enzymatic activity might involve reactive oxygen species generated by phagocytes. Myeloperoxidase, a heme protein secreted by neutrophils, monocytes, and macrophages, uses hydrogen peroxide to generate hypochlorous acid (HOCl). We demonstrate that HOCl inhibits the activity of human matrilysin (MMP-7) *in vitro*, suggesting that it might limit proteolytic activity during inflammation. When MMP-7 was exposed to HOCl generated by myeloperoxidase, the proteinase lost activity. High performance liquid chromatographic analysis of the tryptic digest of the HOCl-treated proteinase demonstrated the absence of two peptides that were present in the untreated enzyme. Tandem mass spectrometric analysis revealed that both of the lost peptides contained methionine and tryptophan-glycine residues. The methionine residue of one of the peptides had been oxidized to methionine sulfoxide. In contrast, the major product from the other peptide was 4 atomic mass units smaller than its precursor (WG−4). This novel oxidation product was derived though modification of adjacent tryptophan and glycine residues in the catalytic domain of the enzyme. Loss of proteolytic activity was associated with conversion of the precursor peptide to WG−4 but not with methionine oxidation. In contrast, hydrogen peroxide failed to oxidize MMP-7 or to inactivate the enzyme. Thus, HOCl inactivates MMP-7, perhaps by site-specific conversion of tryptophan-glycine to WG−4. This inactivation mechanism is distinct from the well studied mechanisms involving tissue inhibitors of metalloproteinases. Our findings suggest that local pericellular production of HOCl by phagocytes is a physiological mechanism for governing MMP activity during inflammation.

Matrix metalloproteinases (MMPs)[1] play a central role in the turnover of the extracellular matrix as well as the processing of numerous proteins (1–6). These zinc endopeptidases are produced as latent proteins; hence, mechanisms that convert pro-MMPs to catalytically active MMPs are essential for regulating enzyme function. Once activated, MMPs can be inactivated by endogenous tissue inhibitors of metalloproteinases (5) or by internalization (7, 8). During regulated proteolysis, a balance exists between activation and inhibition of MMPs. Dysregulation of this balance is implicated in the pathogenesis of a variety of diseases, such as atherosclerosis, aneurysm formation, emphysema, and tumor growth and metastasis (1–6, 9).

The MMP prodomain contains a highly conserved thiol residue within the consensus sequence PRC*XX*PD (5). In the inactive state, this conserved thiol binds to the zinc atom of the catalytic domain preventing the latent enzyme from becoming inappropriately active. A pro-MMP can be activated *in vitro* by proteolytic cleavage of its prodomain, which releases the thiol-zinc interaction and consequently frees the catalytic site to interact with substrate. However, activation of pro-MMPs can be mediated by processes that disrupt the thiol-zinc interaction, and it is likely that proteolysis is not the sole mechanism controlling MMP activity (10).

Reactive oxygen and nitrogen species can regulate MMP activity *in vitro* (11–14), suggesting that the generation of such species by inflammatory cells (15, 16) controls MMP activation and inactivation *in vivo*. Myeloperoxidase, a heme protein secreted by neutrophils, monocytes, and some populations of macrophages, is one potential source of reactive oxygen and nitrogen species (17, 18). This enzyme uses hydrogen peroxide (H_2O_2) to generate hypochlorous acid (HOCl) (19).

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O \qquad \text{(Eq. 1)}$$

HOCl is a potent cytotoxic oxidant that converts pro-collagenase-2, pro-gelatinase B, and pro-matrilysin (MMP-7) into their proteolytically active forms (11, 20, 21). HOCl reacts rapidly and nearly quantitatively with thioethers and thiols (22–24). Tryptophan residues in proteins also appear to be important targets for damage (25). Therefore, HOCl might control MMP activity by covalently modifying key amino acid residues in the enzyme (26).

Pro-MMP-7, pro-collagenase-2, and pro-gelatinase B exposed to increasing concentrations of HOCl initially increase their proteolytic activities (11, 20, 21). We recently demonstrated that HOCl converts the thiol residue of the cysteine switch

* This work was supported by National Institutes of Health Grants AG021191, HL64344, HL56419, and HL29594. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MMP, matrix metalloproteinase; MMP-7, matrilysin; LC, liquid chromatography; HPLC, high performance liquid chromatography; M, peptide; MS, mass spectrometry; MS/MS, tandem mass spectroscopy; *m/z*, mass to charge ratio; WG−4, tryptophan-glycine oxidation product lacking 4 atomic mass units; Mca peptide, 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Leu-β-(2,4-dinitrophenylamino)Ala-Ala-Arg-NH_2.

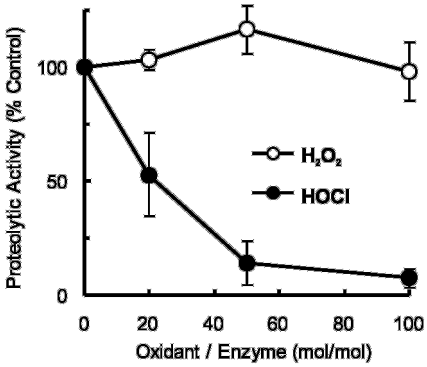


FIG. 1. **Effect of HOCl or H_2O_2 on proteolytic activity of MMP-7.** MMP-7 was exposed to the indicated ratio of oxidant/protein (*mol/mol*) in buffer B for 30 min at 37 °C. The reaction was initiated by adding oxidant and terminated by adding methionine (10:1, mol/mol, thiol/oxidant). Enzyme activity was then determined using Mca peptide. Control experiments demonstrated that methionine does not affect the proteolytic activity of MMP-7. Results represent the mean ± S.E. of two or three independent experiments, with two determinations per experiment.

domain of pro-MMP-7 to the corresponding sulfinic acid (21). Thiol oxidation is associated with autolytic cleavage of pro-MMP-7, strongly suggesting that oxygenation activates the latent enzyme. We have proposed that this pathway plays a critical role in the rupture of atherosclerotic lesions (18, 21, 27). These observations suggest that HOCl provides an oxidative mechanism for activating latent MMPs in vascular disease (28).

The oxidative footprints of myeloperoxidase have been found in human disorders ranging from atherosclerosis to lung disease, indicating that HOCl contributes to tissue damage during inflammation (29). However, hypercholesterolemic mice deficient in myeloperoxidase develop more atherosclerosis than do wild-type mice (30), and MMPs can be inactivated by reactive intermediates (12, 14, 21, 31), suggesting that myeloperoxidase may also restrain pathological tissue injury. In the current studies, we have proposed potential mechanisms for enzyme inactivation and demonstrated that HOCl, but not H_2O_2, oxidizes methionine and tryptophan residues of active MMP-7. When a specific tryptophan is converted to a novel oxidation product, the active enzyme loses catalytic activity. These observations reveal a molecular mechanism for inactivating MMPs.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise indicated, all materials were purchased from Sigma. Sodium hypochlorite (NaOCl), H_2O_2, HEPES, trifluoroacetic acid, and HPLC-grade CH_3CN were obtained from Fisher. Human matrilysin (MMP-7) was obtained from Chemicon International Inc. (Temecula, CA) and Calbiochem. Myeloperoxidase (A_{430}/A_{280} ratio > 0.8) was purified from HL-60 cells by sequential lectin affinity, ion exchange, and size-exclusion chromatographies (32, 33). Myeloperoxidase concentrations were determined spectrophotometrically (ϵ_{430} = 0.178 M^{-1} cm^{-1}) (34).

Methods

Reaction Conditions—Reactions were carried out for 30 min at 37 °C in buffer A (150 mM NaCl, 10 mM HEPES, pH 7.4, 5 mM $CaCl_2$) or buffer B (phosphate-buffered saline, pH 7.4) supplemented with 3.0 μM MMP-7. Reactions were initiated by adding oxidant and terminated by adding a 10-fold molar excess (relative to oxidant) of L-methionine. Concentrations of HOCl and H_2O_2 were determined spectrophotometrically (ϵ_{292} = 350 M^{-1} cm^{-1} and ϵ_{240} = 39.4 M^{-1} cm^{-1}) (35, 36).

Proteolytic Digestion—MMP-7 (100 pmol) was incubated overnight at 37 °C with sequencing grade modified trypsin (Promega, Madison, WI) at a ratio of 25:1 (w/w) MMP-7:trypsin in buffer C (10% CH_3CN, 50 mM NH_4HCO_3, pH 8.0). Digestion was halted by freezing the mixture or acidifying (pH 2–3) it with formic acid or trifluoroacetic acid.

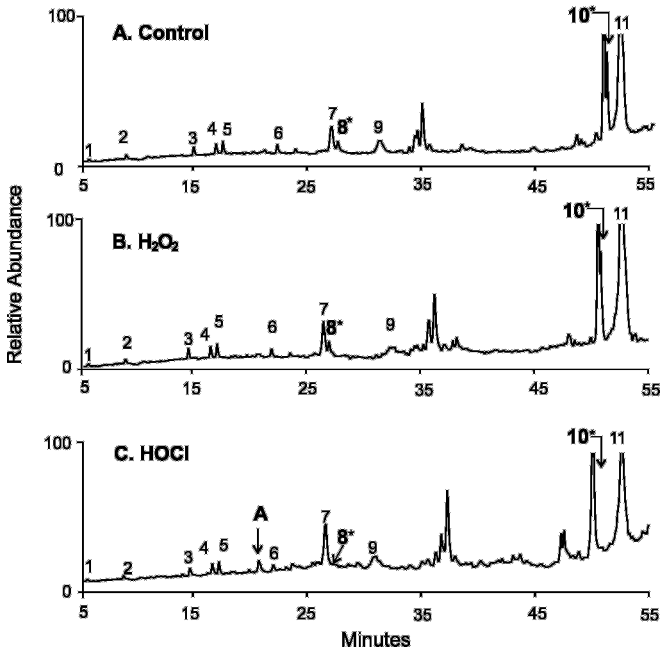


FIG. 2. **Total ion chromatogram of tryptic peptides of MMP-7 exposed to H_2O_2 or HOCl.** MMP-7 was incubated alone (*Control*) (*A*) or with oxidant (50:1, mol/mol, oxidant/protein) (*B* and *C*) for 30 min at 37 °C in buffer A. After the reaction was terminated with methionine, the protein was digested with trypsin overnight at 37 °C, and the tryptic digest was analyzed by LC-MS. Note that LC-MS identified all of the MMP-7 peptides containing methionine or tryptophan (M or W, underlined) and that MS/MS confirmed the identities of these peptides: peptide *1*, WTSK; peptide *2*, LYGK; peptide *3*, VVTYR; peptide *4*, IVSYTR; peptide *5*, LSQDDIK; peptide *6*, EIPLHFRK; peptide *7*, DLPHITVDR; peptide *8*, ALNMWGK; peptide *9*, YSLFPNSPK; peptide *10*, VVWGTADIMIGFAR; peptide *11*, GAHGDSYPFDGPGNTLAHAFAPGTGLGGD AHFDEDERWTDGSSLGINFLYAATHEL GHSLGMGHSSDPNAVMYPTYGNGDPQNFK. *8** and *10**, peptides lost upon HOCl exposure; *A* ↓, peptide generated upon HOCl exposure.

Liquid Chromatography (LC) Electrospray Ionization Mass Spectrometry (MS)—LC-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 (21). Tryptic peptides were separated at a flow rate of 0.2 ml/min on a reverse-phase column (Vydac C18 MS column; 2.1 × 25 mm) using solvent A (0.2% formic acid in water) and solvent B (0.2% formic acid in 80% CH_3CN, 20% water). Peptides were eluted using the following linear gradient: 0 to 15% B over 15 min; 15 to 30% B over 25 min; then 30 to 65% B over 20 min. The electrospray needle was held at 4500 volts. Nitrogen, the sheath gas, was set at 80 units. The collision gas was helium. The temperature of the heated capillary was 220 °C.

Assay of MMP-7 Proteolytic Activity—MMP-7 activity was assayed using Invitrogen precast casein zymogram gels or in solution (21, 37) with the fluorescent Mca peptide (7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Leu-β-(2,4-dinitrophenylamino)Ala-Ala-Arg-NH_2) as the substrate. MMP-7 (15–30 ng) was added to individual wells of a 96-well microtiter plate containing 500 μl of buffer C (0.2 M NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 0.02% NaN_3, 0.05% w/v Brij35) and 2 μM Mca peptide and incubated for 20 min at 37 °C. Fluorescence (λ_{ex} = 328 nm, λ_{em} = 392 nm) was monitored using a microplate reader (SPECTRAmax GEMINI XS, Molecular Devices, Sunnyvale, CA).

RESULTS

HOCl Inactivates MMP-7—To determine whether oxidants inactivate MMP-7 *in vitro*, we exposed activated enzyme to HOCl or H_2O_2 for 30 min at 37 °C in buffer B. Then we determined whether MMP-7 could cleave Mca, a synthetic fluorescent peptide. MMP-7 exposed to increasing concentrations of HOCl lost its proteolytic activity (Fig. 1). Zymographic analysis

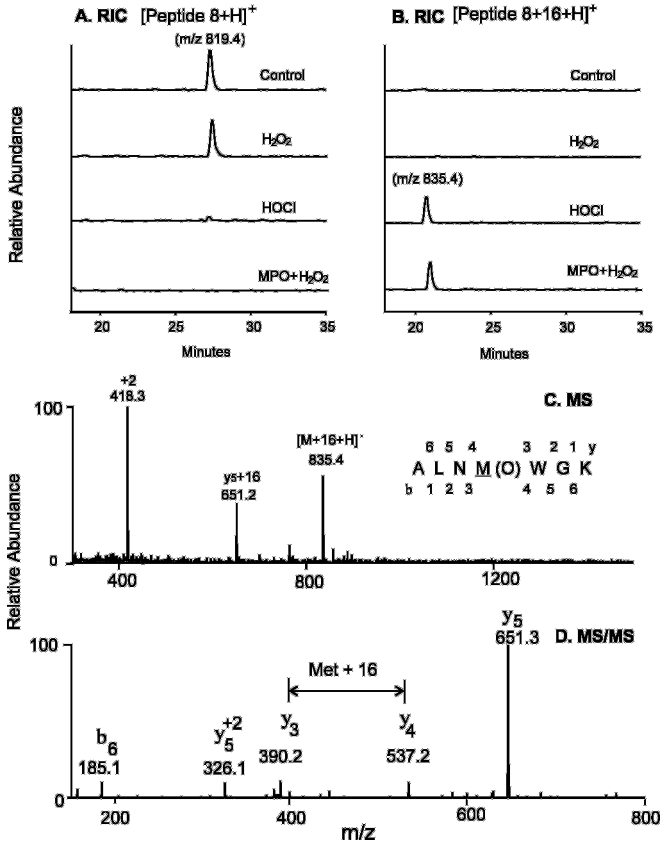


FIG. 3. **LC-MS and MS/MS analyses of peptide 8 in a tryptic digest of MMP-7 exposed to H_2O_2, HOCl, or HOCl generated by myeloperoxidase (*MPO*).** MMP-7 was incubated for 30 min at 37 °C in buffer A alone (*Control*) or buffer A supplemented with H_2O_2, HOCl, or H_2O_2 containing 5 nM myeloperoxidase (50:1, mol/mol, oxidant/protein). Following the addition of methionine, the protein was digested with trypsin overnight at 37 °C, and the tryptic peptides were analyzed by LC-MS. *A*, reconstructed ion chromatogram (*RIC*) with monitoring of ions of *m/z* 819.4, the predicted *m/z* of singly protonated unmodified peptide 8. *B*, reconstructed ion chromatogram with monitoring ions of *m/z* 835.4, the predicted *m/z* of singly protonated peptide 8 plus 16 atomic mass units. *C* and *D*, full-scan MS analysis (*C*) and MS/MS analysis (*D*) of the ion of *m/z* 835.4 observed in the tryptic digest in MMP-7 exposed to H_2O_2 plus myeloperoxidase.

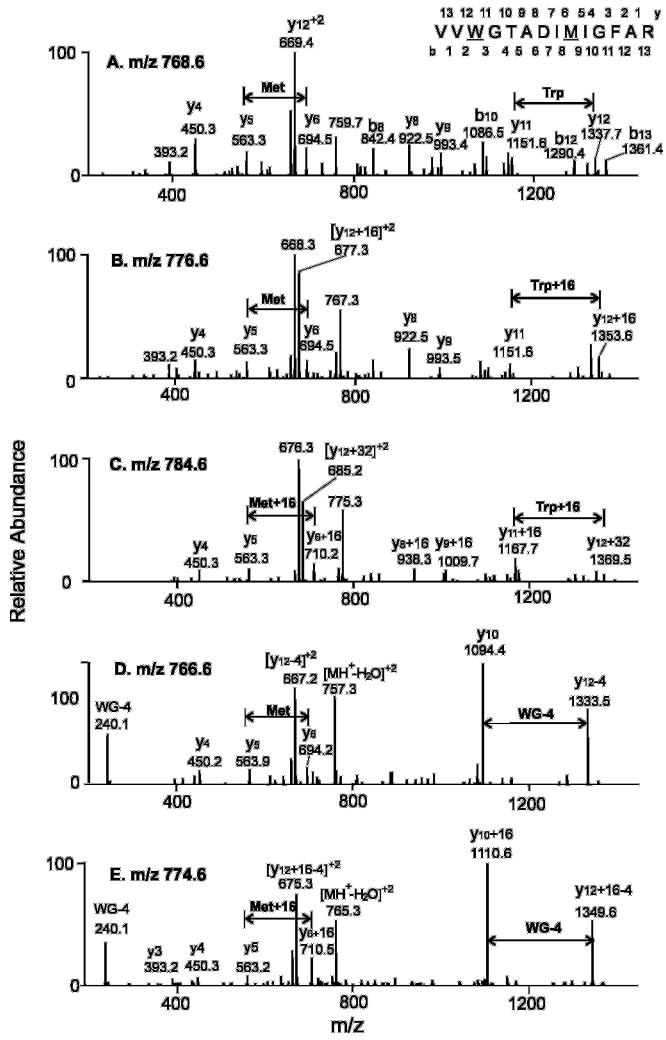


FIG. 4. **MS/MS analysis of peptide 10 in a tryptic digest of MMP-7 exposed to HOCl.** MMP-7 was incubated with HOCl (50:1, mol/mol, oxidant/protein) for 30 min at 37 °C in buffer A. Following the addition of methionine, the protein was digested with trypsin overnight at 37 °C, and the tryptic peptides were analyzed by LC-MS. MS/MS analysis of ions of: *A*, *m/z* 768.6; *B*, *m/z* 776.6; *C*, *m/z* 784.6; *D*, *m/z* 766.6; *E*, *m/z* 774.6.

of MMP-7 with casein also showed that the enzyme lost activity when it was exposed to HOCl. MMP-7 was inactivated by the complete myeloperoxidase system, consisting of enzyme, H_2O_2, and chloride ion (data not shown). In contrast, the same concentrations of H_2O_2 did not inhibit the proteolytic activity of MMP-7 (Fig. 1). These observations indicate that HOCl generated by myeloperoxidase can convert active MMP-7 to an enzymatically inactive form.

HOCl Oxidizes Specific Residues in MMP-7—To investigate the molecular basis for the oxidative inactivation of MMP-7, we first digested unmodified enzyme with trypsin and identified the resulting peptides through LC-MS and MS/MS analysis (Fig. 2). The 11 peptides detected by LC-MS accounted for 92% of the protein sequence. Importantly, they included all of the amino acid residues of MMP-7 known to be highly susceptible to oxidation by HOCl or H_2O_2 (tryptophan (W) and methionine (M); (Fig. 2, peptides *1*, *8*, *10*, and *11*). Because of poor retention on the HPLC column, we were unable to identify small peptides and single amino acid residues.

To determine whether HOCl modifies MMP-7 at specific sites, we exposed MMP-7 to a concentration of oxidant (50:1, mol/mol, oxidant/protein) that resulted in ~80% inhibition of the enzyme and then analyzed a tryptic digest of the enzyme by LC-MS. When the oxidant was H_2O_2, the total ion chromatogram of the tryptic digest was indistinguishable from that of the tryptic digest obtained from the native protein (compare Fig. 2, *A* and *B*). In striking contrast, peptides 8 and 10 were selectively absent from the tryptic digest of MMP-7 exposed to HOCl (Fig. 2*C*, *8** and *10**). Loss of the two peptides was associated with the appearance of one major peak of material (termed peptide A) and four peaks of material that were poorly seen in the total ion chromatogram. These observations suggest that HOCl modified specific amino acids of MMP-7 converting two of the original peptides of the enzyme to five or more new peptides.

HOCl Selectively Oxygenates the Methionine Residue in Peptide 8—To identify the site at which HOCl modifies peptide 8, we exposed MMP-7 to H_2O_2, HOCl, or H_2O_2 supplemented with myeloperoxidase for 30 min at 37 °C in buffer A. We then digested the protein with trypsin and analyzed the digest by LC-MS. The reconstructed ion chromatogram of unmodified MMP-7 revealed a peak of material with the anticipated mass to charge ratio (*m/z*) of singly protonated peptide 8 (Fig. 3*A*, *m/z 819.4*, $[M + H]^+$, retention time 27.5 min). MS/MS analysis confirmed the sequence of the peptide, ALNMWGK, which contained methionine and tryptophan residues (underlined letters) potentially susceptible to oxidation. The abundance of this

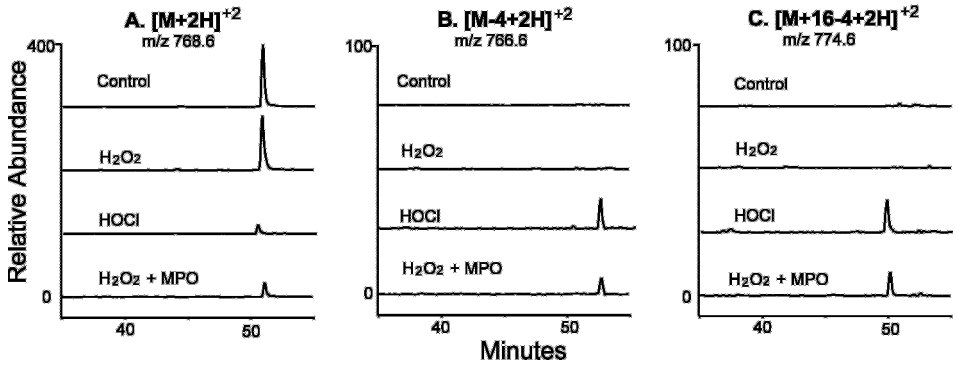


FIG. 5. **LC-MS analysis of peptide 10 in a tryptic digest of MMP-7 exposed to H_2O_2, HOCl, or HOCl generated by myeloperoxidase (*MPO*).** MMP-7 was incubated for 30 min at 37 °C in buffer A alone (*Control*) or in buffer A supplemented with H_2O_2, HOCl, or H_2O_2 plus 5 nM myeloperoxidase (50:1, mol/mol, oxidant/protein). Following the addition of methionine, the protein was digested with trypsin overnight at 37 °C, and the tryptic peptides were analyzed by LC-MS. *A*, reconstructed ion chromatogram with monitoring of ions of *m/z* 768.6, the predicted *m/z* of doubly protonated unmodified peptide 10. *B*, reconstructed ion chromatogram with monitoring of ions of *m/z* 766.6, the predicted *m/z* of doubly protonated peptide 10 minus 4 atomic mass units ($[M - 4 + 2H]^{+2}$). *C*, reconstructed ion chromatogram with monitoring of ions of *m/z* 774.6, the predicted *m/z* of doubly protonated peptide 10 plus 16 atomic mass units minus 4 atomic mass units ($[M + 16 - 4 + 2H]^{+2}$).

peptide barely changed when MMP-7 was exposed to H_2O_2 alone. In contrast, when MMP-7 was exposed to HOCl alone or to H_2O_2 supplemented with myeloperoxidase, peptide 8 was absent from the tryptic digest, and a new peak of material was seen (compare Fig. 3, *A* and *B*). The retention time of this singly charged material resembled that of peptide A, and its *m/z* was 835.4 (Fig. 3*C*), which is consistent with the addition of 16 atomic mass units to peptide 8 ($[M + 16 + 2H]^{+}$). MS/MS analysis of the modified peptide indicated that the methionine residue of peptide 8 had gained 16 atomic mass units (Fig. 3*D*). These observations demonstrate that HOCl generated by the myeloperoxidase-H_2O_2-Cl^- system, but not H_2O_2 alone, converts the alkylated thiol group of the methionine residue in peptide 8 of MMP-7 to the sulfoxide. LC-MS and MS/MS analysis indicated that the tryptophan residue adjacent to this methionine residue remained intact.

HOCl Converts Peptide 10 to Four Products—Peptide 10 (VVWGTADIMIGFAR) also contains tryptophan and methionine residues that might be vulnerable to HOCl. We therefore used LC-MS and MS/MS to determine the relative abundance of peptide 10 and its potential oxidation products in tryptic digests of untreated MMP-7 and MMP-7 exposed to oxidant. MS/MS analysis confirmed the sequence of doubly protonated peptide 10 in the digest of unmodified MMP-7 (Fig. 4*A*). The relative abundance of this peptide (Fig. 2, *A* and *B*) changed little when MMP-7 was exposed to H_2O_2 alone. In contrast, when MMP-7 was exposed to HOCl or to H_2O_2 supplemented with myeloperoxidase, peptide 10 disappeared and several small peaks of material appeared in the total ion chromatogram of the tryptic digest (Fig. 2*C*). LC-MS analysis with reconstructed ion chromatograms showed that the tryptic digest contained four new peaks of material that appeared to derive from peptide 10 (data not shown): two major products of *m/z* 766.6 ($[M - 4 + 2H]^{+2}$) and *m/z* 774.6 ($[M + 12 + 2H]^{+2}$); and two minor products of 776.6 ($[M + 16 + 2H]^{+2}$) and 784.6 ($[M + 32 + 2H]^{+2}$).

HOCl Oxygenates the Tryptophan Residue in Peptide 10—We used MS/MS to determine which amino acid residues had been modified in the two minor oxidation products of peptide 10 (Fig. 4, *B* and *C*). This approach indicated that when peptide 10 gained 16 atomic mass units ($[M + 16 + 2H]^{+2}$) the tryptophan residue was oxygenated. When peptide 10 gained 32 atomic mass units, both the methionine residue and the tryptophan residue became oxygenated ($[M + 32 + 2H]^{+2}$). These observations suggest that the tryptophan residue in peptide 10 is more readily oxygenated than the methionine residue. These observations indicate that tryptophan and methionine residues in different regions of MMP-7 are differentially susceptible to oxidation by HOCl, perhaps because of differences in flanking sequences or the secondary or tertiary structure in the two regions of the enzyme.

HOCl Converts the Tryptophan Residue of Peptide 10 to a Novel Oxidation Product—We next characterized the two major products that we had detected in the tryptic digest of MMP-7 exposed to HOCl. MS/MS analysis of the peptide of *m/z* 766.6 ($[M - 4 + 2H]^{+2}$) (Fig. 4*D*) confirmed that this material was derived from peptide 10; it also revealed two important features: (i) loss of the y_{12} (Trp) and y_{11} ions (Gly) found in native peptide 10 and (ii) major ions at y_{10} (*m/z* 1094.4), and $y_{12} - 4$ (*m/z* 1333.5). These observations suggest that peptide 10 is modified by a reaction that removes 4 atomic mass units from WG. Detection of an ion of *m/z* 240.1 ($[WG - 4 + 2H]^{+}$) by MS/MS (Fig. 4*D*) supported this interpretation.

MS/MS analysis of the material of *m/z* 774.6 ($[M + 12 + 2H]^{+2}$) demonstrated a ladder of y ions (Fig. 4*E*). This result was similar to that observed in the material of *m/z* 766.6 ($[M - 4 + 2H]^{+2}$) except that the methionine residue had gained 16 atomic mass units ($[M + 16 - 4 + 2H]^{+2}$). These observations indicate that the tryptophan residue in peptide 10 was more susceptible than that peptide's methionine residue was to oxidation by HOCl. They also strongly suggest that HOCl oxidized WG by removing 4 atomic mass units.

HOCl Generated by Myeloperoxidase Converts WG to WG−4 in MMP-7—To determine whether HOCl generated by myeloperoxidase also oxidizes WG of peptide 10, we exposed MMP-7 to H_2O_2 alone, HOCl alone, or H_2O_2 supplemented with myeloperoxidase (H_2O_2 + MPO) for 30 min at 37 °C in buffer A. Reconstructed ion chromatograms were used to monitor the ions derived from peptide 10 in the tryptic digest of the enzyme. The doubly charged peptide 10 (*m/z* 768.6, $[M + 2H]^{+2}$) was present in the digests of the native enzyme and of MMP-7 exposed to H_2O_2 alone (Fig. 5*A*). When the enzyme was exposed to HOCl or H_2O_2 supplemented with myeloperoxidase, peptide 10 disappeared almost completely. Its disappearance was associated with the appearance of product peptides (Fig. 5, *B* and *C*) that had either lost 4 atomic mass units (*m/z* 766.6, $[M - 4 + 2H]^{+2}$) or had both lost 4 atomic mass units and gained 16 atomic mass units (*m/z* 774.6, $[M - 4 + 16 + 2H]^{+2}$). These results indicate that HOCl generated by the myeloperoxidase-H_2O_2-Cl^- system, but not H_2O_2 alone, can convert WG in peptide 10 of MMP-7 to a product that has lost 4 atomic mass units.

WG Is a Site-specific Target for Modification by HOCl—Both peptide 8 and peptide 10 contained methionine and trypto-

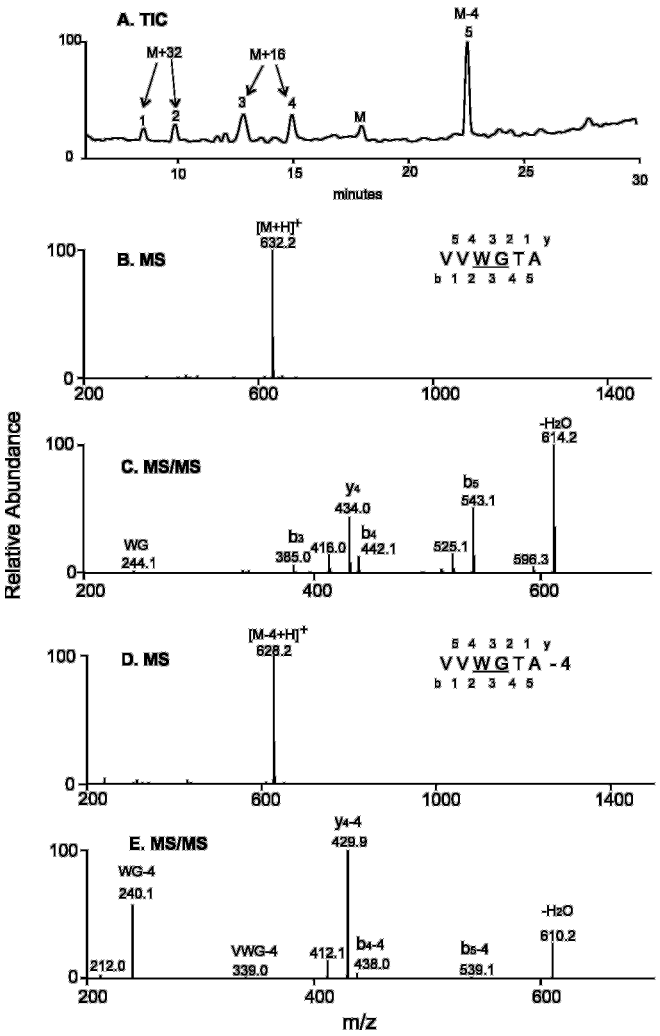


FIG. 6. **LC-MS and MS/MS analyses of the synthetic peptide VVWGTA exposed to HOCl.** Peptide VVWGTA (50 μM) was incubated with HOCl (5:1, mol/mol, oxidant/peptide) for 30 min at 37 °C in buffer A and incubated in buffer C overnight. *A*, total ion chromatogram (TIC) of the reaction mixture. *B* and *C*, MS (*B*) and MS/MS (*C*) analyses of the precursor peptide (*M*). *D* and *E*, MS (*D*) and MS/MS (*E*) analyses of the major peak of material, which eluted with a retention time of 22.5 min.

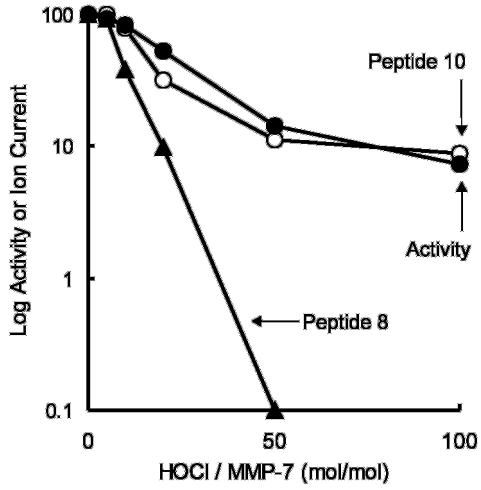


FIG. 7. **Relationship between proteolytic activity and the relative intensity of peptides 8 and 10 in the tryptic digest of MMP-7 exposed to HOCl.** MMP-7 was incubated at different mol ratios of HOCl/protein for 30 min at 37 °C in buffer B, and the reaction was terminated with methionine. Proteolytic activity of intact enzyme was immediately assessed, using Mca peptide as substrate, or the protein was digested with trypsin and subjected to LC-MS.

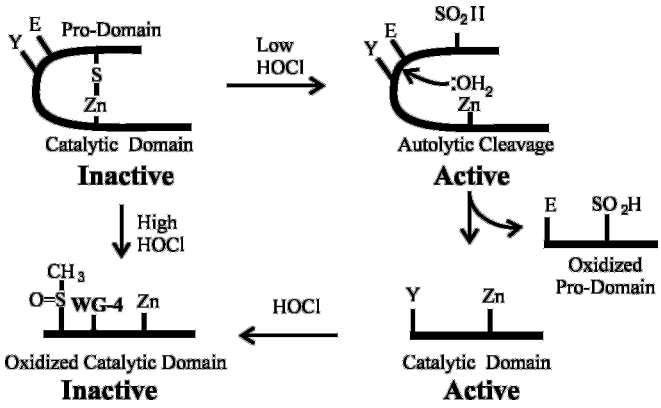
phan-glycine residues, but only the tryptophan residue in peptide 10 was oxidized when MMP-7 was exposed to HOCl. The selective oxidation of tryptophan in this peptide might reflect a site-specific reaction. To investigate this possibility, we synthesized a synthetic peptide, VVWGTA, which duplicates the region of peptide 10 that contains the vulnerable tryptophan residue. However, the peptide presumably lacks the secondary and tertiary structure of that part of the enzyme. After exposing the synthetic peptide to HOCl for 30 min at 37 °C in buffer A, the reaction mixture was analyzed by LC-MS and MS/MS. The total ion chromatogram of the reaction mixture revealed almost complete loss of the precursor peptide (Fig. 6*A*, peak *M*) and evidence of the appearance of one major and four minor product peptides (peaks 1–5). The molecular mass (*m/z* 632.2, $[M + H]^+$) and sequence of the unmodified peptide were confirmed by LC-MS and MS/MS analysis (Fig. 6, *B* and *C*). LC-MS analysis of the major peak of new material (peak 5) demonstrated a peptide of *m/z* 628.2 (Fig. 6*D*), indicating that the precursor peptide had lost 4 atomic mass units ($[M - 4 + H]^+$). MS/MS analysis of peak 5 revealed a series of ions consistent with loss of 4 atomic mass units from the precursor peptide, including VWG − 4, $b_5 - 4$, $b_4 - 4$, and $y_4 - 4$ (Fig. 6*E*). Importantly, MS/MS analysis of peak 5 showed a prominent ion of *m/z* 240.1, which is consistent with loss of 4 atomic mass units from WG (compare Fig. 6, *C* and *E*). LC-MS analysis of the minor products of the reaction (peaks 1–4) revealed two ions of *m/z* 648 and two ions of *m/z* 664, suggesting that the precursor peptide (*m/z* 632.2) had been converted to two different products by addition of 1 and 2 oxygen atoms, respectively. Collectively, these observations strongly suggest that WG in peptide 10 is susceptible to site-specific modification by HOCl and that the major product lacks 4 atomic mass units (WG−4). They also suggest that this peptide can be oxygenated into products that contain either one or two oxygen atoms.

When MMP-7 Is Exposed to HOCl, Oxidation of Peptide 10 Is Associated with Loss of Enzymatic Activity—To determine whether oxidation of specific amino acid residues in peptide 8 or peptide 10 might inactivate MMP-7, we examined the relationship between the disappearance of each peptide and loss of proteolytic activity. Because enzymes exposed to high concentrations of oxidant might undergo stochastic oxidative modifications, we focused our studies on MMP-7 that was ≤90% inactivated. Under these conditions, there should be a linear relationship between loss of proteolytic activity and peptide modification if oxidation played a causal role in regulating enzyme activity.

Peptide 8 disappeared completely from the tryptic digest of MMP-7 exposed to HOCl, but there was little correlation between its loss and the proteolytic activity of the enzyme (Fig. 7). In contrast, loss of peptide 10 was strongly associated with loss of proteolytic activity. These observations indicate that peptide 8 is more sensitive to oxidation than peptide 10 but that its modification does not diminish enzyme activity. In contrast, oxidative modification of peptide 10 is associated with loss of MMP-7 activity and therefore is likely involved in enzyme inactivation.

DISCUSSION

We demonstrated that HOCl generated by myeloperoxidase inactivates MMP-7. Tandem MS analysis of tryptic digests of MMP-7 exposed to HOCl revealed that only two peptides in the catalytic domain of the enzyme are significantly modified under these conditions. Both contained methionine and tryptophan residues known to be sensitive to oxidation. Despite similarities in the amino acid compositions of the peptides,



SCHEME 1. **Proposed mechanism for the sequential activation and then inactivation of MMP-7 activity by HOCl.**

different amino acids were selectively modified. In peptide 8, only methionine was oxygenated, but tryptophan was the major target in peptide 10. Loss of proteolytic activity in MMP-7 exposed to HOCl was strongly associated with modification of peptide 10 but not with modification of peptide 8. Thus, HOCl oxidized different amino acid residues in different peptides, and loss of enzymatic activity apparently involved oxidation of an amino acid residue in a single localized region of MMP-7.

Importantly, our observations indicate that, in peptide 10 of MMP-7, HOCl generates a major product of unknown structure involving tryptophan and an adjacent glycine residue. MS/MS analysis demonstrated that tryptophan-glycine was oxidized to an intermediate that had lost 4 atomic mass units (WG−4). Moreover, LC-MS and MS/MS studies of the synthetic peptide VVWGTA, which mimics this region of MMP-7, confirmed that HOCl specifically targeted the tryptophan-glycine site to generate WG−4. Thus, our observations indicate that HOCl converts tryptophan-glycine to a novel oxidation product, WG−4.

When we exposed MMP-7 to HOCl, oxidation of peptide 10, but not of peptide 8, was associated strongly with loss of proteolytic activity. LC-MS and MS/MS analyses of peptide 10 revealed that WG−4 was a major product, but we also detected oxygenated methionine and tryptophan residues. These observations suggest that formation of WG−4 (or other oxidation products derived from tryptophan and methionine) inactivates MMP-7. Consistent with this mechanism, H_2O_2 failed to inactivate MMP-7, and LC-MS analysis demonstrated that neither peptide 8 nor peptide 10 was modified by this oxidant. The methionine in peptide 8 was more sensitive to oxidation by HOCl than was WG in peptide 10, suggesting that the methionine residue could act as an endogenous antioxidant in the proteinase (38). It is unclear how oxidation of peptide 10 inactivates MMP-7. The crystal structure of the enzyme reveals that peptide 10 lies in a region that is far from the active site of the enzyme (39). In future studies, it will be important to determine the structure of WG−4 and to explore how this novel modified dipeptide and other oxidized amino acids in peptide 10 affect MMP-7 activity.

HOCl produced by myeloperoxidase might not be the only oxidant capable of inactivating MMPs (17, 18, 21). A related phagocyte enzyme, eosinophil peroxidase, converts bromide ion to hypobromous acid, which modifies tryptophan residues in lipoproteins more efficiently than HOCl (40, 41). Other possible candidates include brominating oxidants produced by myeloperoxidase (42, 43) and peroxynitrite, nitrogen dioxide radical, and hypothiocyanate (16, 17, 21, 44).

We showed previously that HOCl oxygenates the thiol residue of the cysteine switch domain activating latent MMP-7 (21). Tandem MS analysis revealed that the cysteine of pro-MMP-7 is the primary target for oxidation at low concentrations of HOCl. However, when pro-MMP-7 was exposed to higher concentrations of HOCl, we also detected WG−4 (data not shown). These observations suggest that the thiol of the cysteine switch domain is the preferred site of oxidation in pro-MMP-7. However, both pro-MMP-7 and MMP-7 become targets for the formation of WG−4 at higher concentrations of HOCl. Thus, oxidants generated by phagocytes *in vivo* might contribute to both activation and inactivation of MMPs (Scheme 1). Which action predominates might depend on the local concentration of HOCl at a particular time. Because phagocytes store MMPs and myeloperoxidase in secretory compartments, degranulation of these components could create high local concentrations of both enzymes near the cell surface (4, 17, 18). Because the phagocyte NADPH oxidase is associated with the plasma membrane, production of H_2O_2 is also localized to the cell surface (45). Moreover, because pro-MMP-7, pro-collagenase-2, and pro-gelatinase B are activated by low concentrations of HOCl but disabled by higher concentrations of oxidant (11, 20, 21, 31), traditional enzyme kinetics cannot fully explain the regulation of MMP activity by reactive oxygen species in tissue or extracellular fluids (4). Instead, regulation is likely to be highly localized both temporally and spatially by rapid local changes in proteinase and oxidant concentrations. By analogy, it is noteworthy that neutrophils produce evanescent "quantum bursts" of pericellular proteolytic activity when activated *in vitro* (4). Therefore, regulation of proteolytic activity might depend critically on local concentrations of oxidants and MMPs near the phagocyte surface.

Our suggestion that HOCl inactivates MMP-7 by oxidizing specific amino acid residues provides an alternative pathway to the tissue inhibitor of metalloproteinases mechanism traditionally associated with MMP inactivation (5). This new mechanism implicates phagocytes, the cellular hallmark of inflammation, in protecting matrix from degradation by MMPs. Moreover, HOCl converts latent pro-MMP-7 to MMP-7, suggesting that post-translational oxidative modifications control both activation and deactivation of enzyme activity (11, 12, 21, 26, 31). Generation of oxidants by myeloperoxidase *in vivo* could confine MMP activation to bursts of pericellular proteolysis that are highly regulated in space and time. Because dysregulation of matrix degradation has been implicated in many pathological disorders, our findings could have broad implications for understanding and potentially preventing the degradation of healthy tissue.

REFERENCES

1. Woessner, J. F. (1998) in *Matrix Metalloproteinases* (Parks, W. C., and Mecham, R. P., eds) pp. 1–13, Academic Press, San Diego, CA
2. Halpert, I., Roby, J. D., Sires, U. I., Potter-Perigo, S., Wight, T. N., Welgus, H. G., Shapiro, S. D., Wickline, S. A., and Parks, W. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 9748–9753
3. Carmeliet, P., Moons, L., Lijnen, R., Baes, M., Lemaitre, V., Tipping, P., Drew, A., Eeckhout, Y., Shapiro, S., Lupu, F., and Collen, D. (1997) *Nat. Genet.* **17,** 439–444
4. Campbell, E. J., Campbell, M. A., Boukedes, S. S., and Owen, C. A. (1999) *J. Clin. Invest.* **104,** 337–344
5. Woessner, J. F., and Nagase, H. (2000) *Matrix Metalloproteinases and TIMPs*, Oxford University Press, New York
6. Coussens, L. M., Tinkle, C. L., Hanahan, D., and Werb, Z. (2000) *Cell* **103,** 481–490
7. Walling, H. W., Chan, P. T., Omura, T. H., Barmina, O. Y., Fiacco, G. J., Jeffrey, J. J., and Partridge, N. C. (1998) *J. Cell. Physiol.* **177,** 563–574
8. Jiang, A., Lehti, K., Wang, X., Weiss, S. J., Keski-Oja, J., and Pei, D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 13693–13698
9. Pyo, R., Lee, J. K., Shipley, J. M., Curci, J. A., Mao, D., Ziporin, S. J., Ennis, T. L., Shapiro, S. D., Senior, R. M., and Thompson, R. W. (2000) *J. Clin. Invest.* **105,** 1641–1649
10. Van Wart, H. E., and Birkedal-Hansen, H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5578–5582
11. Weiss, S. J., Peppin, G., Ortiz, X., Ragsdale, C., and Test, S. T. (1985) *Science* **227,** 747–749
12. Rajagopalan, S., Meng, X. P., Ramasamy, S., Harrison, D. G., and Galis, Z. S. (1996) *J. Clin. Invest.* **98,** 2572–2579
13. Okamoto, T., Akaike, T., Nagano, T., Miyajima, S., Suga, M., Ando, M.,

Ichimori, K., and Maeda, H. (1997) *Arch. Biochem. Biophys.* **342,** 261–274
14. Owens, M. W., Milligan, S. A., Jourd'heuil, D., and Grisham, M. B. (1997) *Am. J. Physiol.* **273,** L445–L450
15. Fridovich, I. (1975) *Annu. Rev. Biochem.* **44,** 147–159
16. Ignarro, L. J. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30,** 535–560
17. Hurst, J. K., and Barrette, W. C., Jr. (1989) *Crit. Rev. Biochem. Mol Biol.* **24,** 271–328
18. Daugherty, A., Dunn, J. L., Rateri, D. L., and Heinecke, J. W. (1994) *J. Clin. Invest.* **94,** 437–444
19. Harrison, J. E., and Schultz, J. (1976) *J. Biol. Chem.* **251,** 1371–1374
20. Peppin, G. J., and Weiss, S. J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 4322–4326
21. Fu, X., Kassim, S. Y., Parks, W. C., and Heinecke, J. W. (2001) *J. Biol. Chem.* **276,** 41279–41287
22. Peskin, A. V., and Winterbourn, C. C. (2001) *Free Radic. Biol. Med.* **30,** 572–579
23. Winterbourn, C. C. (1985) *Biochim. Biophys. Acta* **840,** 204–210
24. Levine, R. L., Moskovitz, J., and Stadtman, E. R. (2000) *IUBMB Life* **50,** 301–307
25. Finley, E. L., Dillon, J., Crouch, R. K., and Schey, K. L. (1998) *Protein Sci.* **7,** 2391–2397
26. Stadtman, E. R. (1992) *Science* **257,** 1220–1224
27. Sugiyama, S., Okada, Y., Sukhova, G. K., Virmani, R., Heinecke, J. W., and Libby, P. (2001) *Am. J. Pathol.* **158,** 879–891
28. Steinberg, D. (1997) *J. Biol. Chem.* **272,** 20963–20966
29. Heinecke, J. W. (1999) *FASEB J.* **13,** 1113–1120
30. Brennan, M. L., Anderson, M. M., Shih, D. M., Qu, X. D., Wang, X., Mehta, A. C., Lim, L. L., Shi, W., Hazen, S. L., Jacob, J. S., Crowley, J. R., Heinecke, J. W., and Lusis, A. J. (2001) *J. Clin. Invest.* **107,** 419–430
31. Michaelis, J., Vissers, M. C., and Winterbourn, C. C. (1992) *Arch. Biochem. Biophys.* **292,** 555–562
32. Heinecke, J. W., Li, W., Daehnke, H. L., 3rd, and Goldstein, J. A. (1993) *J. Biol. Chem.* **268,** 4069–4077
33. Hope, H. R., Remsen, E. E., Lewis, C., Jr., Heuvelman, D. M., Walker, M. C., Jennings, M., and Connolly, D. T. (2000) *Protein Expression Purif.* **18,** 269–276
34. Morita, Y., Iwamoto, H., Aibara, S., Kobayashi, T., and Hasegawa, E. (1986) *J. Biochem.* (*Tokyo*) **99,** 761–770
35. Morris, J. C. (1966) *J. Phys. Chem.* **70,** 3798–3805
36. Nelson, D. P., and Kiesow, L. A. (1972) *Anal. Biochem.* **49,** 474–478
37. Woessner, J. F., Jr. (1995) *Methods Enzymol.* **248,** 485–495
38. Levine, R. L., Mosoni, L., Berlett, B. S., and Stadtman, E. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 15036–15040
39. Browner, M. F., Smith, W. W., and Castelhano, A. L. (1995) *Biochemistry* **34,** 6602–6610
40. Weiss, S. J., Test, S. T., Eckmann, C. M., Roos, D., and Regiani, S. (1986) *Science* **234,** 200–203
41. Carr, A. C., Decker, E. A., Park, Y., and Frei, B. (2001) *Free Radic. Biol. Med.* **31,** 62–72
42. Gaut, J. P., Yeh, G. C., Tran, H. D., Byun, J., Henderson, J. P., Richter, G. M., Brennan, M. L., Lusis, A. J., Belaaouaj, A., Hotchkiss, R. S., and Heinecke, J. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 11961–11966
43. Henderson, J. P., Byun, J., Williams, M. V., Mueller, D. M., McCormick, M. L., and Heinecke, J. W. (2001) *J. Biol. Chem.* **276,** 7867–7875
44. Winterbourn, C. C., Vissers, M. C., and Kettle, A. J. (2000) *Curr. Opin. Hematol.* **7,** 53–58
45. Babior, B. M. (1999) *Blood* **93,** 1464–1476