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Hypochlorous Acid Reacts with the N-Terminal Methionines of Proteins To Give Dehydromethionine, a Potential Biomarker for Neutrophil-Induced Oxidative Stress[†]

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ABSTRACT: Electrophilic halogenating agents, including hypohalous acids and haloamines, oxidize free methionine and the N-terminal methionines of peptides and proteins (e.g., Met-1 of anti-inflammatory peptide 1 and ubiquitin) to produce dehydromethionine (a five-membered isothiazolidinium heterocycle). Amide derivatives of methionine are oxidized to the corresponding sulfoxide derivatives under the same reaction conditions (e.g., Met-3 of anti-inflammatory peptide 1). Other biological oxidants, including hydrogen peroxide and peroxynitrite, also produce only the corresponding sulfoxides. Hypothiocyanite does not react with methionine residues. We suggest that dehydromethionine may be a useful biomarker for the myeloperoxidase-induced oxidative stress associated with many inflammatory diseases.

Neutrophils, which typically comprise 33−75% of all leukocytes in humans, possess both oxidative and nonoxidative defense mechanisms; the former mechanisms are implicated in the oxidative stress that is associated with many inflammatory diseases (e.g., reperfusion injury, acute respiratory distress syndrome, diabetes mellitus, inflammatory bowel disease, rheumatoid arthritis, asthma, emphysema, vasculitis, and many rarer diseases) (*1*). Neutrophilic myeloperoxidase (MPO),[1] the only mammalian enzyme that is capable of oxidizing Cl^- to hypochlorous acid (HOCl) at a significant rate under physiological conditions, accounts for ~5% of total protein in the leukocyte. MPO is believed to play a pivotal role in many inflammatory diseases (*2*−*5*). Paradoxically, HOCl is capable of modulating the inflammatory response by both stimulating and repressing inflammatory mediators. Measured inflammatory response is critical to the resolution of infection, but overstimulation of neutrophils likely contributes to host tissue damage. Thus, equally valid hypotheses could be founded on the contribution of beneficial or deleterious properties of HOCl to the progression of diseases. Accordingly, there has been considerable interest in identifying biomarkers that uniquely assess the involvement of HOCl in vivo (*6*). Winterbourn and Kettle have recently reviewed the advantages and disadvantages of existing biomarkers for MPO-derived HOCl, which include chlorinated tyrosines (3-TyrCl and 3,5-$TyrCl_2$), lipid chlorohydrins, 5-chlorocytosine, protein carbonyls, and a sulfonamide derivative of glutathione (GSA) (Chart 1) (*7*). In the interim, 5-hydroxybutyrolactam derivatives of glutathione have also been identified as possible biomarkers (M-45 and M-90) (*8*). GSA (*9*) is a particularly attractive potential biomarker because thiols (e.g., Cys) and thioethers (e.g., Met) are among the first chemical targets of HOCl in a biological setting (*10*). For Met, it has been suggested that the sulfoxide (MetO) is the final product of oxidation by hypohalous acids (*11*−*18*). However, on the basis of previous studies of the I_3^- oxidation of Met, we suspected that dehydromethionine (DHM) might also be an intermediate (*19*−*25*). Indeed, we report herein that Met generally reacts with electrophilic halogenating agents to give high yields of DHM. Furthermore, oligopeptides and proteins with Met at the N-terminus also yield DHM.

MATERIALS AND METHODS

Reagents. All chemicals were ACS-certified grade or better and were used without further purification; 18.2 MΩ cm water, obtained from a Millipore Milli-Q A10 ultrapure water purification system, was used for all experiments. Unless otherwise noted, all solutions contained 0.1 M phosphate buffer (pH 7.4), which was prepared from $NaH_2PO_4 \cdot H_2O$ and Na_2HPO_4 (used as received from Mallinckrodt). Deuterium oxide (99.9%) and acetonitrile-d_3 were obtained from Cambridge Isotope Laboratories. Ubiquitin from bovine erythrocyte (≥98% by SDS−PAGE, essentially salt-free, lyophilized powder), chloramine-T hydrate (98%), DL-methionine (>99%), taurine (99%), anti-inflammatory peptide 1 (antiflammin-1), hydrogen peroxide (30 wt %), urea, calcium chloride (powder, >97%), and bromine were all obtained from Sigma-Aldrich. *N*-Acetyl-L-methionine (>99%) was obtained from Fluka. Sodium iodide, sodium bromide, and sodium nitrite were obtained from EMD Chemicals. Iodine was obtained from Fisher Scientific. DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) was obtained from TCI chemicals. Sequencing grade trypsin (specific activity > 18900 units/mg) was obtained from Promega. The N-terminal hexapeptide NH_2-Met-Gln-Ile-Phe-Val-Lys-COOH (Ub1−6) (>95% pure) was synthesized by the Molecular Biology Proteomic Facility at the University of Oklahoma Health Science Center. pH and pD were adjusted with NaOH, NaOD, HCl, or DCl as required.

pH Measurements. The $[H^+]$ of the buffered solutions was determined with an Orion model EA920 ion analyzer using a Ag/

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[1]Abbreviations: antiflammin-1, anti-inflammatory peptide 1; DHM, dehydromethionine; GSA, sulfonamide derivative of glutathione; MetO, methionine sulfoxide; MPO, myeloperoxidase; TauX (X = Cl or Br), halotaurine; $TauX_2$ (X = Cl or Br), dihalotaurine; 3-TyrCl, 3-chlorotyrosine; 3,5-$TyrCl_2$, 3,5-dichlorotyrosine; Ub, ubiquitin; Ub1−6, first six residues of Ub (NH_2-Met-Gln-Ile-Phe-Val-Lys-COOH).

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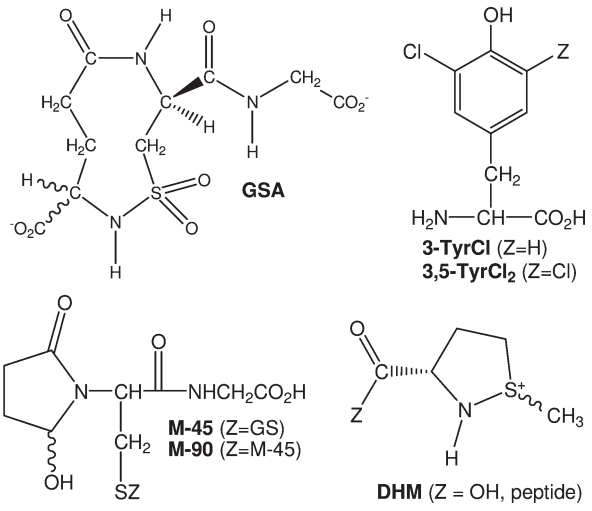
Chart 1: Some Proposed Biomarkers for HOCl-Induced Oxidative Stress



AgCl combination pH electrode. pD measurements in D_2O were taken using the same pH electrode by adding 0.4 unit to the measurement (*26*).

UV−Vis Spectroscopy. Electronic spectra were recorded using a Hewlett-Packard 8452A diode array spectrophotometer with quartz cells calibrated for 1 mm, 2 mm, 1 cm, and 10 cm path lengths at 20 °C. The 10 cm cell was used to measure the concentration of $OSCN^-$ in stock solutions (vide infra).

^{1}H NMR Measurements. For the studies involving the oxidation of free methionine by various oxidants, spectra were recorded with a Varian Mercury VX-300 spectrometer at 20 °C. For the studies involving the oxidation of antiflammin-1 and Ub1−6, spectra were recorded with a Varian VNMRS-400 NMR spectrometer at 20 °C. The chemical shifts (parts per million) were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, δ 0.015). The S-CH_3 groups of methionine, methionine sulfoxide, and dehydromethionine have characteristic chemical shifts (*20*, *27*). Integration of the peaks corresponding to the S-CH_3 groups was used to determine the Met:MetO:DHM ratio.

Preparation of Oxidant Stock Solutions. The concentration of chloramine-T in stock solutions was determined by mass. The concentration of H_2O_2 in stock solutions was determined spectrophotometrically (ε_{240} = 43.6 M^{-1} cm^{-1}) (*28*). Stock solutions of NaOCl were prepared by sparging Cl_2 into a 2.1 M solution of NaOH. The sparging was stopped when the OCl^- concentration reached ~0.99 M (pH >12), as determined spectrophotometrically at 292 nm (ε_{292} = 350 M^{-1} cm^{-1}) (*29*). Stock solutions of NaOBr were prepared by two methods. In both cases, the solutions of OBr^- were standardized spectrophotometrically at 329 nm (ε_{329} = 332 M^{-1} cm^{-1}) (*29*). The first method consisted of adding a 5-fold excess of NaBr to a solution of NaOCl. This reaction mixture was stored in the dark for 5 min before its concentration was determined spectrophotometrically. The second method consisted of addition of Br_2 to an ice-cold solution of NaOH. All solutions of OBr^- were kept at 0 °C and used within 2 h of their preparation to minimize errors due to decomposition (*30*). Stock solutions of $ONOO^-$ were prepared by a method adapted from Koppenol et al. (*31*). Briefly, ice-cold solutions of $NaNO_2$ in water and H_2O_2 in 1 M HCl were rapidly combined with a hand mixer (comprised of two Hamilton syringes and a T-mixer). The effluent of the hand mixer was added directly to a stirring solution of 1.1 M NaOH. The concentration of $ONOO^-$ was determined spectrophotometrically (ε_{302} = 1670 M^{-1} cm^{-1}) (*32*). Aqueous I_3^- stock solutions were prepared by addition of I_2 to a stirring solution of a 10-fold excess of NaI. After all the I_2 dissolved, the concentration of I_3^- was determined spectrophotometrically (ε_{353} = 26400 M^{-1} cm^{-1}) (*33*). Taurine monohaloamine (TauX, X = Cl or Br) and dihaloamine ($TauX_2$, X = Cl or Br) stock solutions were prepared by methods similar to that of Thomas et al. (*34*). Briefly, TauX (X = Cl or Br) was prepared by dropwise addition of the hypohalite solution (OBr^- was prepared by the Br_2/NaOH method as described previously) into an equimolar amount of vortexed taurine in 0.1 M NaOH. $TauX_2$ (X = Cl or Br) was prepared by dropwise addition of the hypohalite solution to a half-molar amount of vortexed taurine in water. Immediately after the solution had been mixed, the pH was lowered to <5 by addition of HCl, to encourage the disproportionation of TauX to $TauX_2$. After 30 min in the dark, the concentrations of the haloamines were determined spectrophotometrically: $\varepsilon(TauCl)_{252}$ = 429 M^{-1} cm^{-1} (*34*), $\varepsilon(TauCl_2)_{300}$ = 370 M^{-1} cm^{-1} (*34*), $\varepsilon(TauBr)_{288}$ = 430 M^{-1} cm^{-1} (*35*), and $\varepsilon(TauBr_2)_{336}$ = 371 M^{-1} cm^{-1} (*35*). $OSCN^-$ was generated by the LPO-catalyzed oxidation of SCN^- by H_2O_2 at pH 7.4 (*36*). SCN^- and LPO were incubated in 0.1 M phosphate buffer at 20 °C, and the reaction was initiated by the addition of H_2O_2. The concentration of $OSCN^-$ was determined spectrophotometrically (ε_{376} = 26.5 M^{-1} cm^{-1}) (*36*).

Reaction of Free Methionine with Oxidants. A 75 mM stock solution of methionine was prepared in 0.5 M phosphate buffer (pH 7.4). While the solution was being vortexed, 400 μL of the oxidant (diluted to the desired concentration with water prior to the reaction) was added dropwise to 400 μL of the methionine solution. After a period of time (<10 min for halogenating reagents and ca. 3 h for $ONOO^-$, $OSCN^-$, and H_2O_2), 200 μL of D_2O (20% deuterium to lock and shim) containing DSS (the NMR internal standard) was added. The final pH of the solutions was between 7.4 and 7.6.

Reaction of Antiflammin-1 and Ub1−6 with Oxidants. We prepared stock solutions of the peptides by dissolving the lyophilized peptide in a 50:50 mixture of water and deuterated acetonitrile-d_3. The peptides were further diluted (to achieve a peptide concentration of ca. 100 μM) by addition of a 0.1 M deuterated phosphate buffer (pH 7.4), made by adding DCl to D_2O solutions of anhydrous Na_2HPO_4. The formation of the sulfoxide derivative of the peptide was achieved by addition of excess H_2O_2 (*37*). The DHM peptide derivative was obtained by reaction of a ca. 10% molar excess of I_3^- over the peptide.

Reactions and Digestion of Ubiquitin. Ubiquitin (Ub, 1.9 mg) was dissolved in 0.1 M phosphate buffer (pH 7.4, 100 μL). This solution was subsequently divided into three parts, and equal volumes of water (a control), excess H_2O_2 in water, and 10 molar equiv (cf. Results) of HOCl in water (prepared by diluting a stock solution of NaOCl in 0.1 M NaOH) were added to each part. The resulting three solutions were placed in the dark for approximately 20 min before the tryptic digestion was initiated. The method used for digestion of the modified Ub samples was adapted from that of Cox et al. (*38*). Briefly, a trypsin solution [7 μg of enzyme in 310 μL of digestive buffer consisting of 50 mM Tris-HCl (pH 7.9), 6.5 M urea, and 10 mM $CaCl_2$] was added to each of the Ub samples [650 μg of Ub in 33 μL of 0.1 M PBS (pH 7.4)]. After this first addition of the trypsin, the samples were incubated in a shaking water bath at 37 °C for 2−4 h; then a second trypsin solution (7 μg of enzyme in 110 μL of digestive

buffer) was added to each Ub sample. After the second aliquot of trypsin was added, the digestion was allowed to continue for 4 h in a shaking water bath at 37 °C. The digested samples were divided into 50 μL aliquots and stored at −20 °C until they were used. The LC chromatograms of digested samples that were stored at −20 °C for up to 1 month showed no changes.

LC−MS of Tryptic Digested Ubiquitin. LC separation of the tryptic digests of native and oxidized Ub was performed using a BAS (Bioanalytical Systems Inc., West Lafeyette, IN) 200B HPLC system equipped with a BAS Unijet reversed phase microbore column (ODS-18, 100 mm × 1 mm, 3 μm particle size) and a Unijet guard column (ODS-18, 10 mm × 1 mm, 3 μm particle size). Mobile phase A consisted of 0.05% TFA and 0.05% TEA in deionized water. Mobile phase B consisted of 0.05% TFA and 0.05% TEA in 40% acetonitrile. These mobile phase solutions were filtered and vacuum-degassed prior to use. A binary gradient was applied as follows: 90% solvent A and 10% solvent B from 0 to 2 min, linear gradient to 30% solvent A and 70% solvent B from 2 to 28 min, linear gradient to 100% solvent B from 28 to 35 min, 100% solvent B from 35 to 65 min, and linear gradient to return the mobile phase to 90% solvent A and 10% solvent B from 65 to 75 min, which was maintained for an additional 32 min before the next sample was injected. The microbore column eluent was split (zero dead volume T) to a BAS UV-116A UV−visible detector set at 205 nm (final flow rate of 6 μL/min) and to a Micromass/Waters (Bedford, MA) Q-Tof-1 mass spectrometer operating in ESI (+) mode (final flow rate of 13 μL/min). The MS operating conditions were as follows: capillary voltage, 3.0 kV; cone voltage ramp, 10−85 V; source block temperature, 120 °C; desolvation temperature, 150 °C; and desolvation gas flow rate, 200 L/h.

RESULTS

Oxidation of Methionine. The products were quantified using their characteristic ^{1}H NMR spectra; note that because the sulfoxide and DHM derivatives are stereotopic at S, they comprise two diastereomers that are typically formed in a ca. 1:1 ratio (Table 1 and Figure S1 of the Supporting Information) (*20*). Table 2 summarizes the chemical yields of MetO and DHM that were obtained for the reactions of various oxidants with a 20% excess of Met (based upon the amount of oxidant that was employed). Hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) produced only MetO, as expected for agents that oxidize by two-electron O atom transfer (Table 2). Hypothiocyanite ($OSCN^-$) does not react with Met. In contrast, all of the halogenating agents produced significant amounts of DHM, in addition to MetO (Table 2).

Oxidation of Met-Containing Peptides. As a consequence of electronic conjugation with an adjacent carbonyl, amide nitrogen atoms are not sufficiently nucleophilic to form isothiazolidinium rings. Thus, the reactions of amide derivatives of Met with the oxidants of Table 2 yield only MetO. For example, oxidation of *N*-acetylmethionine with I_3^- in phosphate buffer (pH 7.4) produced the sulfoxide derivative (data not shown). A similar oxidation of anti-inflammatory peptide 1 (Met-Gln-Met-Lys-Lys-Val-Leu-Asp-Ser) with a molar excess of I_3^- produced DHM-Gln-MetO-Lys-Lys-Val-Leu-Asp-Ser, where the N-terminal Met was converted to a DHM derivative and the internal Met was oxidized to a sulfoxide (Figure S2 of the Supporting Information).

Table 1: Observed Chemical Shifts (relative to DSS) for the Methyl Resonance of Methionine and Its Derivatives in 0.1 M PBS (pH 7.4)

	δ (ppm)		
	methionine	methionine sulfoxide	dehydromethionine
free amino acid	2.14	2.76	2.84, 2.85
antiflammin-1 (Met-1)	2.13	2.76, 2.77	2.88, 2.89
antiflammin-1 (Met-3)	2.12	2.75, 2.75	−
Ub1−6 hexapeptide	2.12	2.72, 2.73	2.87, 2.88

Table 2: Product Distribution (%) for the Reaction of Various Oxidants with GSH To Give GSSG and GSA and with Met To Give MetO and DHM[a]

oxidant[b]	GSSG[e]	GSA[e]	MetO	DHM
H_2O_2	100	nd	100	0
$ONOO^-$	73	2	100	0
$OSCN^-$	100	0	0	0
avg (non-X^+)			**100**	**0**
HOCl	40	32	56	44
TauCl	66	nd	56	44
$TauCl_2$	−	−	57	43
chloramine-T	−	−	58	42
avg (Cl^+)			**57**	**43**
HOBr (HOCl/Br^-)[c]	−	−	26	74
HOBr (Br_2/NaOH)[c]	44	4	22	78
TauBr	−	−	23	77
$TauBr_2$	−	−	24	76
avg (Br^+)			**24**	**76**
I_3^-	97	nd	3	97
I^- with chloramine-T (5:1)[d]	−	−	10	90
avg (I^+)			**6**	**94**

[a]Conditions: 25 mM oxidant and 30 mM Met at pH 7.4 (0.2 M PBS) and 20 °C. [b]The oxidant was added to Met. [c]Prior to its addition to Met, HOBr was generated in situ by addition of HOCl to Br^- or by addition of Br_2 to base, followed by neutralization. [d]I^- was added to Met prior to the addition of chloramine-T. [e]These data from ref (*62*).

Oxidation of Met-1 in Ubiquitin. Reaction of Ub with 5 molar equiv of HOCl at pH 7.4 did not result in oxidation of Met-1, although some other modifications occurred, as indicated by changes in the chemical shifts in the aromatic region of the ^{13}C−^{1}H HSQC NMR spectrum (unpublished results). However, upon treatment of Ub with 10 molar equiv of HOCl at pH 7.4, followed by tryptic digestion and LC−MS analysis (Figure S3 of the Supporting Information), we identified two modifications of the N-terminal hexapeptide Met-Gln-Ile-Phe-Val-Lys (monoisotopic mass of 764.4, observed mass of 764.4 + H^+ in MS-ES$^+$) (Figure 1, top). One of the modifications was the oxidation of Met to the sulfoxide, MetO-Gln-Ile-Phe-Val-Lys (monoisotopic mass of 780.4, observed mass of 780.4 + H^+ in MS-ES$^+$) (Figure 1, top), as evidenced using an authentic sample of Ub that was selectively oxidized by H_2O_2 to MetO (Figure 1, middle) (*37*). The other modification was attributed to the DHM derivative DHM-Gln-Ile-Phe-Val-Lys (monoisotopic mass of 762.4, observed mass of 762.4 + H^+ in MS-ES$^+$) (Figures 1, top, and Figure S4 of the Supporting Information).

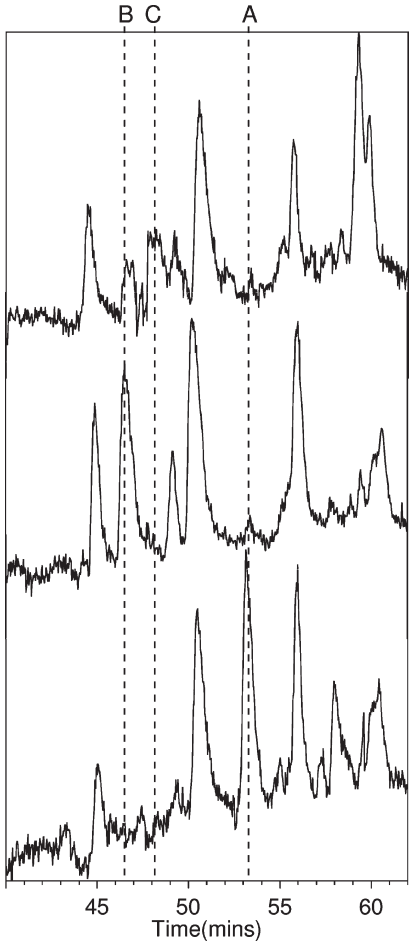


FIGURE 1: LC−MS-ESI$^+$ chromatogram of trypsin-digested Ub (bottom), a digest of Ub that was oxidized with a 10% molar excess of H_2O_2 (middle), and a digest of Ub that was oxidized with 10 molar equiv of HOCl (top). The time scale shown is for native Ub. The other chromatograms were aligned by best fit of the retention time and fragment m/z values. On the basis of the observed m/z values, the following modifications of the Met-Gln-Ile-Phe-Val-Lys (residues 1−6) peptide of Ub have been assigned: (A) Met-Gln-Ile-Phe-Val-Lys (retention time of ca. 53 min, m/z 764.4 + H^+), (B) MetO-Gln-Ile-Phe-Val-Lys (retention time of ca. 46−47 min, m/z 780.4 + H^+), and (C) DHM-Gln-Ile-Phe-Val-Lys (retention time of ca. 48 min, m/z 762.4 + H^+).

Confirmation of the latter assignment was achieved by independent synthesis of Met-Gln-Ile-Phe-Val-Lys, and subsequent reaction with I_3^-, followed by ^{1}H NMR of the synthesized DHM-Gln-Ile-Phe-Val-Lys [which exhibited the characteristic pattern of the two diastereomeric methyl groups of DHM (Figure 2)]. To further confirm the assignment, the tryptic digest of Ub treated with 10 molar equiv of HOCl was spiked with an authentic sample of DHM-Gln-Ile-Phe-Val-Lys, which exhibited the same chromatographic retention time and mass spectrum as the corresponding hexapeptide in the tryptic digest of oxidized Ub (Figure S5 of the Supporting Information, which also shows the products of excess I_3^- oxidation of a Tyr-containing peptide and fragments of the trypsin autodigest).

DISCUSSION

General Mechanisms of Formation of DHM. Remarkably, the halogenating agents can be grouped by the yields of DHM produced (Table 2). All of the chlorinating agents gave approximately equimolar amounts of MetO and DHM (42−44% DHM); the brominating agents produced higher yields

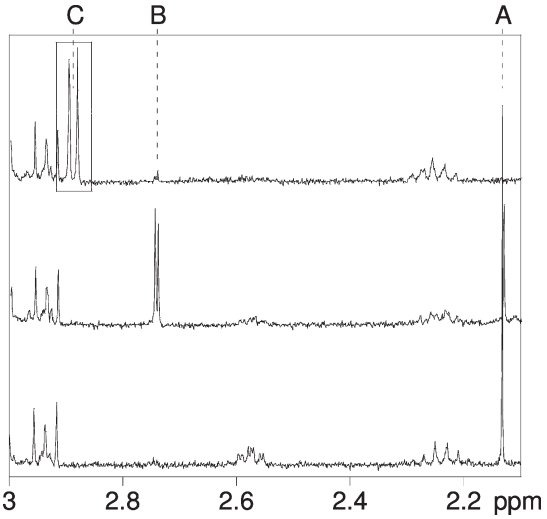
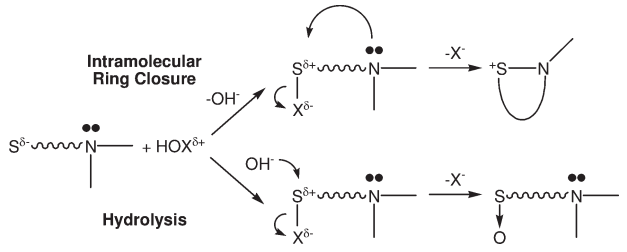


FIGURE 2: ^{1}H NMR spectra of hexapeptide Ub1−6 (40 μM) in 0.1 M PBS (pD 7.4): native peptide (bottom), peptide oxidized with a 10% molar excess of H_2O_2 (middle), and peptide oxidized with a 10% molar excess of I_3^- (top). The labeled resonances are S-CH_3 of methionine (A), methionine sulfoxide (B), and dehydromethionine (C).

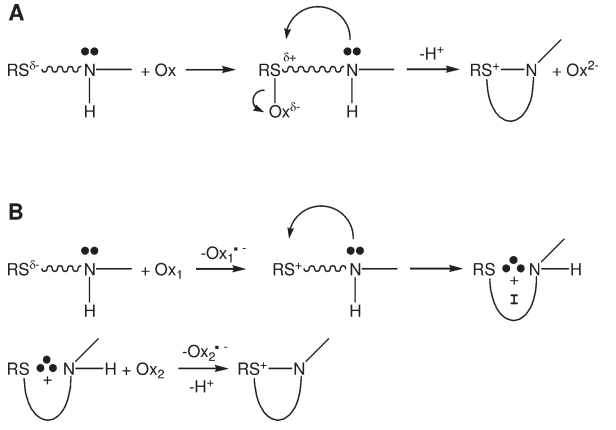
of DHM (74−78% DHM), and the iodinating agents gave nearly stoichiometric yields of DHM (90−97% DHM) (Table 2). Grouped by halogen, the chemical yields indicate a common branching mechanism, most likely intramolecular nucleophilic attack of the amine on a sulfonium intermediate to give DHM, or alternatively hydrolysis of the sulfonium intermediate to give MetO (Scheme 1). On the basis of analogous halosulfonium species (*39*−*48*), it follows that the relative order of stability toward hydrolysis would be as follows: I > Br > Cl (which reflects the trends observed in the yields of DHM relative to MetO). We note that the product yields for GSA exhibit the opposite trend, with larger amounts of GSA observed for Cl followed by Br followed by I (Table 2). However, it is important to point out that the oxidation of Met to DHM is a two-electron process (requiring 1 molar equiv of the oxidants of Table 2 through presumably a single halosulfonium intermediate), whereas the oxidation of glutathione to GSA is a six-electron process (requiring 3 molar equiv of oxidant vis-à-vis multiple intermediates) (*9*).

As a consequence of electronic conjugation with an adjacent carbonyl (*49*), amide nitrogen atoms are apparently not sufficiently nucleophilic to form isothiazolidinium rings. Thus, the reactions of amide derivatives of Met with the oxidants of Table 2 yield only MetO. For example, oxidation of *N*-acetylmethionine with I_3^- produces only the sulfoxide derivative (unpublished results). See also the products observed for the oxidation of anti-inflammatory peptide 1 that is described in Results. Because the start codon for eukaryotic protein synthesis encodes Met (*50*), mammalian proteins are produced in vivo with Met at the N-terminus. As a consequence of post-translational modifications, roughly 40% of the N-terminal Met residues are cleaved, but the majority remain after protein processing (*51*). The highly conserved 8.5 kDa eukaryotic regulatory protein ubiquitin (*52*) was selected for investigation of whether DHM is formed in proteins because it contains only one Met (at the N-terminus) and no Cys residues (a competitive target of HOCl) (*53*). Our findings for Ub suggest the N-terminal Met residues of the protein also yield DHM derivatives when reacted with electrophilic halogenating agents.

Scheme 1: Schematic Branching Mechanisms via a Common Sulfenyl Halide Intermediate that Yield S−N and S−O Derivatives



Scheme 2: Two-Electron (A) and One-Electron (B) Pathways That Produce DHM



Additional reaction pathways that yield DHM exist. Scheme 2 illustrates generic two-electron (A) and sequential one-electron (B) reaction mechanisms that produce isothiazolidinium heterocycles. The reactions of HOX (X = Cl or Br) with Met (Scheme 1) are examples of the two-electron pathway (Scheme 2A). Photooxygenation of Met by singlet oxygen ($^1O_2$) produces a persulfoxide intermediate that subsequently undergoes intramolecular ring closure with extrusion of H_2O_2, which is another example of a two-electron oxidation (Scheme 2A, Ox = $^1O_2$, $Ox^{2-}+2H^+ = H_2O_2$) (*54*, *55*). In contrast, the mechanism of Scheme 2B involves two sequential one-electron steps. Hydroxyl radical and halogen radical anions are capable of oxidizing thioethers to a sulfide radical cation (Scheme 2B, Ox_1 = $^\bullet$OH or $X_2^{\bullet-}$, and X = Cl or Br) (*56*, *57*), which in the case of Met is stabilized intramolecularly via a three-electron S−N bond. In the absence of a reaction partner (Ox_2), the intermediate decomposes rapidly, typically on the microsecond time scale. To produce DHM via pathway B of Scheme 2, the one-electron oxidized intermediate must be trapped before it has the chance to decompose. The most efficient trapping agents appear to be radicals. Superoxide ($Ox_2 = O_2^{\bullet-}$) reacts with the intermediate to produce sulfoxides (and $Ox_2^- + 2H^+ = H_2O_2$) (*55*). However, triplet oxygen ($Ox_2 = {}^3O_2$) reacts with the intermediate to yield DHM (and $Ox_2^- = O_2^{\bullet-}$) (*58*). Besides the title reaction, it appears likely to us that only the reaction of Met with $^\bullet$OH, followed by trapping of the one-electron oxidized intermediate by $^3O_2$, has some likelihood in vivo. However, $^\bullet$OH exhibits promiscuous reaction chemistry [the rate constant of the reaction of $^\bullet$OH with free amino acids are all $\sim 10^9$ M^{-1} s^{-1} at pH 7 (*59*)], whereas HOCl exhibits selectivity for the sulfur-containing amino acids [$\sim 10^7$ M^{-1} s^{-1} for Met and Cys, with a 7 order of magnitude range of rate constants for all of the amino acids (*10*)]. Thus, it is not clear whether the $^\bullet$OH/$^3O_2$ reaction is kinetically competent to produce DHM in vivo. There have been no reports of the production of DHM in proteins upon reaction with $^\bullet$OH.

Stability of DHM Derivatives. At neutral pH in the absence of catalysts, the pseudo-first-order rate constant for hydrolysis of DHM is 6×10^{-9} s^{-1}, which corresponds to a hydrolysis half-life of ~3.6 years; the rate increases markedly at both lower and higher pH (*25*). DHM can be reduced by thiols; the half-life for the reaction of DHM (Chart 1, Z = OH) with cytosolic concentrations of glutathione is on the order of hours, although the rate in most physiologic fluids would be significantly lower (*19*, *23*). Accordingly, DHM (Chart 1, Z = OH) is potentially sufficiently long-lived in vivo to be detectable if it is formed. However, the concentration of free Met in the plasma of healthy individuals is only 10−40 μM (*60*, *61*). The real potential of DHM as a biomarker may be for protein derivatives, as identification of DHM modifications of specific proteins could afford insight into the amounts of electrophilic halogenating agents (the sum of hypohalous acids and the haloamines that are derived from them) that are produced in different microenvironments. We have not thus far detected hydrolysis of the DHM derivative of Ub. It is conceivable that DHM (Chart 1, Z = peptide) moieties could be more or less stable than free DHM (Chart 1, Z = OH), depending upon whether the protein environment stabilizes the DHM moiety with respect to hydrolysis (e.g., in a hydrophobic fold) and reduction (e.g., by steric confinement), and upon whether adjacent residues catalyze such reactions (as is frequently the case for acid/base-catalyzed reactions). DHM (prepared by oxidation of free Met with a 10% molar excess of I_3^-) that was treated with trypsin, using the same procedure that was employed for oxidized Ub, underwent 40% hydrolysis to the sulfoxide during the digestive process. Thus, it is conceivable that the MetO derivative of Ub1−6 is due to conversion of the DHM derivative during digestion. Further investigation will be required to establish whether DHM moieties are found in vivo upon insult with hypohalous acids and their derivatives.

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We appreciate the assistance of Dr. S. L. Nimmo with the NMR experiments.

SUPPORTING INFORMATION AVAILABLE

Additional NMR and MS data (Figures S1−S5). This material is available free of charge via the Internet at http://pubs.acs.org.

NOTE ADDED IN PROOF

Some parts of this study have been independently reported (*63*).

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