

Human Neutrophils Employ the Myeloperoxidase-Hydrogen Peroxide-Chloride System to Oxidize α -Amino Acids to a Family of Reactive Aldehydes

MECHANISTIC STUDIES IDENTIFYING LABILE INTERMEDIATES ALONG THE REACTION PATHWAY*

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We have recently demonstrated that neutrophils oxidize nearly all of the amino acids commonly found in plasma to a corresponding family of aldehydes in high yield. The reaction is mediated by hypochlorous acid (HOCl), the major oxidant generated by the myeloperoxidase-H₂O₂-Cl⁻ system of phagocytes. We now present evidence for the underlying mechanism of this reaction, including the structural requirements and reaction intermediates formed. Utilizing mass spectrometry and isotopically labeled amino acids, we rule out hydrogen atom abstraction from the α -carbon as the initial event in aldehyde formation during amino acid oxidation, a pathway known to occur with ionizing radiation. Aldehyde generation from amino acids required the presence of an α -amino moiety; β - and ϵ -amino acids did not form aldehydes upon oxidation by either the myeloperoxidase system or HOCl, generating stable monochloramines instead. UV difference spectroscopy, high pressure liquid chromatography, and multinuclear (¹H, ¹⁵N) NMR spectroscopy established that the conversion of α -amino acids into aldehydes begins with generation of an unstable α -monochloramine, which subsequently decomposes to yield an aldehyde. Precursor product relationships between α -amino acid and α -monochloramine, and α -monochloramine and aldehyde were confirmed by high pressure liquid chromatography purification of the reaction intermediate and subsequent ¹H and ¹⁵N NMR spectroscopy. Collectively, these results detail the chemical mechanism and reaction intermediates generated during conversion of amino acids into aldehydes by myeloperoxidase-generated HOCl.

We have recently shown that the enzyme can use H₂O₂ and chloride ions (Cl⁻) to oxidize nearly all of the common amino acids into a family of aldehydes (3). Because these products react readily with biological constituents and may be generated in significant quantities at sites of inflammation, we set out to elucidate the chemical mechanism by which they are generated.

Our study explored two potential mechanisms (Scheme I). Pathway A, known to be a route by which amino acids are oxidized by ionizing radiation, involves initial hydrogen atom abstraction to generate an α -carbon-centered radical (4–9). This short-lived intermediate decomposes into carbon dioxide and an imine, which then may form an aldehyde in the presence of H₂O₂ through liberation of ammonia (4–9). Metal-catalyzed oxidation of amino acids may similarly convert amino acids into aldehydes (9, 10), as first suggested by Dakin (11–13) nearly a century ago. Pathway B involves initial chlorination of the α -amino moiety, generating an α -monochloramine. This reaction is plausible because myeloperoxidase generates hypochlorous acid (HOCl) from H₂O₂ and Cl⁻ (14), and monochloramines form readily when HOCl reacts with amines (15–17). Zgliczynski and colleagues (18) first suggested that monochloramines of common amino acids might serve as precursors in aldehyde formation. These conclusions, however, were drawn from indirect evidence and confirmed neither the structures of products formed nor the putative monochloramine intermediates. In fact, monochloramines are generally thought to be relatively stable compounds under physiological conditions (15–17, 19–23). The stability of the presumed monochloramine generated from taurine is exploited as a quantitative assay for HOCl production (16). Despite the widely held belief that the myeloperoxidase-H₂O₂-Cl⁻ system chlorinates primary amines to form monochloramines, structural characterization of these species has been indirect (*e.g.* UV absorbance) rather than direct (*e.g.* NMR).

In this study, we use a variety of techniques to explore in detail the chemical mechanism of myeloperoxidase-mediated amino acid oxidation. We rule out hydrogen atom abstraction (a requirement for Pathway A) through mass spectrometric analysis of isotopically labeled amino acids. We then demonstrate that aldehydes can be generated only from amino acids that have free α -amino and α -carboxylic acid groups. We show that chlorination of α -amino acids by HOCl yields an unstable α -monochloramine, as in Pathway B, utilizing a variety of techniques including UV spectroscopy, ¹H and ¹⁵N NMR, and HPLC. The monochloramine then loses both α -carboxylic acid

Activated phagocytes both secrete the heme enzyme myeloperoxidase and generate hydrogen peroxide (H₂O₂)¹ (1, 2).

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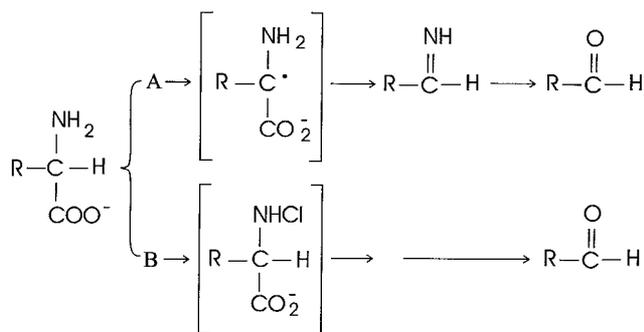
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¹ The abbreviations used are: H₂O₂, hydrogen peroxide; HOCl, hypochlorous acid; HPLC, high performance liquid chromatography; M⁺, molecular

ion; PFB, pentafluorobenzyl; pHA, *p*-hydroxyphenylacetaldehyde.



SCHEME I. Potential pathways of amino acid oxidation to yield aldehydes.

and NHCl groups to yield an aldehyde. Collectively, these results describe the underlying mechanism for aldehyde formation from α -amino acids by the phagocytic oxidant HOCl. The relative abundance of α -amino acids in plasma (24), interstitial fluid, and intracellular compartments (25) suggests that aldehydes derived from these precursors should be readily formed at sites of inflammation.

EXPERIMENTAL PROCEDURES

Materials

HPLC solvents were purchased from Baxter (McGaw Park, IL). Sodium phosphate, ethyl acetate, H₂O₂, and sodium hypochlorite were obtained from Fisher. D₂O, L-[¹⁵N]tyrosine, and L-[*d*₈]phenylalanine were purchased from Cambridge Isotopes, Inc. (Andover, MA). All other materials were purchased from Sigma, except where indicated.

Methods

General Procedures—Aldehydes were synthesized and analyzed by electron impact gas chromatography/mass spectrometry and reverse phase HPLC as described (3).

UV Absorbance Spectroscopy—UV absorbance spectra were obtained on a Beckman DU7 spectrophotometer equipped with a thermostatically controlled cuvette holder. UV difference spectra of amino acid-derived monochloramines were performed at the indicated temperature with the starting amino acid used as a reference. Kinetic studies of monochloramines were carried out similarly by monitoring the time-dependent changes in absorbance at 254 nm.

Glycolaldehyde Production—Glycolaldehyde production was measured by reverse phase HPLC following derivatization with 3-methyl-2-benzothiazolinone hydrazone hydrochloride as described previously (26).

Multinuclear NMR Studies—Monochloramines for NMR experiments were synthesized by dropwise addition of NaOCl (1:1; mol/mol) to the indicated amino acid in 20 mM sodium phosphate buffer (pH 7.0) in 10% D₂O, 90% H₂O (v/v) at 0 °C with constant mixing. NMR spectra were acquired on a Varian Unity-Plus 500 spectrometer (499.843 MHz for ¹H). A Nalorac indirect detection probe was employed for the ¹H and ¹⁵N two-dimensional NMR studies. Sample temperature was maintained (± 0.1 °C) with an Oxford Instruments temperature controller. ¹H chemical shifts were referenced to external sodium 3-(trimethylsilyl)propionate-2,2,3,3,3-*d*₄ in D₂O. The intense HOD signal was attenuated by transmitter pre-irradiation, and digital signal processing was employed to suppress phase distortions for ¹H spectra.

The proton NMR spectrum of L-tyrosine α -monochloramine was recorded from 32 transients under the following conditions: pre-acquisition delay = 1 s, acquisition time = 1.89 s (37,760 complex data points), pulse width = 5 μ s (62° flip angle) and spectral width = 10,000 Hz. The free induction decays were processed with a combination of gaussian and exponential weighting functions.

¹⁵N NMR chemical shifts of L-[¹⁵N]tyrosine and L-[¹⁵N]tyrosine α -monochloramine were established through heteronuclear multiple bond correlation spectroscopy experiments collected at 0 °C. Collection conditions for the *t*₂ domain (¹H) include transmitter presaturation of the intense water signal, a spectral width of 6,000 Hz, and collection of eight transients containing 2,048 complex data points. The *t*₁ domain (¹⁵N) included 128 increments over a spectral width of 7,000 Hz (evolution time \sim 18 ms). The 90° pulse duration was 8 and 26 μ s for the ¹H and ¹⁵N channels, respectively. Data were collected and processed in the hypercomplex mode employing sine bell and gaussian weighting in

TABLE I
Aldehyde production and monochloramine half-life of amino compounds oxidized by myeloperoxidase and reagent HOCl

Aldehyde production from non-aromatic compounds was quantified by reverse phase HPLC of the PFB-oxime derivatives (3). pHA production from tyrosine and its analogues was determined as described (3). Aldehydes were prepared by incubating the indicated compounds (100 nmol) individually with myeloperoxidase (20 pmol), H₂O₂ (100 nmol), and NaCl (100 μ mol) in 50 mM sodium phosphate buffer (pH 7.0; final volume 1 ml) within gas-tight reaction vials at 37 °C for 60 min. Identities of amino acid-derived aldehydes and their oxime derivatives were confirmed by mass spectrometry (3). The half-life of individual monochloramines was determined spectrophotometrically (*A*₂₅₄). Monochloramines were prepared by drop-wise addition of HOCl (1.5 mM final) to each amino acid (1.5 mM each) in 50 mM sodium phosphate buffer (pH 7.0) at 0 °C. Reaction mixtures were briefly (15 s) warmed to 37 °C in a H₂O bath and then transferred to thermostatically controlled cuvettes (37 °C).

Amino compound	Aldehyde yield ^a	Monochloramine half-life
	%	min
α -Alanine	94	6.8
β -Alanine	<1	>1500
<i>N</i> ^{α} -Acetyllysine	85	9.0
<i>N</i> ^{α} -Acetyllysine	<1	>1500
Taurine	<1	>1500
Ethanolamine	<1	>1500
L-Tyrosine	94	7.3
D-Tyrosine	87	7.2
<i>N</i> ^{α} -Acetyltyrosine tyrosinamide	<1	— ^b
	<1	— ^b
<i>N</i> ^{α} -Acetyltyrosine ethyl ester	<1	— ^b
Tyrosine methyl ester	<1	— ^b

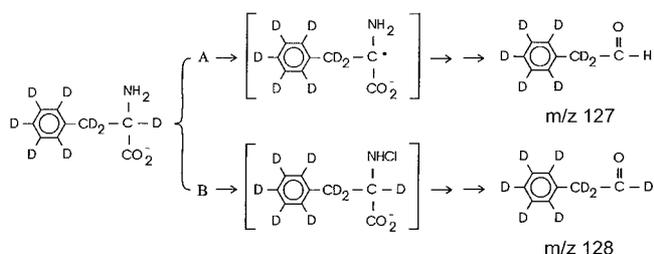
^a Yield relative to oxidizing substrate (H₂O₂).

^b UV spectra not examined.

*t*₂ and *t*₁, respectively. The ¹⁵N chemical shifts were established through 3-bond connectivities to the benzylic protons and were referenced to L-[¹⁵N]tyrosine arbitrarily set to 0 ppm.

RESULTS

Only Amino Acids with Free Amino and Carboxylic Acid Groups on the α -Carbon Yield Aldehydes When Oxidized by the Myeloperoxidase-H₂O₂-Cl⁻ System—To explore the reaction mechanism by which the myeloperoxidase system generates aldehydes during oxidation of α -amino acids, we first determined which structural features the amino acids must possess. Incubation of α -alanine (1-aminopropanoic acid) with the complete myeloperoxidase-H₂O₂-Cl⁻ system resulted in the near quantitative conversion of the α -amino acid into its corresponding aldehyde, acetaldehyde (Table I) (3). In contrast, incubation of β -alanine (2-aminopropanoic acid) with the complete myeloperoxidase system generated no detectable aldehyde. We next examined aldehyde yield during regiospecific chlorination of either the α - or ϵ - amino moieties of lysine. Incubation of the ϵ -amino blocked analog (*N* ^{ϵ} -acetyllysine) with the myeloperoxidase-H₂O₂-Cl⁻ system resulted in aldehyde formation in high yield (85%; Table I). In contrast, incubation of the α -amino blocked analog (*N* ^{α} -acetyllysine) with the complete myeloperoxidase system failed to generate detectable aldehyde. No aldehyde was formed when the myeloperoxidase system oxidized taurine (β -aminosulfonic acid) or ethanolamine (Table I). Thus, only amino acids that have both the primary amino and carboxylic acid groups in the α -position are oxidized to aldehydes by the myeloperoxidase-H₂O₂-Cl⁻ system. There were no significant differences in overall aldehyde yield from D- or L-tyrosine. Finally, aldehyde formation required that both the α -amino and α -carboxylic acid groups be free since no detectable *p*-hydroxyphenylacetaldehyde (pHA) was formed during



SCHEME II. Experimental strategy to discriminate between proposed mechanisms of aldehyde formation.

myeloperoxidase-catalyzed oxidation of N^α -acetyltyrosine, tyrosinamide, N^α -acetyltyrosine ethyl ester, or tyrosine methyl ester (Table I).

The Myeloperoxidase System Does Not Abstract a Hydrogen Atom When It Oxidizes α -Amino Acids—The finding that amino acids must contain an α -amino moiety to serve as substrates for aldehyde generation during amino acid oxidation suggested either the initial formation of an unstable α -monochloramine (Pathway B) or an alternative catalytic strategy involving hydrogen atom abstraction from the tertiary α -carbon (Pathway A). To determine which of these reaction mechanisms mediated aldehyde formation, we analyzed the products generated when myeloperoxidase acted on an amino acid that was isotopically labeled with deuterium at the α -carbon position (Scheme II). Oxidation of isotopically labeled L-[d_8]phenylalanine in an aqueous solution would be anticipated to generate an aldehyde with a molecular ion (M^+) of mass to charge ratio (m/z) 127, if hydrogen atom abstraction of the α -carbon occurred. Chlorination of the primary amino group generating an α -monochloramine intermediate would instead lead to a product with a M^+ of m/z 128.

Electron impact gas chromatography/mass spectrometric analysis of L-phenylalanine and L-[d_8]phenylalanine oxidized by the myeloperoxidase- H_2O_2 - Cl^- system revealed that the aldehyde formed with the d_8 analogue possessed an M^+ of m/z 128 (Fig. 1) and was identical in isotopic composition to the parent amino acid. Examination of pentafluorobenzyl (PFB)-oxime derivatives of the aldehydes formed from the precursor deuterated and non-deuterated amino acids revealed molecular ions of m/z 323 and m/z 315, respectively. The 8 mass unit increase in the derivatized aldehyde derived from myeloperoxidase-catalyzed oxidation of L-[d_8]phenylalanine confirms that the deuterium at the α -carbon position was retained, as in Pathway B. In contrast, L-[d_8]phenylalanine oxidized by a hydroxyl radical-generating system (2 mM H_2O_2 and 100 μ M $CuSO_4$) produced only trace amounts of aldehyde, as well as other products that likely reflect HO^\bullet addition products to the ring (27, 28). The low abundance of the aldehyde formed prevented its mass analysis by electron impact mass spectrometry; however, gas chromatography-mass spectrometry analysis of the PFB-oxime derivative confirmed that the aldehyde lost the α -carbon deuterium (M^+ of m/z 322; data not shown). Thus, aldehyde generation by myeloperoxidase does not involve hydrogen (or deuterium) atom abstraction from the α -carbon of L-phenylalanine, in contrast to amino acid oxidation with a hydroxyl radical generating system.

Oxidation of α -Amino Acids by HOCl Yields Unstable Intermediates with UV Spectral Features Consistent with α -Monochloramines—Preliminary studies demonstrated that oxidation of several common amino acids (glycine, alanine, isoleucine, phenylalanine, and serine) by the myeloperoxidase- H_2O_2 - Cl^- system generated a labile intermediate with a UV absorbance band at 252–254 nm, the absorbance maximum of monochloramines (15–17). To explore the possibility of an unstable α -monochloramine as a reaction intermediate, amino

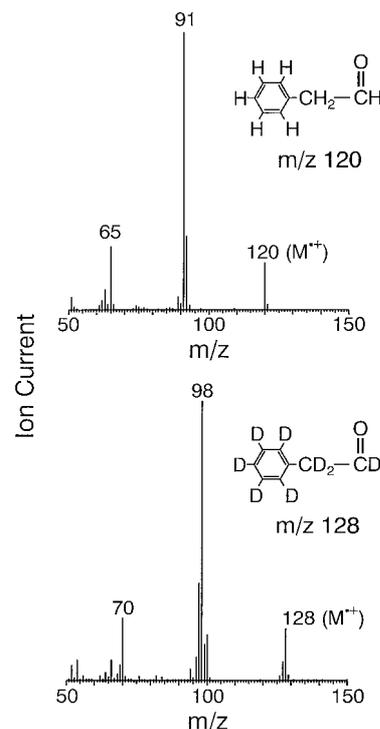


FIG. 1. Electron impact mass spectra of L-phenylalanine and L-[d_8]phenylalanine oxidation products generated by the myeloperoxidase- H_2O_2 - Cl^- system. L-Phenylalanine (200 μ M) or L-[d_8]phenylalanine (200 μ M) were incubated with myeloperoxidase (6 nM), H_2O_2 (200 μ M), and sodium chloride (100 mM) in buffer A (50 mM sodium phosphate (pH 7.0)) within gas-tight reaction vials at 37 $^\circ$ C for 30 min. Oxidation products were extracted into ethyl acetate (1:1, v/v) and analyzed by gas chromatography-mass spectrometry in the electron impact mode with an electron ionization energy of 70 eV. Gas chromatographic separations were carried out utilizing a Restek RTX-200 column (15 m, 0.33 mm inner diameter, 1- μ m film thickness) in the splitless mode with helium as the carrier gas. The column was run with the following temperature gradient: 70–150 $^\circ$ C at 10 $^\circ$ C/min; 150–280 $^\circ$ C at 20 $^\circ$ C/min. **Upper panel**, L-phenylalanine oxidized by the myeloperoxidase- H_2O_2 - Cl^- system. Prominent ions in the mass spectrum include the molecular ion at m/z 120 (M^+), m/z 91 (M^+ -CHO) and m/z 65 (M^+ -CHO- C_2H_5). **Lower panel**, L-[d_8]phenylalanine oxidized by the myeloperoxidase- H_2O_2 - Cl^- system. The molecular ion of m/z 128 (M^+) indicates that the α -carbon deuterium is not abstracted during aldehyde formation. Prominent fragment ions in the mass spectrum at m/z 98 and m/z 70 represent (M^+ -CDO) and (M^+ -CDO- C_2D_2), respectively.

acids were incubated with HOCl at 0 $^\circ$ C, and their UV difference spectra were determined. A characteristic absorbance band was observed at 254 nm, suggesting monochloramine formation. Comparisons of the stability of α -, β - and ϵ -monochloramines of amino acids, as assessed by the disappearance of absorbance at 254 nm at 37 $^\circ$ C, demonstrated that only α -monochloramines were thermally labile (Table I). Parallel experiments with each of these same α -, β -, and ϵ -amino acids reacted with purified myeloperoxidase, chloride, and H_2O_2 also demonstrated that only α -monochloramines were unstable, as assessed by loss of an absorbance band at 254 nm (data not shown).

The differing stabilities of α - and ϵ -monochloramines are illustrated in the experiment depicted in Fig. 2. Either the α -amino or ϵ -amino moiety of L-lysine was regiospecifically chlorinated with HOCl using the appropriate N -acetylated parent compound as substrate, and the thermal stability of the resulting α - or ϵ -monochloramine was determined. Chlorination of the α -amino group of N^ϵ -acetyllysine generated a labile α -monochloramine that demonstrated a $t_{1/2}$ of \sim 9 min at 37 $^\circ$ C (Fig. 2A). In striking contrast, chlorination of the ϵ -amino

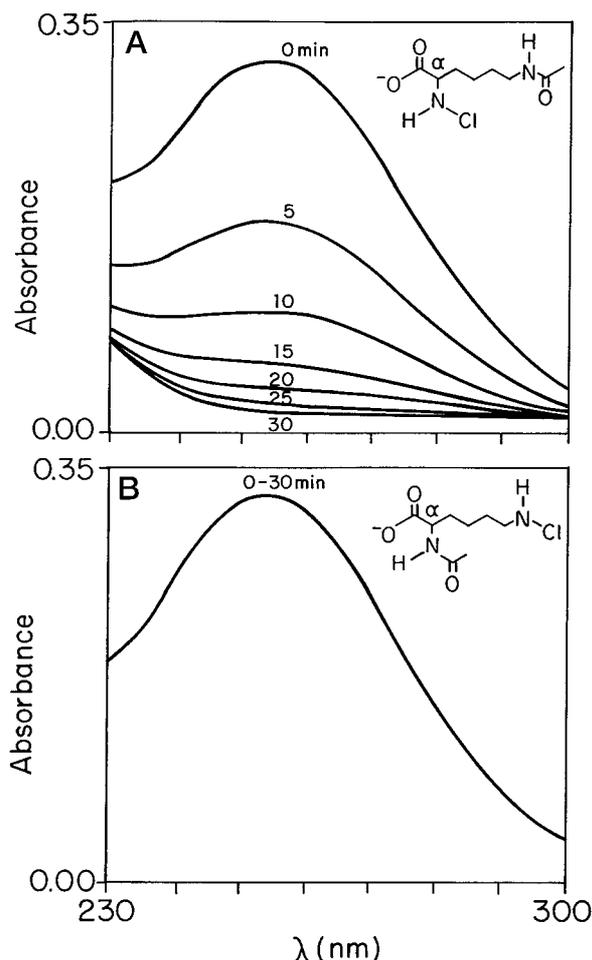


FIG. 2. Oxidation of N^α -acetyllysine and N^ϵ -acetyllysine by HOCl. Monochloramines of N^ϵ -acetyl lysine (A) or N^α -acetyllysine (B) were generated by dropwise addition of HOCl (1.5 mM) to reaction mixtures of the amino acids (1.5 mM) in buffer A at 0 °C. Reaction mixtures were rapidly warmed to 37 °C, transferred to thermostatically controlled cuvettes (37 °C), and the initial rate of monochloramine decay monitored spectrophotometrically ($A_{254 \text{ nm}}$). Note that at $t = 0$ min, quantitative chlorination of both the α - and ϵ -amino moieties results in near identical UV absorbance profiles. Upon warming to 37 °C, the α -monochloramine is unstable and decomposes with first-order kinetics, whereas the ϵ -monochloramine is stable. Insets in A and B represent the proposed structures of the monochloramines.

group of N^α -acetyllysine as substrate generated a ϵ -monochloramine that was apparently stable (Fig. 2B).

Examination of L-lysine oxidized with HOCl (1:1, mol/mol) revealed an initial first-order loss of monochloramine (as monitored by the change in A_{254}) followed by persistence of $\sim 30\%$ of the original absorbance, despite prolonged incubation (>10 h at 37 °C). The UV spectrum of the reaction mixture demonstrated an absorbance band at 254 nm, suggesting that a stable ϵ -monochloramine was still present.

The HOCl-dependent oxidation of acidic (L-glutamate), alcoholic (L-serine), aliphatic (L-leucine), amide (L-glutamine), aromatic (L-phenylalanine), and basic (L-lysine) amino acids all resulted in the formation of a labile intermediate with UV spectral features consistent with an α -monochloramine (Table II). Interestingly, chlorination of the secondary amino group of L-proline also led to formation of an unstable monochloramine, as assessed by loss of an absorbance band at 254 nm (Table II). However, this monochloramine did not generate an aldehyde during its decomposition, since derivatization of the product yielded neither a PFB-oxime nor a DNPH derivative; moreover, the reaction products failed to exhibit a positive Tollen's test, a

TABLE II

Half-life of monochloramines of each class of α -amino acids

Monochloramines of amino acids were prepared as described in the legend to Table I, and the initial rate of monochloramine decay was monitored spectrophotometrically as the decrease in absorbance at 254 nm.

Amino acid	Half-life
	<i>min</i>
Acidic	
Glu	7.6
Alcoholic	
Ser	6.0
Aliphatic	
Gly	35
Leu	7.8
Amides	
Gln	5.7
Aromatic	
Phe ^a	6.3
Basic	
Lys ^b	9.2
Secondary	
Pro	7.8
Thiols	
Cys	ND ^c

^a Following an initial first-order loss of monochloramine, $\sim 10\%$ of the original absorbance at 254 nm remained, despite prolonged incubation (>10 h) at 37 °C. The UV spectrum of the reaction mixture, however, demonstrated an absorbance maximum at 230 nm and no absorbance band at 254 nm.

^b Following an initial first order loss of monochloramine, $\sim 30\%$ of the original absorbance at 254 nm remained, despite prolonged incubation (>10 h) at 37 °C. The UV spectrum of the reaction mixture still demonstrated an absorbance maxima at 254 nm, consistent with monochloramine formation.

^c No detectable monochloramine was observed.

classic qualitative assay for a reactive carbonyl (29, 30). These results are consistent with our recent observation that no aldehyde was formed when proline (an imino acid that contains a secondary amino group) was oxidized by the myeloperoxidase- $\text{H}_2\text{O}_2\text{-Cl}^-$ system (3). Collectively, these results suggest that unstable primary α -monochloramines serve as intermediates when the myeloperoxidase- $\text{H}_2\text{O}_2\text{-Cl}^-$ system of phagocytes oxidizes α -amino acids to form aldehydes.

Intermediates with UV Spectral Features Consistent with a Monochloramine Are Precursors for α -Amino Acid-derived Aldehydes—The labile intermediates formed during HOCl-mediated oxidation of α -amino acids are stabilized at low temperatures and have UV spectral features consistent with an α -monochloramine; aldehydes were also formed when these reaction mixtures were warmed (Table I). Direct demonstration of this precursor-product relationship was achieved by isolating the unstable intermediate and demonstrating its conversion into an aldehyde. L-Tyrosine was first cooled to 0 °C, and aliquots of HOCl were added. Examination of the reaction products by reverse-phase HPLC at 0 °C demonstrated a product whose retention time was longer than that for the anticipated end product of the reaction, pHA (Fig. 3A). UV difference spectroscopy of the intermediate demonstrated an absorbance band at 254 nm, suggesting the formation of an α -monochloramine. A precursor-product relationship between the presumed α -monochloramine intermediate and pHA was confirmed by gradually warming the isolated intermediate and subjecting the solution to reverse phase HPLC analysis. As the putative α -monochloramine disappeared, the aldehyde, pHA, was formed (Fig. 3B). A parallel set of experiments with L-[¹⁴C]tyrosine and scintillation counting demonstrated that production of the presumed α -monochloramine intermediate from L-tyrosine and HOCl was nearly quantitative at 0 °C (Fig. 4, left panel); loss of the intermediate upon warming was accounted for by the generation of pHA (Fig. 4, right panel). Collectively,

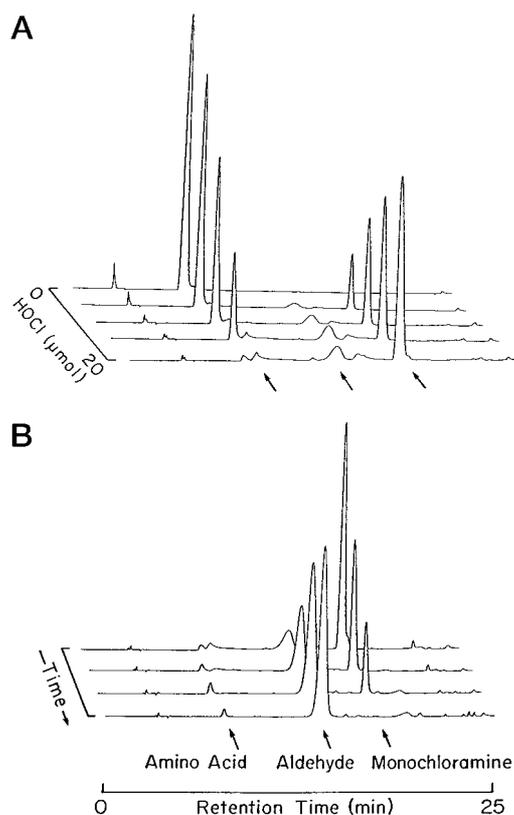


FIG. 3. Reverse phase HPLC analysis of L-tyrosine oxidation products generated by HOCl. A, 4 aliquots of HOCl (5 μmol) were added dropwise with constant stirring to L-tyrosine (20 μmol) in 10 ml of ice-cold buffer A. Following each addition, the reaction mixture was analyzed by reverse phase HPLC with the column and sample loop packed in ice. Addition of HOCl resulted in the near quantitative consumption of L-tyrosine yielding a product whose retention time was slightly longer than that for *p*-hydroxyphenylacetaldehyde. B, the ice-cold reaction mixture containing L-tyrosine α -monochloramine was warmed repeatedly to 37 $^{\circ}\text{C}$ for 10 min, cooled to 0 $^{\circ}\text{C}$ to halt monochloramine decomposition, and then analyzed by reverse phase HPLC as described above. Note the consumption of the L-tyrosine α -monochloramine and production of the aldehyde, *p*-hydroxyphenylacetaldehyde, upon sample warming. Identity of the intermediate as L-tyrosine α -monochloramine was confirmed utilizing multinuclear NMR spectroscopy as described under "Results."

these results demonstrate that oxidation of α -amino acids by myeloperoxidase-generated HOCl produces an unstable intermediate with UV spectral features consistent with an α -monochloramine. This labile intermediate then serves as a precursor for the ultimate generation of a reactive aldehyde.

NMR Spectroscopy Confirms That α -Monochloramines Are Intermediates in Aldehyde Formation during HOCl-mediated Oxidation of α -Amino Acids—Because UV difference spectroscopy cannot establish unequivocally the structure of a reaction intermediate, we utilized multinuclear NMR to confirm the structure of the thermally labile intermediate generated during incubation of an α -amino acid and HOCl. ^{15}N -Labeled L-tyrosine was employed since the presumed α -monochloramine of the aromatic amino acid is readily resolved at 0 $^{\circ}\text{C}$ on reverse phase HPLC (Fig. 3) and since the ^{15}N resonance position should serve as a non-perturbing and sensitive probe into the immediate chemical environment at the α -amino nitrogen atom. L- ^{15}N]Tyrosine was incubated with HOCl (1:1, mol/mol) in 50 mM sodium phosphate buffer (pH 7.0) at 0 $^{\circ}\text{C}$. The intermediate formed was then analyzed by ^1H NMR and $^1\text{H}, ^{15}\text{N}$ two-dimensional NMR spectroscopy. The chemical shifts, integrated areas, and coupling constants of resonances in the ^1H NMR spectrum of the compound all were consistent with the

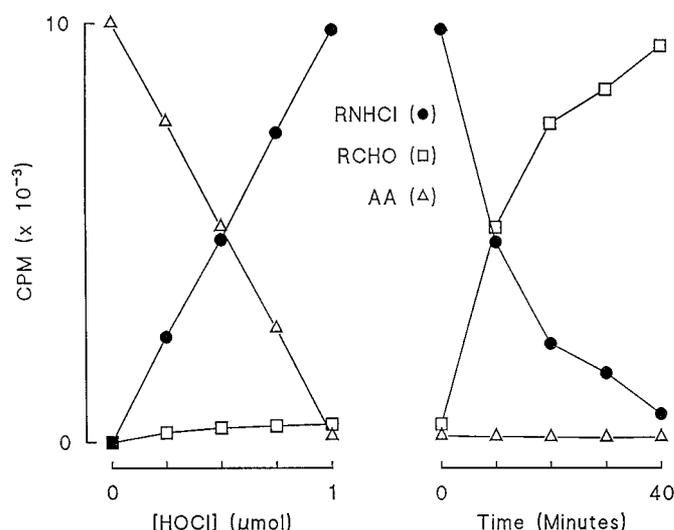


FIG. 4. Progress curve of L-tyrosine oxidation by HOCl. Aliquots of L- ^{14}C tyrosine (1 μmol) in buffer A (1 ml) were cooled to 0 $^{\circ}\text{C}$. The indicated amounts of HOCl were then added (left panel), and the reaction products were analyzed by reverse phase HPLC as described in the legend to Fig. 3. Fractions containing L-tyrosine (AA), L-tyrosine α -monochloramine (RNHCl), and *p*-hydroxyphenylacetaldehyde (RCHO) were collected, dried, and quantified by scintillation spectrometry. Right panel, L-tyrosine α -monochloramine was prepared at 0 $^{\circ}\text{C}$, warmed to 37 $^{\circ}\text{C}$ for the indicated times, and then rapidly cooled to 0 $^{\circ}\text{C}$. Products were then isolated by reverse phase HPLC and quantified by scintillation spectrometry as described in the legend to Fig. 3. Note that warming results in the loss of monochloramine and the near quantitative production of *p*-hydroxyphenylacetaldehyde.

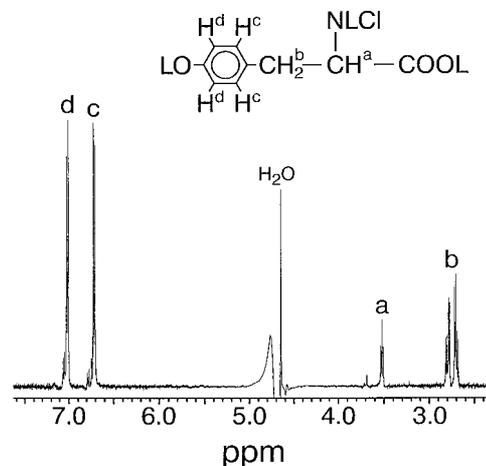


FIG. 5. ^1H NMR spectrum of L-tyrosine α -monochloramine. An ice-cold solution of L- ^{15}N]tyrosine (1.8 mM) in a 20 mM sodium phosphate buffer (pH 7.0), supplemented with D_2O (10%, v/v) was converted to its monochloramine at 0 $^{\circ}\text{C}$ as described in the legend to Fig. 3. The reaction mixture was then analyzed by ^1H NMR as described under "Experimental Procedures." To avoid temperature-dependent shifts in proton resonances, the spectrum was obtained immediately after warming the sample to 25 $^{\circ}\text{C}$. Peak assignments are depicted (inset). The benzylic protons (H^b) are not degenerate due to the distinct chemical environments generated by the adjacent chiral α -carbon. Protons not observed due to rapid exchange with solvent are designated (L). The spectral feature at ~ 4.7 ppm is an artifact resulting from suppression of the intense water signal.

formation of L-tyrosine α -monochloramine (Fig. 5). The chemical environment of the ^{15}N atom of the initial L- ^{15}N]tyrosine/HOCl oxidation product was interrogated by heteronuclear multiple bond correlation spectroscopy. The chemical shift of the ^{15}N resonance of the thermally labile intermediate was consistent with a monochloramine (data not shown); the electron withdrawing halide deshielded the ^{15}N atom, shifting the resonance to a characteristic downfield position. Thus,

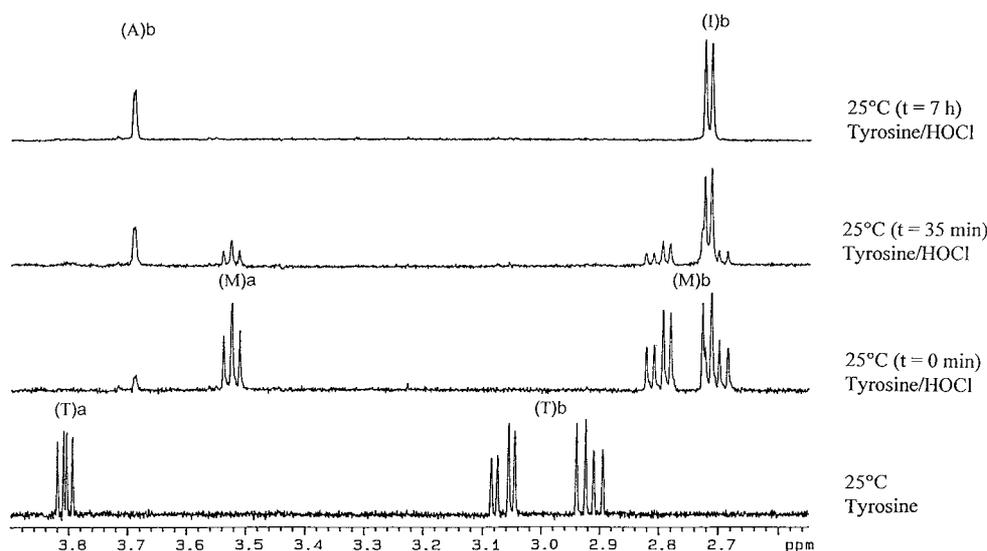


FIG. 6. ^1H NMR spectra of L-tyrosine and its HOCl oxidation products. The ^1H NMR spectra of L-tyrosine, L-tyrosine α -monochloramine, and the thermal decomposition products of L-tyrosine α -monochloramine are illustrated over the spectral region where benzylic protons (H^b ; Scheme III) and protons α to the benzylic protons (H^a ; Scheme III) are observed. L-Tyrosine (2 mM; 25 $^\circ\text{C}$) is depicted in the bottom spectrum. L-Tyrosine α -monochloramine was formed as described in the legend to Fig. 5, and its ^1H NMR spectrum obtained immediately following brief warming to 25 $^\circ\text{C}$ (to avoid temperature-dependent shifts observed in proton resonances). Prolonged warming (45 min) at 25 $^\circ\text{C}$ results in significant disappearance of the α -monochloramine and formation of both the aldehyde and another species (putative structures *I*) illustrated in Scheme III). The ^1H spectra obtained after incubation of the α -monochloramine at 25 $^\circ\text{C}$ for 7 h is also shown. Note that the aldehydic proton (9.58 ppm) of pHA is not observed in this spectral region but was observed to grow as the monochloramine disappeared (data not shown). Resonances in the full ^1H spectra for L-tyrosine (*T*), tyrosine α -monochloramine (*M*), pHA (*A*), and the compound(s) in equilibrium with pHA (*I*) are described in Table III. Resonance assignments are illustrated in Scheme III.

multinuclear NMR studies directly confirm that the α -monochloramine is a reaction intermediate in aldehyde formation.

To confirm a precursor-product relationship between L-tyrosine α -monochloramine and pHA, we performed an NMR experiment similar to the HPLC study described in Figs. 3 and 4. L-Tyrosine α -monochloramine was warmed from 0 to 25 $^\circ\text{C}$ in the NMR spectrometer with continuous ^1H spectra acquisition. A time- and temperature-dependent disappearance of the α -monochloramine was observed (Fig. 6). Disappearance of the α -monochloramine occurred in concert with the appearance of pHA and a second compound that exhibited distinct spectral features (Fig. 6).

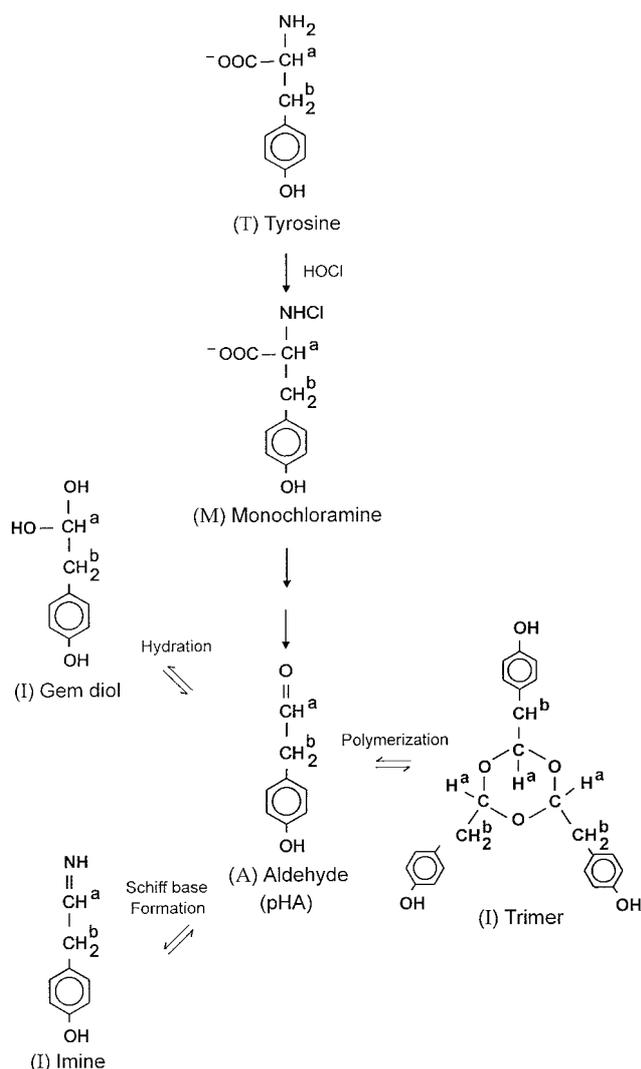
Aldehydes Generated during Amino Acid Oxidation by HOCl Exist in a Complex Equilibrium between Monomeric Aldehyde and Other Species Such as the Gem Diol, Aldehydic Condensation Products, and Schiff Base Adducts—Aldehydes react with amines to form Schiff bases; they also produce gem diols and condensation products in aqueous solution (31, 32). The ^1H NMR spectrum obtained following the thermal decomposition of tyrosine α -monochloramine (Fig. 6) suggested that the monomeric pHA product was in equilibrium with one or more of these species (termed *I* in Scheme III). To test this hypothesis, we used ^1H NMR to analyze the distribution of monomeric pHA and its equilibrium product(s) as a function of temperature. Warming the reaction mixture greatly increased the proportion of monomeric pHA, and cooling it increased the proportion of its equilibrium product(s), confirming the reversible nature of the equilibrium (Fig. 7). In another experiment similar to that shown in Fig. 3B, we monitored the ^1H NMR peaks that appeared as reagent HOCl oxidized L-tyrosine. A peak for α -monochloramine was clearly present, and it was replaced by a pHA peak as the reaction proceeded. Resonances in the full ^1H spectra for L-tyrosine (*T*), tyrosine α -monochloramine (*M*), pHA (*A*), and the compound(s) in equilibrium with pHA (*I*) are summarized in Table III. Collectively, these results demonstrate that the conversion of L-tyrosine to pHA involves a monochloramine intermediate. The resulting monomeric aldehyde then exists in equilibrium with a second compound or

compounds.

We used a variety of approaches to determine whether the Schiff base, the gem diol, or the trimer accounted for the compound that is in equilibrium with pHA (Scheme III). These species cannot be distinguished by ^1H NMR because the proton resonances at the benzylic and α -carbon positions of each compound possess nearly identical chemical shifts. However, even ^1H , ^{15}N two-dimensional NMR analysis failed to detect a ^{15}N correlation with the ^1H resonances attributed to the equilibrium compound(s). This suggests that either the Schiff base is not a component of the reaction mixture or that the heteronuclear coupling constants were too small for detection. We also examined the reaction products by electrospray ionization mass spectrometry, finding a major ion of m/z 136, the mass of monomeric pHA. Tandem mass spectrometry with collisional activated dissociation confirmed that the ion was the M^+ of pHA (data not shown). There was no evidence for ions derived from the three equilibrium products suggested in Scheme III, the Schiff base (gem diol or trimer) suggesting that these compounds, if produced, are thermally labile, even under the gentle ionization conditions of electrospray.

When we analyzed an aqueous solution of reagent phenylacetaldehyde, the aldehyde that forms when the myeloperoxidase system oxidizes phenylalanine (3), we detected ^1H NMR peaks corresponding to both the monomeric form of the aldehyde and a second compound. The latter had distinctive spectral features that resembled those of the benzylic and α -carbon proton resonances assigned to the compound in equilibrium with pHA (labeled *I* in Fig. 6 and Scheme III). Because phenylacetaldehyde has no amino groups available for Schiff base formation under these conditions, the equilibrium compound is likely to be a gem diol and/or trimer. ^1H NMR analysis of reagent phenylacetaldehyde or pHA extracted into an aprotic solvent (CDCl_3) also demonstrated spectral features consistent with the monomeric form of each aldehyde (data not shown). These observations again suggest that a gem diol and/or trimer are the equilibrium products.

To determine whether Schiff base formation may at least



SCHEME III. Potential species in equilibrium with pHA. Oxidation of tyrosine with HOCl generates an unstable α -monochloramine, which subsequently decomposes into the aldehyde pHA. Species in equilibrium with monomeric aldehyde in aqueous solution might include the hydrated form (gem diol), the Schiff base adduct (imine), or a polymerized form such as a trimer (31, 32). Benzylic protons (H^b) and α -carbon protons (H^a) are labeled to facilitate assignment of resonances in the 1H NMR spectra shown in Fig. 6 and are summarized in Table III.

partly account for the resonances observed in the 1H NMR spectrum of the compound(s) in equilibrium with pHA, we examined the effect on the 1H NMR spectrum of adding NH_4OH to phenylacetaldehyde in aqueous solution at neutral pH. Our rationale was that ammonia is present as an end product after HOCl oxidizes an amino acid to an aldehyde (18). We found that adding NH_4OH decreased the resonances attributed to monomeric aldehyde and increased the relative intensity of the non-monomeric 1H resonances (data not shown). These results strongly suggest that the 1H spectra of the compound(s) in equilibrium with monomeric aldehyde are nearly identical to that of an imine. It therefore appears that a complex equilibrium between a monomeric aldehyde, its gem diol, aldehydic condensation products, and Schiff base adducts exists in aqueous solution.

Halide Dependence of Glycolaldehyde Production from Serine by the Myeloperoxidase- H_2O_2 -Halide System—Myeloperoxidase and eosinophil peroxidase use Cl^- , Br^- , and I^- as substrates to generate a variety of hypohalous acids (1, 14, 33). Previous studies have suggested that Cl^- is the only halide

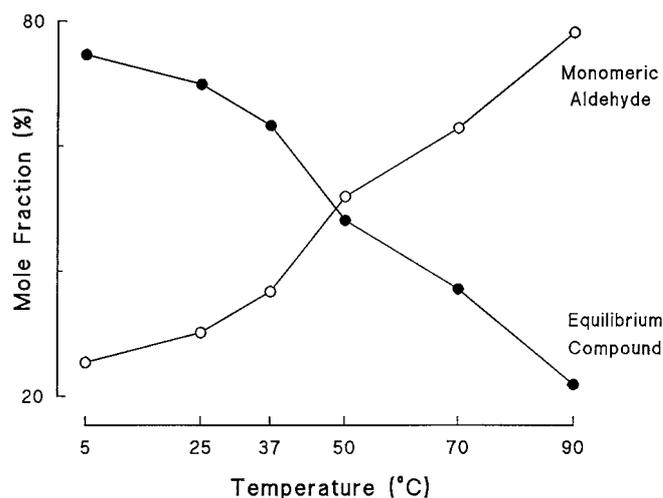


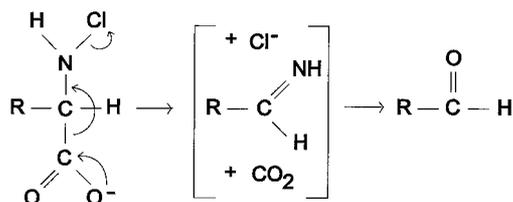
FIG. 7. Temperature dependence of equilibrium between monomeric pHA and other species formed upon α -monochloramine decomposition. L-Tyrosine α -monochloramine was prepared as described in the legend to Fig. 5 and incubated for 7 h at 25 °C. Under these conditions, pHA and a second compound(s) (termed "equilibrium compound") were apparent by 1H NMR spectroscopy with monitoring of their benzylic proton resonances (H^b ; see Scheme III and Fig. 6). The temperature of the reaction mixture varied between 5 and 90 °C with continuous monitoring of the relative mole fraction of each species. Quantitation of the monomeric aldehyde and the equilibrium species was performed by integration of their benzylic proton resonances (H^b ; Scheme III).

TABLE III
 1H NMR spectral features of L-tyrosine and its HOCl oxidation products

The 1H NMR spectra of L-tyrosine and its HOCl oxidation products (Figs. 6 and 7) are described. Samples were prepared as described in the legend to Fig. 7. All spectra were performed at 25 °C as described under "Methods." d, doublet; t, triplet; and dd, doublet-doublet.

	Benzylic protons		Protons α to benzylic protons		Aromatic protons	
L-Tyrosine (T)						
Chemical shift (ppm)	2.92	3.07	3.81	6.76	7.06	
Multiplicity	dd	dd	dd	d	d	
Coupling constant (Hz)	14.5, 7.8	14.5, 5.3	5.3, 7.8	8.5	8.5	
Monochloramine (M)						
Chemical shift (ppm)	2.71	2.80	3.52	6.72	7.02	
Multiplicity	dd	dd	dd	d	d	
Coupling constant (Hz)	14.2, 7.4	14.2, 6.6	6.6, 7.4	8.5	8.5	
Monomeric Aldehyde (A)						
Chemical shift (ppm)		3.69	9.58	6.78	7.03	
Multiplicity		d	t	d	d	
Coupling constant (Hz)		1.5	1.5	7.9	7.9	
Equilibrium compound (I)						
Chemical shift (ppm)		2.72	5.07	6.74	7.06	
Multiplicity		d	t	d	d	
Coupling constant (Hz)		5.9	5.9	7.94	7.94	

with which myeloperoxidase converts L-tyrosine into pHA, however (34). To explore this halide specificity in greater detail, we studied the oxidation of L-serine by the enzyme. HOCl generated by myeloperoxidase converts L-serine to glycolaldehyde, an α -hydroxy aldehyde lacking functional groups that can scavenge reactive chlorinating species (26). We quantified this aldehyde by HPLC following derivatization with 3-methyl-2-benzothiazolinone hydrazone hydrochloride (26). Exposing L-serine to either the myeloperoxidase- H_2O_2 - Br^- system or hypobromous acid, the primary oxidant of eosinophil peroxidase (33, 35, 36), generated glycolaldehyde (data not shown). In contrast to HOCl (26, 34), hypobromous acid generated aldehyde from L-serine only after prolonged incubation (>1 h at 37 °C). Other halides (I^- , F^-) or the pseudohalide SCN^- failed



SCHEME IV. Proposed reaction mechanism for conversion of an α -monochloramine into an aldehyde via an imine.

to generate glycolaldehyde in this system, despite incubations of up to 24 h at 37 °C (data not shown). These results suggest that hypobromous acid reacts with amino acids to form an α -monobromamine intermediate that is more stable to deamination and decarboxylation than its α -monochloramine analogue.

DISCUSSION

We have recently demonstrated that human neutrophils use the myeloperoxidase- H_2O_2 - Cl^- system to convert common α -amino acids to an array of reactive aldehydes (3). Here, we provide evidence for the mechanism of aldehyde production. Free primary amino and carboxylic acid groups on the α -carbon of an amino acid were required for aldehyde generation. Mass spectrometric studies with isotopically labeled amino acids demonstrated that hydrogen atom abstraction from the α -carbon (Scheme I, *Pathway A*), a mechanism described for aldehyde formation by ionizing radiation (4–9) and Fenton systems (9–13), did not occur. Rather, several independent analytical methods (UV spectroscopy and ^1H and ^1H , ^{15}N two-dimensional NMR spectroscopies) indicate that chlorination of the α -amino group by HOCl (a product of the myeloperoxidase pathway) appears to be the initial event. This generates a labile α -monochloramine which rapidly decomposes to an aldehyde (Scheme I, *Pathway B*). Examination of HOCl oxidation products of multiple α -, β -, and ϵ -amino acids confirmed that α -monochloramines, but not β - or ϵ -monochloramines, are labile.

We were unable to confirm the structural identity of a stable intermediate between the α -monochloramine and aldehyde for any of the amino acids examined. A likely explanation is that the half-lives of the intermediates are too short for detection under the methods employed. For example, a short-lived imine might form during monochloramine decomposition directly in a single step by the concerted loss of CO_2 and Cl^- ; subsequent loss of ammonia would then yield an aldehyde. A plausible mechanism for this rearrangement is illustrated in Scheme IV. Indeed, a short-lived imine has been proposed as a reaction intermediate in the oxidation of amino acids by ionizing radiation (Scheme I; Ref. 9). Our identification of α -monochloramines as reaction intermediates in myeloperoxidase-mediated oxidation of α -amino acids suggests that these labile intermediates could also have biological functions at sites of inflammation. By analogy, this may also be true for α -monobromamines formed by either myeloperoxidase or eosinophil peroxidase, particularly at sites of an allergic inflammatory response where an eosinophilic infiltrate is a characteristic finding (37).

The ^1H NMR studies clearly suggest that aldehydes formed by the myeloperoxidase system *in vivo* are likely to exist in equilibrium with hydrated and condensed forms (31, 32). Aldehydes are also likely to interact with thiols and primary amino groups, generating an array of Michael adducts (32) and Schiff bases (9, 31, 32). We recently have identified the reduced Schiff base adduct between pHA and the ϵ -amino groups of protein lysine residues in inflamed human tissues (34). It is likely that adducts between other amino acid-derived aldehydes and proteins, lipids, and nucleic acids will also be found. Autoantibod-

ies that recognize proteins modified by acrolein, an α - β unsaturated aldehyde formed when myeloperoxidase oxidizes L-threonine (26), are present in animals and humans with atherosclerosis (38, 39). Anti-acrolein antibodies also recognize epitopes in atherosclerotic lesions and in lipoproteins recovered from human aorta (38, 40), suggesting that the myeloperoxidase system generates reactive aldehydes in the artery wall. Indeed, the enzyme is abundant and catalytically active in human atherosclerotic lesions (41, 42). The selective reactivity of aldehydes, and their ability to form covalent adducts, suggests that amino acid-derived aldehydes generated at sites of inflammation may damage cellular targets. The reversible nature of many of these adducts also may serve to prolong their half-lives. Because reversible covalent modification has served as a paradigm for biological signaling processes, labile α -monochloramines and amino acid-derived aldehydes have the potential to serve as signaling molecules at sites of inflammation, perhaps to coordinate the oxidative capacity of phagocytes with the response of other immune cells of the host's defense system.

Collectively, these observations identify the reaction mechanism for aldehyde formation by HOCl and strongly suggest that free α -amino acids, which are present at a concentration of 4–5 mM in plasma and even higher intracellularly (24, 25), may be major substrates for oxidation by activated phagocytes. The ability of aldehydes to react with nucleophilic moieties on proteins, lipids, and DNA suggests that the generation of such species may represent an important mechanism for damage of biological targets and the transduction of biological signals at sites of inflammation.

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