Human Neutrophils Employ Myeloperoxidase To Convert α-Amino Acids to a Battery of Reactive Aldehydes: A Pathway for Aldehyde Generation at Sites of Inflammation†

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ABSTRACT: We have recently demonstrated that activated phagocytes employ the heme protein myeloperoxidase, H_2O_2, and Cl^- to oxidize the aromatic amino acid L-tyrosine to the reactive aldehyde *p*-hydroxyphenylacetaldehyde. We now present evidence for the generality of this reaction by demonstrating that neutrophils employ the myeloperoxidase-H_2O_2-Cl^- system to oxidize nearly all of the common α-amino acids to yield a family of reactive aldehydes. Chemical characterization suggested that reactive carbonyl moieties were generated during amino acid oxidation by myeloperoxidase. The structures of amino-acid-derived aldehydes were confirmed using a variety of mass spectrometric methods. Aldehyde production required myeloperoxidase, H_2O_2, Cl^-, and an amino acid; it was inhibited by heme poisons and catalase. Hypochlorous acid was the apparent oxidizing intermediate because its addition to α-amino acids resulted in the formation of the anticipated aldehyde. Stimulated human neutrophils likewise generated aldehydes from all classes of α-amino acids by a pathway inhibited by heme poisons and catalase, implicating myeloperoxidase and H_2O_2 in the cell-mediated reaction. Aldehyde production accounted for a significant fraction of the H_2O_2 generated by stimulated neutrophils at physiological concentrations of amino acids. Collectively, these results suggest that amino-acid-derived aldehydes represent a product of reactive oxidant species generated by activated phagocytes.

Reactive aldehydes have been implicated in a wide range of physiological and pathological processes. These activities are likely derived from the selective reactivity of the aldehyde carbonyl with target molecules containing nucleophilic groups, such as thiol and amino moieties (*1*−*4*). For example, 4-hydroxy-2-nonenal, a cytotoxic decomposition product of lipid peroxidation that possesses a reactive α,*β*-unsaturated aldehyde moiety, spontaneously forms Michael adducts with sulfhydryl, histidyl, and lysyl residues (*1*, *2*, *5*, *6*). Similarly, reducing sugars such as glucose covalently modify ϵ- and α-amino groups of proteins to generate Schiff base adducts that may subsequently rearrange to form the Amadori product (*3*, *7*−*10*). Advanced glycation end products derived from this reaction are implicated in the pathogenesis of diabetic vascular disease (*3*, *9*, *10*).

In vivo, aldehydic products are believed to result from reducing sugars, a variety of metabolic pathways, and lipid peroxidation decomposition products (*1*−*4*, *9*, *10*). We have demonstrated that another pathway may involve activated phagocytes, which employ the heme enzyme myeloperoxidase, H_2O_2, and Cl^- to oxidize the aromatic amino acid L-tyrosine, yielding the aldehyde *p*-hydroxyphenylacetaldehyde (*11*). The reaction, which is mediated by HOCl[1] and proceeds readily at neutral pH, accounts for a significant fraction of H_2O_2 generated by optimally stimulated phagocytes under physiological concentrations of L-tyrosine and Cl^- (*11*). We recently showed that this amphipathic aldehyde covalently modifies soluble and membranous proteins of intact cells and is generated in vivo at sites of inflammation (*12*).

Oxidation of aromatic amino acids by high concentrations of HOCl under strongly acidic conditions results in formation of nitriles and trace quantities of chlorinated aldehydes (*13*). Indirect evidence suggests that reactive carbonyls form from common amino acids exposed to myeloperoxidase-generated HOCl (*14*, *15*). HOCl-mediated oxidation of amino acids results in release of ammonia and carbon dioxide from the parent compound (*14*−*16*). However, direct structural identification of the products generated in these reactions is

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[1] Abbreviations: DNPH, 2,4 dinitrophenylhydrazine; DTPA, diethylenetriaminepentaacetic acid; GC, gas chromatography; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; HPLC, high performance liquid chromatography; MS, mass spectrometry; *m*/*z*, mass-to-charge-ratio; $M^{\bullet -}$, molecular anion; $M^{\bullet +}$, molecular cation; NCI, negative-ion chemical ionization; $O_2^{\bullet -}$, superoxide anion; PFB, pentafluorobenzyl; PFBHA, pentafluorobenzylhydroxylamine.

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still lacking. We recently examined the products of myeloperoxidase-catalyzed oxidation of the aliphatic alcoholic amino acids L-serine and L-threonine and demonstrated that α-hydroxy and α,β-unsaturated aldehydes were formed (*17*). The high yield of aldehyde formation during oxidation of L-tyrosine, L-serine, and L-threonine by myeloperoxidase-generated HOCl suggested that other classes of common amino acids might similarly be oxidized to their corresponding aldehyde during phagocyte activation.

Here, we demonstrate that nearly all of the common α-amino acids serve as substrates for the generation of reactive aldehydes by activated human neutrophils through the myeloperoxidase-H_2O_2-Cl^- system. Structures of the amino-acid-derived aldehydes were confirmed using a variety of mass spectrometric methods. In another report (*18*), we present evidence that the reaction mechanism involves initial chlorination of the α-amino group, forming an unstable α-monochloramine intermediate that then decomposes to an aldehyde. These results suggest that phagocytes employ myeloperoxidase to oxidize common α-amino acids into a family of freely diffusible aldehydes.

EXPERIMENTAL PROCEDURES

Materials. HPLC solvents were purchased from Baxter (McGaw Park, IL). Sodium phosphate, ethyl acetate, H_2O_2, and sodium hypochlorite were obtained from Fisher Scientific (St. Louis, MO). Boehringer Mannheim (Indianapolis, IN) provided crystalline catalase (from bovine liver, thymol-free). D_2O and L-[D_8]phenylalanine were purchased from Cambridge Isotopes, Inc. (Andover, MA). Glycolaldehyde was purchased from Fluka Chemical Co. (Ronkonkoma, NY). Amino acids (in free base form) and all other materials were purchased from Sigma Chemical Co. (St. Louis, MO), except where indicated.

Methods. *General Procedures*. All glassware was rendered chlorine-demand free (*19*−*22*) by soaking in NaOCl, extensive rinsing with H_2O, and pyrolysis at 500 °C for >12 h. H_2O was double glass distilled and stored in glassware to avoid low levels of contaminating short-chain aldehydes shed from plastic and deionizing cartridges. Buffers and reagents were prepared and stored in pyrolized glassware capped with Teflon-lined caps. Buffers were demonstrated to be chlorine-demand free (defined as consumption of <3% of 1 mM HOCl in a 10 min incubation at 37 °C within a gastight vial as monitored by the oxidation of iodide to triiodide; ref *22*). Myeloperoxidase (A_{430}/A_{280} ratio of 0.6) was isolated and stored as previously described (*23*, *24*). Enzyme concentration was determined spectrophotometrically (A_{430} = 170 mM^{-1} cm^{-1}; ref *25*). Amino-acid-derived aldehydes were derivatized with 2,4-dinitrophenylhydrazine (DNPH) and subjected to Tollen's Test as described (*26*, *27*). H_2O_2 and NaOCl concentrations were determined spectrophotometrically (A_{240} = 39.4 M^{-1} cm^{-1} and A_{292} = 350 M^{-1} cm^{-1}, respectively; refs *28* and *29*).

Synthesis and Derivatization of Aldehydes. Initial structural and chromatographic characterization of aldehydes was performed using amino acids oxidized with HOCl/ClO^-. NaOCl (1:1, mol:mol, oxidant:amino acid) was added dropwise with constant mixing to ice-cold solutions of each amino acid (2 mM) in sodium phosphate buffer (50 mM, pH 7.0) in sealed vials. Reaction mixtures were then incubated at 37 °C for 1 h. Throughout sample processing, care was taken to avoid losses of volatile aldehydes and derivatives through use of gastight syringes, septa-covered reaction vials, and refrigeration or freezing of samples until analyses were complete. [D_8]Phenylacetaldehyde was synthesized from L-[D_8]phenylalanine with reagent NaOCl to serve as an internal standard for mass spectrometric analyses. Internal standard was added to samples immediately prior to derivatization to correct for potential intersample variations in derivatization and extraction efficiencies.

Amino acids were incubated with the myeloperoxidase-H_2O_2-Cl^- system, or in the presence of human neutrophils, at 37 °C for 60 min as described in the figure and table legends. Reaction products were derivatized with freshly prepared pentafluorobenzylhydroxylamine (PFBHA; 100 mM in H_2O). This derivatization method readily reverses Schiff bases and other reversible covalent adducts (e.g., with thiol residues) forming a stable oxime (*26*, *27*). The final concentration of PFBHA was 4 mM for NaOCl and the purified myeloperoxidase system or 10 mM for neutrophil experiments. NaOH (10 N stock) was then added to a final concentration of 1 N, and the mixture incubated at 65 °C for 1 h in gastight vials. For HPLC analysis, samples were acidified with a 1.1 molar excess (relative to NaOH) of trifluoroacetic acid to permit binding to the C18 matrix and then stored in sealed vials until analysis. Samples analyzed by mass spectrometry were first extracted with ethyl acetate (1:1; vol/vol). The remaining aqueous phase was then acidified with concentrated HCl (final concentration 2 N) and again extracted with ethyl acetate as above. The combined organic extracts were stored in gastight vials until analysis.

Neutrophil Studies. Human neutrophils were isolated by buoyant density centrifugation from plasma anticoagulated with EDTA (5 mM) as described (*24*). Experiments were performed in Hank's Balanced Salt Solution (magnesium-, calcium-, phenol-, and bicarbonate-free; Gibco-BRL) supplemented with 100 μM diethylenetriaminepentaacetic acid (DTPA). Superoxide ($O_2^{\bullet -}$) generation by activated human neutrophils was measured as the superoxide dismutase-inhibitable (10 μg/mL) reduction of ferricytochrome *c* (*30*). Conditions were identical to those employed for amino-acid-derived aldehyde production except that amino acids were omitted and cytochrome *c* (1 mg/mL) was included in the medium. Amino acid oxidation experiments with human neutrophils were performed in gastight vials to prevent loss of volatile aldehydes generated during the prolonged neutrophil incubations. Following incubation, reactions products were derivatized with PFBHA as described above.

High Performance Liquid Chromatography (HPLC). Pentafluorobenzyl (PFB)-oximes of amino-acid-derived aldehydes were analyzed by HPLC using a reversed-phase C18 column (Beckman μPorasil 5 μm resin, 4.6 × 250 mm) equilibrated with solvent A (5% methanol and 0.1% TFA, pH 2.5). Derivatized aldehydes were eluted at a flow rate of 1 mL/min with a nonlinear gradient generated with solvent B (100% methanol and 0.1% TFA, pH 2.5) as follows: 0% B for 5 min; 0−40% solvent B over 5 min; 40−100% solvent B over 40 min; and 100% solvent B for 10 min. Products were monitored spectrophotometrically at 256 nm.

Preliminary experiments utilizing authentic acetaldehyde, acrolein, and glycolaldehyde revealed near identical ultraviolet absorption spectra and extinction coefficients for their

PFB-oxime derivatives. We therefore routinely quantified nonaromatic amino-acid-derived aldehydes by integration of peak absorbance (A_{256}) of the oxime derivatives employing standard curves constructed with the nonvolatile aldehyde glycolaldehyde. *p*-Hydroxyphenylacetaldehyde, the aldehyde derived from L-tyrosine oxidation, was quantified by reversed-phase HPLC as previously described (*11*). Yields of aldehydes derived from the aromatic amino acids L-phenylalanine, L-histidine, and L-tryptophan were estimated by HPLC with monitoring of A_{276}, A_{220}, and A_{276}, respectively, of nonderivatized oxidation products employing standard curves generated from the parent amino acid. These results were in good agreement with HPLC quantification of the amino-acid-derived aldehydes as their DNPH derivatives monitored spectrophotometrically ($\epsilon = 22\,000\ M^{-1}\ cm^{-1}$ at 375 nm; ref *31*).

Gas Chromatography/Mass Spectrometry. Gas chromatography/mass spectrometry (GC/MS) experiments were performed on a Hewlett-Packard 5890 Gas Chromatograph interfaced with a Hewlett-Packard 5988A Mass Spectrometer equipped with extended mass range and a Technivent Vector 1 data system. Electron ionization GC/MS analysis of amino-acid-derived aldehydes (freshly prepared and extracted into ethyl acetate) were carried out utilizing a Restek RTX-200 column (15 m, 0.33 mm i.d., 1 μm film thickness) in the splitless mode with helium as the carrier gas. The column was run with the following temperature gradient: 70 °C to 150 °C at 10 °C/min and 150 °C to 280 °C at 20 °C/min. Gas chromatographic separation of DNPH and PFB-oxime derivatives of aldehydes were also conducted on a Restek RTX-200 column (15 m, 0.33 mm i.d., 1 μm film thickness). The initial column temperature of 70 °C was increased to 100 °C at 10 °C/min. The column temperature was then raised to 280 °C at the rate of 20 °C/min. Identification of PFB-oxime derivatives of aldehydes was confirmed by demonstrating comigration of at least two structurally informative ions and one common ion with an identical retention time to that observed with authentic derivatized material (commercial or generated with HOCl) in the selected ion monitoring mode.

RESULTS

Mass Spectrometric Detection of Aldehydes Derived from Myeloperoxidase-Catalyzed Oxidation of Aromatic and Aliphatic Amino Acids. We have recently shown that phenolic and alcoholic amino acids are converted into aldehydes by myeloperoxidase (*11*, *17*). To determine whether other classes of amino acids might serve as substrates for the generation of reactive aldehydes, we incubated the aromatic amino acid L-phenylalanine and the aliphatic amino acid L-isoleucine individually with the myeloperoxidase-H_2O_2-Cl^- system. The reaction products were then extracted into organic solvent and subjected to electron ionization mass spectrometry, which provides many structurally informative ions. Oxidation of both L-phenylalanine and L-isoleucine by the myeloperoxidase-H_2O_2-Cl^- system yielded the anticipated aldehyde (Figures 1 and 2, respectively). In both cases, the reaction required active myeloperoxidase, H_2O_2, and Cl^-. Aldehyde formation was inhibited by the addition of heme poisons (azide or cyanide) or an H_2O_2 scavenger (catalase).

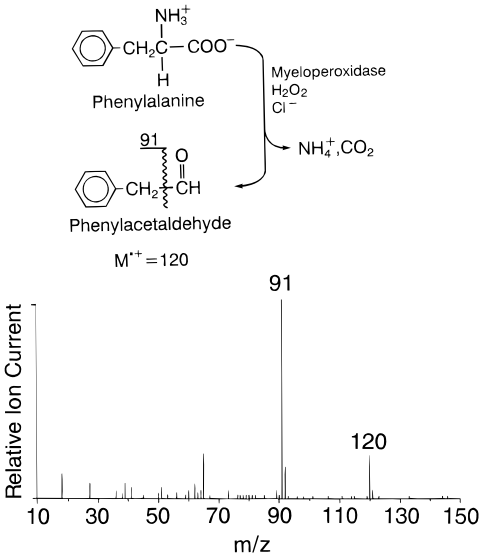


FIGURE 1: Electron ionization mass spectrum of the aldehyde derived from an aromatic amino acid. L-Phenylalanine (200 μM) was incubated with myeloperoxidase (6 nM), H_2O_2 (200 μM), and NaCl (100 mM) in 50 mM sodium phosphate (pH 7.0) within a gastight reaction vial at 37 °C for 30 min. Reaction products were extracted into ethyl acetate (1:1,vol/vol) and analyzed by electron ionization mass spectrometry as described in the Experimental Procedures. Ions in the mass spectrum consistent with the proposed structure of phenylacetaldehyde are shown in the inset. $M^{\bullet+}$, molecular cation.

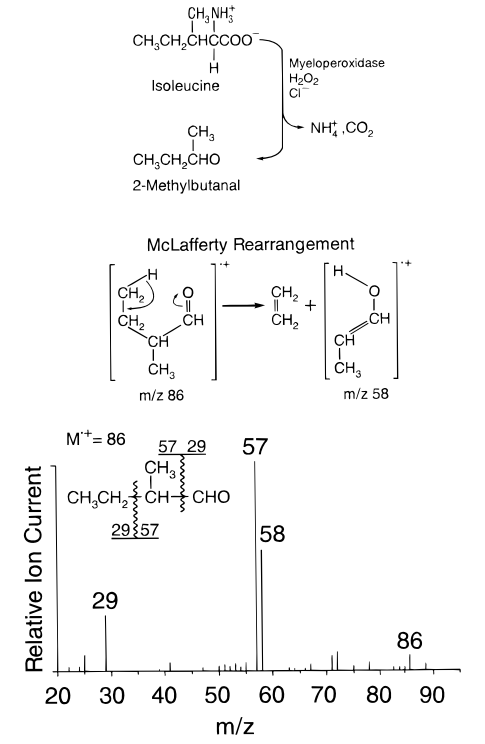


FIGURE 2: Electron ionization mass spectrum of the aldehyde derived from an aliphatic amino acid. L-Isoleucine (200 μM) was oxidized with the myeloperoxidase-H_2O_2-Cl^- system and analyzed by electron ionization mass spectrometry as described in the legend to Figure 1. Ions observed in the mass spectrum consistent with the proposed structure of 2-methylbutanal are shown in the inset.

Myeloperoxidase-Generated HOCl Oxidizes All Classes of Amino Acids To Form Products with Reactive Carbonyl Moieties. In a similar set of experiments, each of the 20 common α-amino acids (1 mM) was individually incubated at 37 °C for 1 h with myeloperoxidase (20 nM), H_2O_2 (100

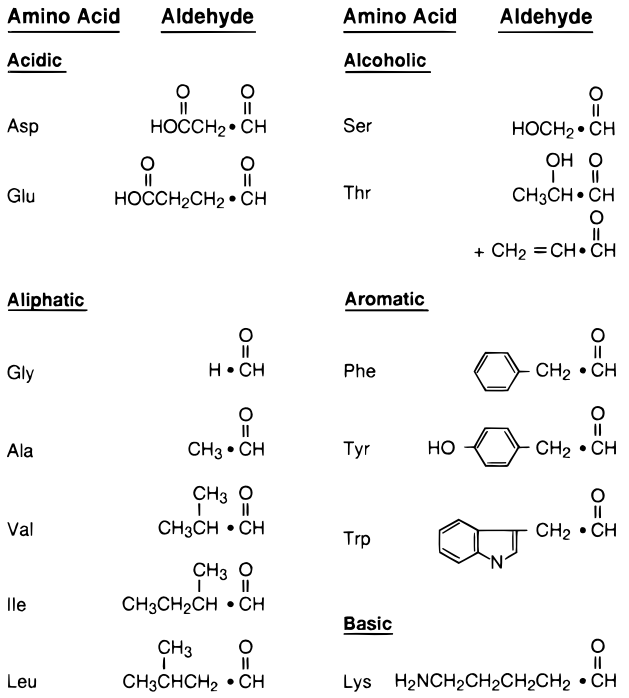
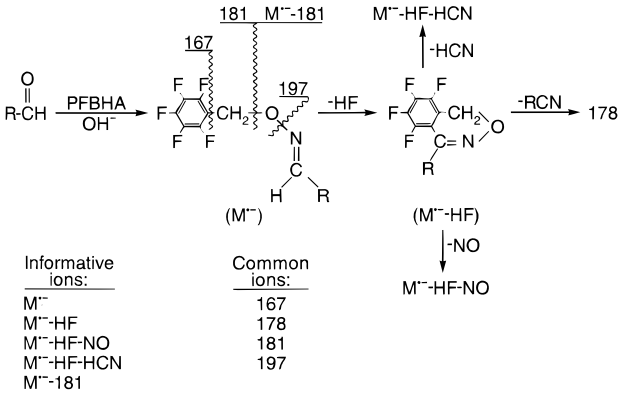


FIGURE 3: Proposed structures of amino acid oxidation products generated by the myeloperoxidase-H_2O_2−Cl^- system.

Scheme 1: Common Ions and Structurally Informative Ions Derived from the PFB-Oxime Derivatives of Aldehydes[a]



[a] The proposed structures of the major ions observed in the electron capture NCI mass spectra and the mechanism of their formation are illustrated. Linked-scanning experiments, collisionally activated dissociation tandem mass spectrometry, and high-resolution molecular mass determination support the proposed structures and will be reported elsewhere (F. F. Hsu, S. L. Hazen, M. L. Gross, J. L. Turk and J. W. Heinecke, submitted). The common ions at *m*/*z* 181 or 178, which represent the base peak of the majority of the derivatized aldehydes, were used for the initial detection of amino acid-derived aldehydes by selected ion monitoring. The presence of each amino acid-derived aldehyde was then confirmed by demonstrating co-migration of two or more structurally informative ions. Descriptions of the mass spectra, along with retention times for each PFB-oxime of the amino acid-derived aldehydes identified are listed in Table 3. Structures of the proposed amino acid-derived aldehydes are shown in Figure 3. $M^{\bullet -}$, molecular anion; R, amino acid functional group.

μM), and NaCl (100 mM) in 50 mM sodium phosphate (pH 7.0). A Tollen's Test, a qualitative assay of carbonyl groups (*27*), was then performed on each of the reaction mixtures. With the exception of the thiol-containing amino acids cysteine and methionine and the imino acid proline, all of the reaction mixtures were positive. In a parallel set of experiments, the same reaction mixtures were derivatized with DNPH, a carbonyl-derivatizing agent, and analyzed spectrophotometrically for hydrazone formation (*31*). Again, all of the common α-amino acids—except cysteine, methionine, and proline—generated a reactive carbonyl when oxidized with myeloperoxidase, H_2O_2, and Cl^-. Comparable results were observed when the amino acids were oxidized with HOCl rather than the complete enzyme system, implicating HOCl in the reaction pathway.

Table 1: Yield of PFBHA-Reactive Products Generated during α-Amino Acid Oxidation by the Myeloperoxidase-H_2O_2-Cl^- System[a]

amino acid	product (nmol)
acidic	
Asp	88
Glu	92
alcoholic	
Ser	87
Thr[b]	90
aliphatic	
Gly	94
Ala	92
Val	96
Leu	89
Ile	87
amide	
Asn[c]	23
Gln[c]	34
aromatic	
Phe	98
Tyr	96
Trp	80
basic	
Arg[c]	72
His[c]	34
Lys	68
thiol	
Cys	<1
Met	<1
imino acid	
Pro	<1

[a] Amino acids (100 nmol) were incubated individually in gastight vials (1 mL) in the presence of myeloperoxidase (20 pmol), H_2O_2 (100 nmol), and NaCl (100 μmol) in sodium phosphate buffer (50 mM; pH 7.0) for 60 min at 37 °C. Products were then derivatized with PFBHA and quantified by reverse phase HPLC as described in the Experimental Procedures. Results are the mean of duplicate determinations (range of <6% of the mean). Similar results were observed in three independent experiments. [b] Combined yield of 2-hydroxypropanal and acrolein PFB-oximes. [c] Structures of these PFBHA-derivatives were difficult to establish by GC/MS (see Results).

Reactive Aldehydes Are Major Products of Amino Acids Oxidized by the Myeloperoxidase-H_2O_2-Cl^- System. Structural characterization and quantification of amino-acid-derived aldehydes in their nonderivatized form proved problematic. Many of the proposed amino acid oxidation products (Figure 3) were anticipated to be highly volatile, while others (aldehydes possessing additional polar functional groups) should partition inefficiently into the organic solvents required for GC/MS studies. To overcome these difficulties, the products were converted to their PFB-oximes with PFBHA, a carbonyl-specific derivatizing reagent (*26*, *27*). The aromatic PFB group then served both as a chromophore for HPLC quantification and as an excellent electron-capturing group for structural characterization using electron capture negative-ion chemical ionization (NCI) GC/MS (Scheme 1).

Table 2: Reaction Requirements for Aldehyde Generation by the Myeloperoxidase- H_2O_2-Cl^- System[a]

reaction condition	aldehyde (nmol)
complete MPO system	
MPO + amino acids + H_2O_2 + Cl^-	61
complete MPO system *minus*	
Cl^-	<1
H_2O_2	<1
MPO	<1
complete MPO system *plus*	
NaN_3	3.3
aminotriazole	1.8
catalase	<1
lactoperoxidase + amino acids + H_2O_2 + Cl^-	<1
HRP + amino acids + H_2O_2 + Cl^-	<1
HOCl + amino acids	55

[a] The reaction mixture (complete system; 1 mL) contained equimolar quantities (5 nmol each; 100 nmol total) of each of the 20 common α-amino acids in sodium phosphate buffer (50 mM; pH 7.0) supplemented with myeloperoxidase (20 pmol), H_2O_2 (100 nmol), and NaCl (100 μmol). Reactions were performed at 37 °C for 60 min within gastight vials. Aldehydes were then converted to their PFB-oximes and quantified by reverse phase HPLC as described in the Experimental Procedures. Where indicated, either NaN_3 (1 mM), aminotriazole (10 mM), catalase (20 μg/mL, 200 U/ml), lactoperoxidase (10 μg/mL, 2 units/mL) or horseradish peroxidase (10 μg/mL, 10 units/mL) were included, or Cl^-, H_2O_2, or myeloperoxidase were omitted from the complete system. HOCl (100 nmol), when present, replaced the MPO−H_2O_2−Cl^- system. HRP, horseradish peroxidase; MPO, myeloperoxidase. Results are the mean of duplicate determinations (range of <8% of the mean). Similar results were observed in two independent experiments.

Incubation of each of the 20 common α-amino acids with the myeloperoxidase-H_2O_2-Cl^- system, followed by HPLC analysis of the PFBHA-derivatized reaction products, revealed that the majority of amino acids were oxidized to form products capable of modification by PFBHA (Table 1). These results were consistent with the formation of aldehydic oxidation products, which subsequently were detected and quantified as their PFB-oxime. GC/MS studies of the HPLC-purified PFB-oxime products confirmed the structures of the majority of the aldehydes formed (see below). For the acidic, alcoholic, aliphatic, and aromatic amino acids, yield of the aldehyde was nearly quantitative with respect to moles of oxidizing substrate (H_2O_2) present in the reaction mixture (Table 1). Smaller yields were observed with basic and amide-containing amino acids, which possess alternative functional groups sensitive to oxidation by HOCl (e.g., amines and amides yield monochloramines and monochloramides, respectively; ref *19*). Neither L-cysteine nor L-methionine was converted to products capable of PFB-oxime formation as long as they

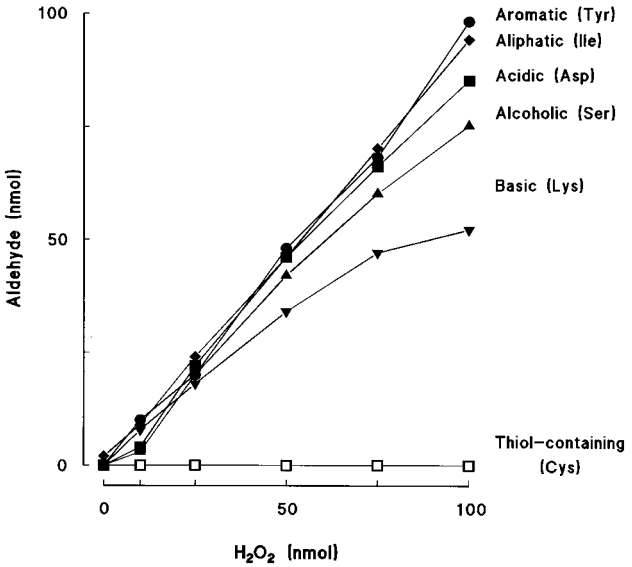


FIGURE 4: Yield of different classes of amino acid-derived aldehydes produced by the myeloperoxidase-H_2O_2-Cl^- system. Amino acids (200 nmol) from each of the indicated classes [(■) acidic, L-aspartic acid; (▲) alcoholic, L-serine; (◆) aliphatic, L-isoleucine; (●) aromatic, L-tyrosine; (▼) basic, L-lysine; or (□) thiol-containing, L-cysteine) were individually incubated in gastight vials (1 mL final volume) with varying amounts of H_2O_2 (0−100 nmol), myeloperoxidase (20 pmol), and NaCl (100 μmol) in sodium phosphate buffer (20 mM; pH 7.0) for 60 min at 37 °C. Aldehydes were then converted to their PFB-oxime derivatives and quantified by reverse phase HPLC as described in the Experimental Procedures. Results are the mean of duplicate determinations (range of <6% of the mean). Similar results were observed in two independent experiments.

Table 3: Electron Capture NCI Mass Spectra and Retention Times of Pentafluorobenzyloxime Derivatives of Aldehydes[a]

		structurally informative ions, *m/z* (abundance)[c]			common ions (abundance)[c]		
amino acid	R-CHO[b]	M	M-HF	M-181	*m/z* 181	*m/z* 178	RT min[d]
Gly	H-	225 (4.2)	205 (9.5)	nd	(100)	(80)	3.13
Ala	CH_3-	239 (0.2, 0.6)	219 (8.9, 9.6)	nd	(100, 100)	(54, 60)	4.24, 4.38
Val	$(CH_3)_2CH$-	267 (0.1, 13)	247 (7, 2.6)	nd	(89, 100)	(100, 41)	5.72, 5.80
Ile	$CH_3CH_2C(CH_3)H$-	281 (0.3, 4.3)	261 (13, 3.7)	nd	(85, 100)	(100, 77)	6.78, 6.82
Leu	$CH_3(CH_2)_3$-	281 (nd, 4.7)	261 (13, 23)	nd	(46, 100)	(100, 90)	6.98, 7.16
Ser	$HOCH_2$-	255 (nd, 4.7)	235 (1.2, 32)	74 (0.4, 50)	(9.6, 0.1)	(100, 100)	8.17, 8.31
Thr	$CH_3C(OH)H$-	269 (nd, 0.3)	249 (37, 12)	88 (0.2, 9)	(nd, 14)	(100, 100)	9.42, 9.56
Asp	$HOOCCH_2$-	283 (nd, 0.1)	263 (57, 17)	102 (1.8, 67)	(2.1, 1.3)	(100, 100)	9.53, 9.69
Lys[e,f]	$NH_2(CH_2)_4$-	269 (<0.1)	276 (21)	nd	(14)	(100)	10.6
Phe	$C_6H_5CH_2$-	315 (nd, 0.5)	295 (1.1, 27)	134 (0.3, 3)	(21, 36)	(100, 100)	11.15, 11.26
Glu[e]	$HOOC(CH_2)_2$-	297 (2)	277 (1.5)	116 (100)	(0.5)	(15)	11.22
Tyr	$HO(C_6H_4)CH_2$-	331 (0.8, nd)	311 (100, 100)	150 (0.7, 0.5)	(nd, 5)	(100, 11)	13.10, 13.21
Trp	$C_8H_6NCH_2$-	354 (0.3, nd)	334 (100, 100)	171 (20, 6.4)	(8.8, 6)	(9, 4.4)	14.62, 14.76

[a] Each amino acid was oxidized by myeloperoxidase as described in the legend to Figure 1 and then derivatized and subjected to GC/MS analysis as described in the Experimental Procedures. Prominent ions observed in the electron capture NCI mass spectra, their relative abundances and the GC retention times for the PFB-oximes are indicated. Precursor amino acids are listed at the far left. RT, retention time; *m/z*, mass-to-charge ratio; nd, not detected. [b] Proposed aldehyde. [c] Numbers in parentheses following the *m/z* of each ion are the estimated relative abundance of the syn and anti isomers (syn, anti), reported as a percent of the base ion. [d] Retention times of syn and anti isomers (syn, anti). [e] The syn and anti isomers of the PFB-oximes derivatives of the aldehydes derived from lysine and glutamate were not baseline resolvable. [f] The abundance of the molecular ion was <0.1% of the base ion. A structurally informative ion (relative abundance 6%) that coeluted with the indicated ions was observed at *m/z* 246 ($M^{\bullet -}$ − HF − NO).

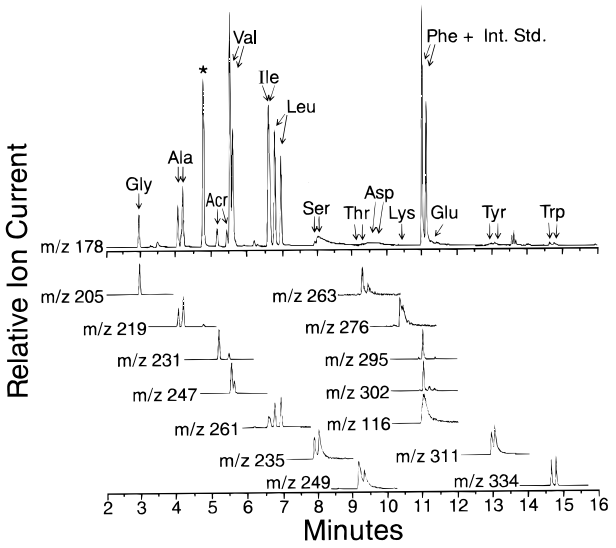


FIGURE 5: GC/MS analysis of amino acid-derived aldehydes generated by the myeloperoxidase-H_2O_2-Cl^- system. An amino acid mixture (1 mL) comprised of equimolar amounts of each of the 20 common α-amino acids (100 μM each) was incubated with myeloperoxidase (20 nM), H_2O_2 (100 μM), and sodium chloride (100 mM) in sodium phosphate buffer (50 mM; pH 7.0) at 37 °C for 60 min within gastight reaction vials. [D_8]Phenylacetaldehyde (3 nmol) was then added as internal standard, and the reaction products were derivatized with PFBHA. PFB-oxime derivatives of aldehydes were analyzed by electron capture NCI GC/MS with selected ion monitoring as described in the Experimental Procedures. (Top) Detection of ions at *m/z* 178, an ion common to PFB-oxime derivatives of aldehydes (Scheme 1). PFB-oxime derivatives of amino acid-derived aldehydes are labeled according to their precursor amino acid [with the exception of acrolein (Acr), the dehydration product of 2-hydroxypropanal derived from threonine; ref *17*]. (Bottom) Co-chromatography of a structurally informative ion for each PFB-oxime derivative of the aldehyde product. Aldehydes derived from the indicated parent amino acid were monitored as ions of *m/z*: L-glycine, 205 ($M^{\bullet -}$ − HF); L-alanine, 219 ($M^{\bullet -}$ − HF); acrolein, 231 ($M^{\bullet -}$ − HF); L-valine, 247 ($M^{\bullet -}$ − HF); L-isoleucine and L-leucine, 261 ($M^{\bullet -}$ − HF); L-serine, 235 ($M^{\bullet -}$ − HF); L-threonine, 249 ($M^{\bullet -}$ − HF); L-aspartic acid, 263 ($M^{\bullet -}$ − HF); L-lysine, 276 ($M^{\bullet -}$ − HF); L-phenylalanine, 295 ($M^{\bullet -}$ − HF); L-[D_8]phenylalanine (internal standard), 302 ($M^{\bullet -}$ − DF); L-glutamic acid, 116 ($M^{\bullet -}$ − 181); L-tyrosine, 311 ($M^{\bullet -}$ − HF); and L-tryptophan, 334 ($M^{\bullet -}$ − HF). (*) Unknown ion. Int. Std., internal standard.

were present in excess relative to the oxidizing substrate; these results are consistent with the potent scavenging ability of thiols for HOCl. Oxidation of the imino acid L-proline also failed to generate an aldehyde, consistent with the critical importance of an α-carbon primary amino group (*18*). Finally, HOCl could be substituted for the complete myeloperoxidase-H_2O_2-Cl^- system, supporting the idea that HOCl (or enzyme-bound ClO^-; refs *32* and *33*) is the oxidizing intermediate in the enzymatic reaction.

When the reaction mixture contained equimolar amounts of all 20 common α-amino acids, the complete myeloperoxidase-H_2O_2-Cl^- system generated a family of reactive aldehydes (Table 2) in overall high yield (60% relative to H_2O_2). The reaction required myeloperoxidase, H_2O_2, and Cl^- (Table 2). Substitution of reagent HOCl for the enzymatic system generated a similar family of aldehydes and in high yield (55% relative to HOCl). Two heme protein inhibitors, sodium azide and 3-aminotriazole, significantly attenuated aldehyde production by the myeloperoxidase system, confirming a critical role for the enzyme in the

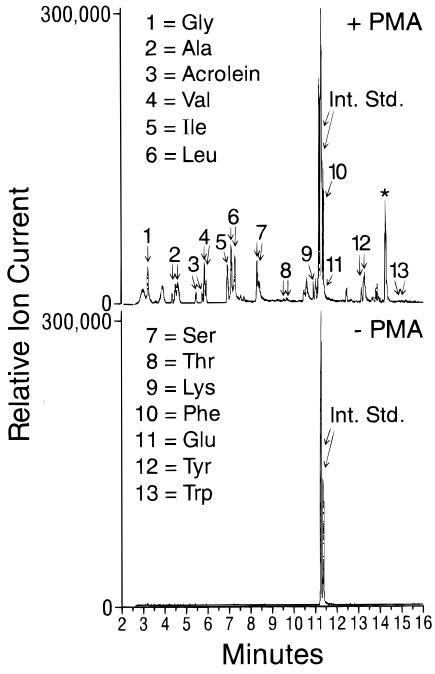


FIGURE 6: Activated human neutrophils employ the myeloperoxidase-H_2O_2-Cl system to convert α-amino acids into aldehydes. Freshly harvested human neutrophils (1×10^6 in 1 mL) were incubated within gastight vials at 37 °C with equimolar concentrations (100 μM) of each of the 20 common α-amino acids in the presence (top panel) or absence (bottom panel) of phorbol myristate acetate (PMA; 200 nM). Cells were maintained in suspension for 60 min by intermittent inversion. [D_8]Phenylacetaldehyde (2 nmol) was then added as internal standard, the reaction products were derivatized with PFBHA, and the PFB-oximes (*m/z* 178) were analyzed by electron capture NIC GC/MS with selected ion monitoring as described in the Experimental Procedures. PFB-oxime derivatives of amino-acid-derived aldehydes are labeled according to their parent amino acid [with the exception of acrolein (Acr)]. (*) Unknown ion. (Int. Std.) Internal standard.

reaction pathway. Finally, both lactoperoxidase and horseradish peroxidase, two peroxidases that do not effectively utilize Cl^- as substrate (*34*), failed to generate amino-acid-derived aldehydes (Table 2). These results strongly suggest that HOCl generated by myeloperoxidase is an intermediate in the synthesis of aldehydes.

Mass Spectrometric Characterization of Amino-Acid-Derived Aldehydes Generated by the Myeloperoxidase System. To facilitate structural characterization of the amino-acid-derived aldehydes, large quantities of the oxidation products were prepared using HOCl and each amino acid, followed by derivatization with PFBHA. Electron capture NCI GC/MS analysis of the majority of the PFBHA-derivatized products confirmed their structures as the anticipated aldehyde. Subsequent GC/MS studies demonstrated that the aldehyde products were identical to those generated by oxidizing amino acids with the complete myeloperoxidase-H_2O_2-Cl^- system. Aldehydes identified by mass spectrometric analysis following myeloperoxidase-catalyzed oxidation of their precursor amino acids are illustrated in Figure 3. Retention times of the derivatized aldehydes and descriptions of their mass spectra (fragment ions and relative abundances) are shown in Table 3. With the exception of formaldehyde, the glycine-derived aldehyde which lacks a chiral α-carbon, and lysine and glutamate, the PFB-oximes exhibited the anticipated syn and anti isomers (Table 3). Analysis of the mass spectra for both series of geometric,

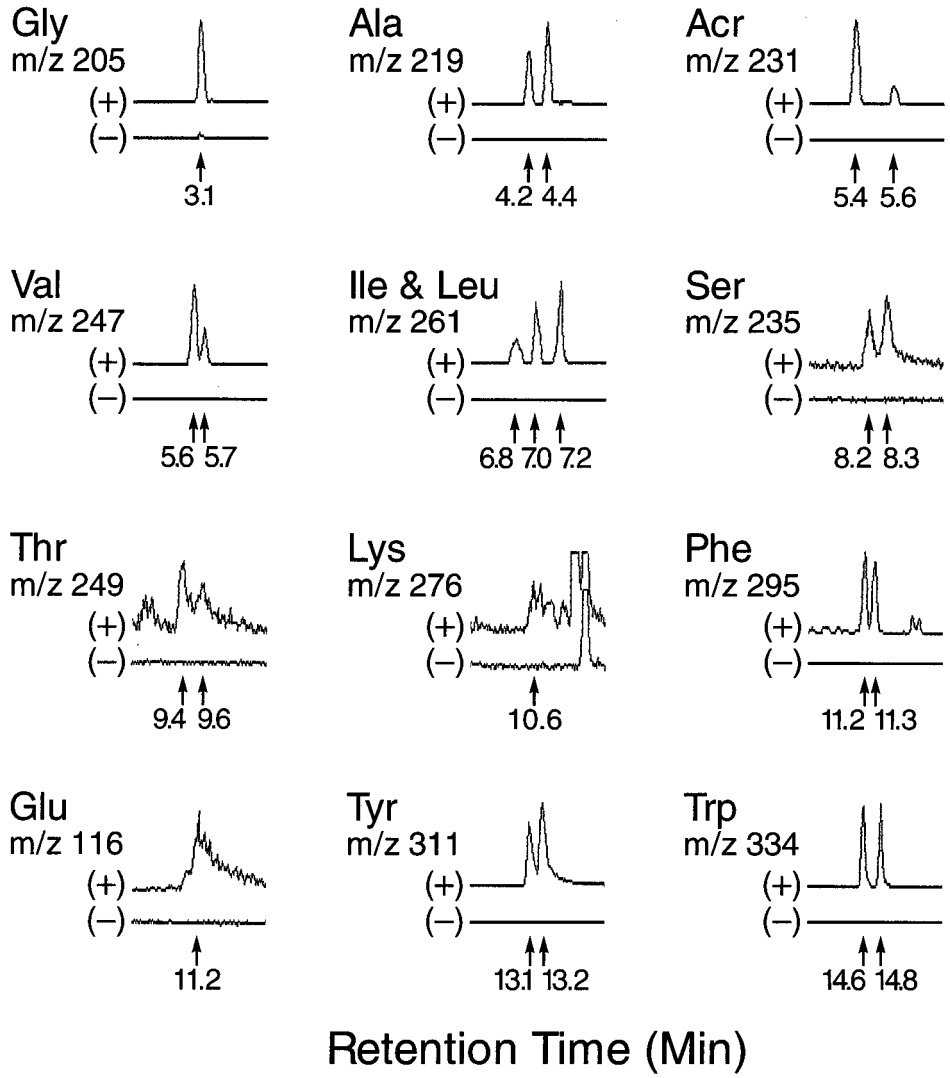


FIGURE 7: GC/MS analysis of α-amino acid-derived aldehydes generated by activated human neutrophils. Incubation media described in Figure 6 were derivatized and analyzed by electron capture NCI GC/MS with selected ion monitoring. Neutrophils were incubated with amino acids in the presence (+) or absence (−) of phorbol myristate acetate, respectively. The *m/z* of a structurally informative ion for each PFB-oxime derivative of the anticipated aldehyde was monitored (Scheme 1). Note that except for trace amounts of the glycine-derived aldehyde, formaldehyde (*m/z* 205; $M^{\bullet -}$ − HF), aldehydes were undetectable in the reaction mixture of cells incubated in the absence of phorbol ester. The ion currents of the PFB-oxime derivative of the internal standard, [D_8]phenylalanine (*m/z* 302, $M^{\bullet -}$ − DF), were similar in cells incubated with and without phorbol ester, indicating that aldehydes in the different incubation media were derivatized and extracted equivalently.

isomers revealed patterns of preferred fragmentation pathways, identifying several common and structurally informative ions that appear to be characteristic for PFB-oxime derivatives of aldehydes. These characteristic ions and their retention times were subsequently used to detect each of the amino-acid-derived aldehydes in complex mixtures (see Scheme 1). The PFBHA-derivatized oxidation products of Arg, Asn, Gln and His observed by HPLC (Table 1) proved problematic to characterize by GC/MS. These difficulties likely reflect a combination of factors, including poor solubility of the derivatized oxidation products in the organic solvents required for GC/MS analysis and the ability of functional groups other than α-carbon amino groups of the amino acids to scavenge reactive chlorinating species, resulting in the generation of a complex and labile mixture of products.

To determine whether amino acids were converted into aldehydes at physiologically plausible levels of H_2O_2, the dependence of the reaction on oxidant concentration was determined (Figure 4). Conversion of representative aromatic (L-tyrosine), aliphatic (L-isoleucine), acidic (L-aspartic acid), and alcoholic (L-serine) amino acids occurred in high yield at all concentrations of H_2O_2 examined (Figure 4). A lower yield of aldehyde was generated during L-lysine oxidation; this likely reflects consumption of HOCl by the ϵ-amino group of the basic amino acid. The curvilinear nature of product yield suggests that the more basic ϵ-amino moiety ($pK_a \approx 10.8$) is a poorer substrate for halogenation than the α-amino group ($pK_a \approx 9.2$); these results also suggest that RNH_2, rather than RNH_3^+, is the target for halogenation. No aldehyde production was observed when L-cysteine was incubated with the myeloperoxidase-H_2O_2-Cl^- system, presumably reflecting rapid scavenging of HOCl by the thiol group of the amino acid (*19*). Collectively, these results suggest that aldehydes are formed from the majority of amino acids following oxidation by the myeloperoxidase system even under conditions where a low concentration of oxidant is present, such as might occur in vivo.

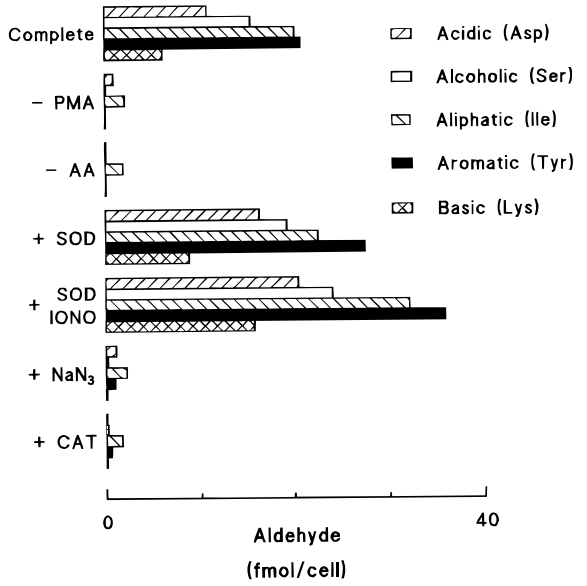


FIGURE 8: Reaction requirements for formation of aldehydes by human neutrophils. The complete system (Complete) consisted of freshly harvested human neutrophils (5×10^5/mL) activated with 200 nM phorbol myristate acetate (PMA) in the presence (200 μM) of either an acidic amino acid (L-aspartic acid), alcoholic amino acid (L-serine), aliphatic amino acid (L-isoleucine), aromatic amino acid (L-tyrosine), or basic amino acid (L-lysine). Following a 60 min incubation at 37 °C, reaction products were quantified by reverse phase HPLC as described in the Experimental Procedures. Where indicated, either PMA or amino acid (AA) was omitted or superoxide dismutase (SOD; 10 μg/mL, 50 units/mL), ionomycin (IONO; 1 μM), NaN_3 (1 mM), or catalase (CAT; 20 μg/mL, 200 units/mL) were included with the complete system. Results are the mean of duplicate determinations (range of <12% of the mean). Similar results were observed in two independent experiments.

Incubation of myeloperoxidase, H_2O_2, and Cl^- with an equimolar mixture of all 20 common α-amino acids resulted in the synthesis of a battery of reactive aldehydes whose PFB-oximes were detected by electron capture NCI GC/MS (Figure 5). Each aldehyde was identified by co-chromatography on selected ion monitoring of the common ion at mass-to-charge ratio (*m*/*z*) 178 (the base ion for the majority of amino acid-derived aldehydes; Table 3) together with a characteristic ion (e.g., $M^- - HF$) (Scheme 1 and Table 3). Co-chromatography of multiple structurally informative ions of each compound (Table 3) confirmed that the parent L-α-amino acid yielded the aldehyde that would be anticipated after deamination and decarboxylation of the α-carbon.

Human Neutrophils Convert α-Amino Acids into a Family of Reactive Aldehydes. Activation of human neutrophils in a balanced salt solution supplemented with all 20 common α-amino acids and the metal chelator DTPA also generated a family of amino-acid-derived aldehydes, as detected by electron capture NCI GC/MS of their PFB-oxime derivatives (Figure 6). The identity of each derivative was confirmed by demonstrating the co-chromatography of at least two structurally informative ions (e.g., *m*/*z* $M^{\bullet -}$, $M^{\bullet -} - HF$, $M^{\bullet -} - 181$, $M^{\bullet -} - HF - HCN$, or $M^{\bullet -} - HF - NO$) and one common ion (e.g., *m*/*z* 178) with an appropriate retention time for the syn and anti isomers (Figure 7). In the absence of phorbol ester, human neutrophils failed to generate detectable levels of nearly all α-amino acid derived-aldehydes (Figure 7). Only trace amounts of formaldehyde, the glycine-derived aldehyde, were observed under these conditions (Figure 7) and likely reflect contamination. Indeed, the exquisite sensitivity of electron capture NCI GC/MS for PFBHA derivatives (detection limit $< 10^{-15}$ mol) resulted in the detection of minute amounts of low molecular weight aldehydes, unless precautionary measures (see Experimental Procedures) were taken to exclude these contaminants from samples.

To further characterize and quantify the relative yield of aldehydes generated by activated neutrophils, we incubated one representative amino acid from each class of amino acids (acidic, alcoholic, aliphatic, aromatic, and basic) with cells and determined the extent of aldehyde production (Figure 8). Human neutrophils required activation to oxidize each of the amino acids, and the reaction was sensitive to inhibition by heme poisons and catalase. Addition of superoxide dismutase to the reaction mixture, which accelerates conversion of $O_2^{\bullet -}$ to H_2O_2 (*30*, *35*), modestly increased aldehyde production (Figure 8). Addition of the calcium ionophore ionomycin further increased aldehyde yield, consistent with enhanced H_2O_2 synthesis and/or myeloperoxidase secretion. Collectively, these results support a role for myeloperoxidase and H_2O_2 in aldehyde formation by phagocytes.

Amino Acid-Derived Aldehydes Quantitatively Account for a Major Fraction of H_2O_2 Generated by Activated Neutrophils. The quantitative significance of amino acid-derived-aldehyde formation was further assessed by monitoring the rate of both $O_2^{\bullet -}$ generation and aldehyde synthesis by stimulated neutrophils (Figure 9). Assuming that 2 mol of $O_2^{\bullet -}$ generate 1 mol of H_2O_2 (*30*, *35*) and that 1 mol of H_2O_2 is required to generate 1 mol of aldehyde from 1 mol of amino acid, up to 70% of the H_2O_2 (depending on the precursor amino acid) derived from $O_2^{\bullet -}$ was used in aldehyde generation (Figure 9). α-Keto acids have been reported to react with H_2O_2 to form the corresponding aldehyde plus CO_2 (*36*). If deamination of α-amino acids by HOCl generates α-keto acids and this reaction competes effectively for H_2O_2 generated by the cells, this reaction may account in part for the lower yield of aldehyde generation by certain of the amino acids. Collectively, these results demonstrate that activated human neutrophils employ the myeloperoxidase-H_2O_2-Cl^- system to convert nearly all of the common α-amino acids into aldehydes at physiological concentrations of amino acids and Cl^-.

DISCUSSION

Reactive aldehydes have long been known to possess potent biological and pharmacological properties. Our results provide evidence that a battery of reactive aldehydes are also generated by human neutrophils through the action of the myeloperoxidase-H_2O_2-Cl^- system on common α-amino acids (Scheme 2).

In plasma, the total concentration of α-amino acids is ~4−5 mM (*37*), while intracellular α-amino acid concentrations can exceed 20 mM (*38*). Given the overall high yield of aldehyde formation by the myeloperoxidase system of neutrophils, this general reaction may represent an important

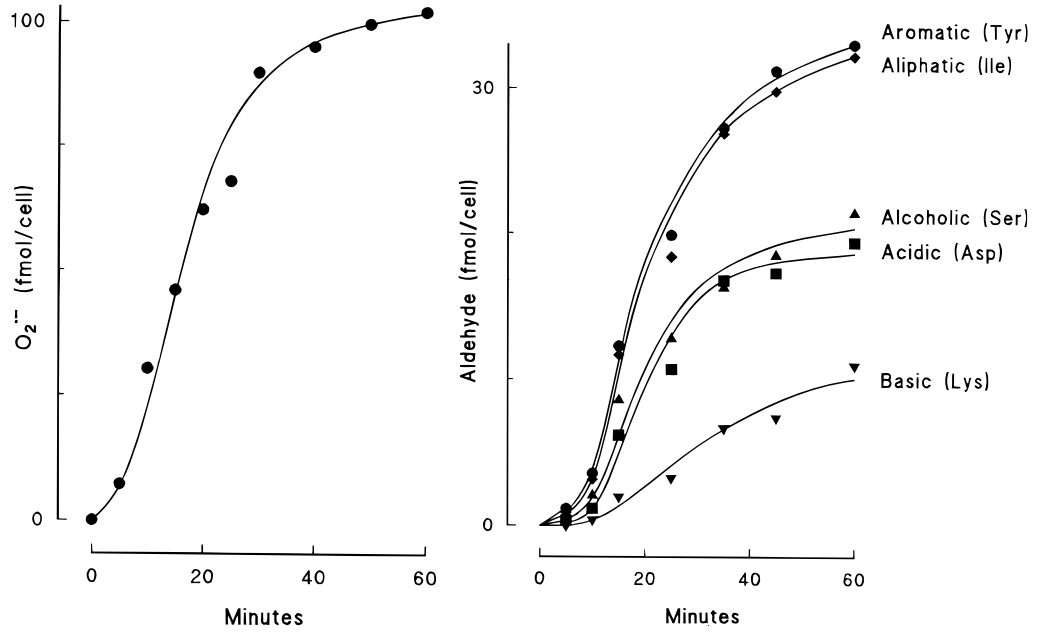
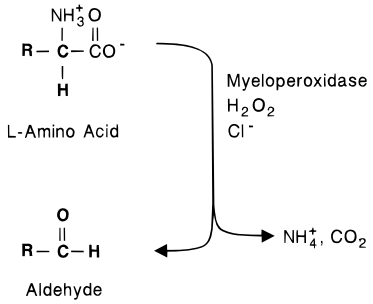


FIGURE 9: Progress curves for the production of $O_2^{\bullet-}$ and amino acid-derived aldehydes by activated human neutrophils. (Right panel) Freshly harvested human neutrophils (5×10^5/mL) were incubated at 37 °C with phorbol myristate acetate (200 nM), superoxide dismutase (50 units/mL) and ionomycin (1 μM) in the presence of each (200 μM) of the indicated classes of amino acids: (■) acidic, L-aspartic acid; (▲) alcoholic, L-serine; (◆) aliphatic, L-isoleucine; (●) aromatic, L-tyrosine; or (▼) basic, L-lysine. Cells were maintained in suspension by intermittent inversion. At the indicated times, aliquots were removed and aldehyde production determined by reverse phase HPLC as described in the Experimental Procedures. (Left panel) In a parallel experiment, $O_2^{\bullet-}$ production (in the absence of amino acids) was determined as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* as described in the Experimental Procedures. Results are the mean of duplicate determinations (range of <9% of the mean). Similar results were observed in two independent experiments.

Scheme 2. Proposed Reaction Pathway for Aldehyde Generation by Myeloperoxidase



oxidative pathway employed by activated phagocytes at sites of inflammation.

The structures of the amino acid-derived aldehydes were confirmed by a variety of independent techniques. (i) Electron ionization GC/MS was performed on several amino acid oxidation products. The resulting spectra demonstrated molecular ions and fragmentation patterns consistent with the structures of the proposed aldehydes. (ii) Electron capture NCI GC/MS studies of the PFB-oxime derivatives of the amino acid oxidation products demonstrated retention times and mass spectra entirely consistent with the proposed aldehyde structures. (iii) The retention times and electron capture NCI mass spectra of the PFB-oxime and DNPH derivatives of the amino-acid-derived aldehydes were also identical to those of commercially available aldehydes, where available (formaldehyde, acetaldehyde, glycolaldehyde, acrolein, and phenylacetaldehyde). Oxidation of common amino acids through the myeloperoxidase-H_2O_2-Cl^- system of phagocytes may thus represents an important pathway for the generation of a battery of reactive aldehydes.

It is interesting to note that many of the aldehydes produced by phagocytes are already known to exert profound biological effects. For example, acetaldehyde, the oxidation product of ethanol, has long been suggested to play a critical role in the toxic effects of ethanol ingestion, including fetal alcohol syndrome, alcoholic cirrhosis, and hepatocellular carcinoma (*39*). This same aldehyde was generated during the oxidation of L-alanine by the myeloperoxidase-H_2O_2-Cl^- system of phagocytes. Formaldehyde, the product of glycine oxidation, is mutagenic and cross-links proteins (*40*).

Many of the aldehydes synthesized by phagocytes also have been demonstrated to possess potent signaling properties, including induction of cytokine production, ion channel regulation and immunomodulation (*41*). Indeed, macrophages bear receptors for proteins modified by formaldehyde, malondialdehyde, and other aliphatic aldehydes (*42*−*44*), though the biological functions of such receptors are unclear. Our studies suggest a physiologically plausible mechanism for generating reactive aldehydes at sites of inflammation and for the subsequent covalent modification of nucleophilic targets, such as amino moieties on proteins, lipids, and nucleic acids.

Myeloperoxidase is found in high abundance within human neutrophils (*45*). Other phagocytic cells such as circulating monocytes (*46*) and some tissue macrophages (*47*) also contain the enzyme. Phagocyte activation at sites of both acute and chronic inflammation will therefore expose amino acids in the immediate environment to the myeloperoxidase-H_2O_2-Cl^- system, potentially resulting in the formation of significant quantities of reactive aldehydes. Indeed, we have recently demonstrated that the L-tyrosine-derived aldehyde, *p*-hydroxyphenylacetaldehyde, covalently modifies proteins in vivo in a variety of inflammatory tissues (*12*). It is likely

that many of the other amino-acid-derived aldehydes will exhibit analogous reactivity with biological targets.

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