Lysine Residues Direct the Chlorination of Tyrosines in YXXK Motifs of Apolipoprotein A-I When Hypochlorous Acid Oxidizes High Density Lipoprotein

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Oxidized lipoproteins may play an important role in the pathogenesis of atherosclerosis. Elevated levels of 3-chlorotyrosine, a specific end product of the reaction between hypochlorous acid (HOCl) and tyrosine residues of proteins, have been detected in atherosclerotic tissue. Thus, HOCl generated by the phagocyte enzyme myeloperoxidase represents one pathway for protein oxidation in humans. One important target of the myeloperoxidase pathway may be high density lipoprotein (HDL), which mobilizes cholesterol from artery wall cells. To determine whether activated phagocytes preferentially chlorinate specific sites in HDL, we used tandem mass spectrometry (MS/MS) to analyze apolipoprotein A-I that had been oxidized by HOCl. The major site of chlorination was a single tyrosine residue located in one of the protein’s YXXK motifs (where X represents a nonreactive amino acid). To investigate the mechanism of chlorination, we exposed synthetic peptides to HOCl. The peptides encompassed the amino acid sequences YKXY, YKXY, or YXXK. MS/MS analysis demonstrated that chlorination of tyrosine in the peptides that contained lysine was regioselective and occurred in high yield if the substrate was KXXX or YXXK. NMR and MS analyses revealed that the Nε amino group of lysine was initially chlorinated, which suggests that chloramine formation is the first step in tyrosine chlorination. Molecular modeling of the YXXK motif in apolipoprotein A-I demonstrated that these tyrosine and lysine residues are adjacent on the same face of an amphipathic α-helix. Our observations suggest that HOCl selectively targets tyrosine residues that are suitably juxtaposed to primary amino groups in proteins. This mechanism might enable phagocytes to efficiently damage proteins when they destroy microbial proteins during infection or damage host tissue during inflammation.

Protein oxidation has been implicated in the pathogenesis of diseases ranging from ischemia-reperfusion injury to atherosclerosis as well as in the aging process itself (1). However, most studies of protein oxidation have focused on the vulnerability of individual amino acid side chains, including the phenolic group of tyrosine and the thiol groups of methionine and cysteine. Remarkably little is known about the influence of nearby residues or of specific sequence motifs on the susceptibility of protein-bound amino acid chains to oxidation.

One example of such effects is the oxidation of specific cysteine residues in tyrosine phosphatases by hydrogen peroxide (2). This reaction, which affects phosphatase activity, is thought to rely on a high local concentration of positively charged amino acid side chains to stabilize the reactive anionic form of the thiol. Another example is the apparent effect of acidic and basic amino acid residues on protein nitrosation (3). However, an extensive study of model proteins oxidized by peroxynitrite revealed few obvious common features among the tyrosine residues that were targeted for nitration (4). Recently, histidine and cysteine residues at the metal binding site have been identified as primary targets during metal-catalyzed oxidation of β-amyloid peptides and iron regulatory protein 2 (5, 6).

Neutrophils, monocytes, and some populations of macrophages use the heme enzyme myeloperoxidase (7–9) to produce hypochlorous acid (HOCl), a potent cytotoxic oxidant (10, 11).

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\text{Cl}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O}
\]

**REACTION 1**

HOCl plays a critical role in destroying microbial pathogens (12, 13). However, it can also react with proteins, lipids, and nucleic acids in host tissues, contributing to inflammatory damage (14, 15).

Because proteins are a major target for HOCl, the oxidant’s reactions with amino acids and peptides have been widely studied (16–21). HOCl readily oxidizes the sulfur-containing amino acids cysteine and methionine, producing disulfides, oxyacids, sulfoxides, and compounds in which sulfur is cross-linked to nitrogen (22–24). However, other reactive species, such as hydroxyl radical and peroxynitrite, can also generate oxygenated sulfur products (25, 26), which therefore cannot serve as definitive markers for HOCl-induced damage. In contrast, *in vitro* and *in vivo* studies have shown that 3-chlorotyrosine is a specific product of myeloperoxidase (13, 27, 28).

Chlorination of the phenolic ring of tyrosine may have physiological relevance because both active myeloperoxidase, the enzyme that generates HOCl, and elevated levels of 3-chlorotyrosine have been detected in human atherosclerotic lesions (8, 9, 14, 29). One important target might be high density lipoprotein (HDL). HDL is believed to inhibit atherosclerosis

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‡ The abbreviations used are: HDL, high density lipoprotein; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; PBS, phosphate-buffered saline; TOF, time-of-flight; LC, liquid chromatography; HPLC, high pressure liquid chromatography.
by mobilizing excess cholesterol from cells of the artery wall, but oxidation of HDL has been proposed to alter its biological properties, thereby contributing to the pathogenesis of atherosclerosis (30, 31). Previous studies have shown that methionine and phenylalanine residues in apolipoprotein A-I, the major protein in HDL, are oxidized by reactive intermediates (32–35). Tyrosine residues are converted to α,α′-dityrosine by a tyrosyl radical (36). However, little is known about the vulnerability to chlorination of specific tyrosine residues in apolipoprotein A-I.

HOCI also reacts with primary amino groups, producing primary (RNClH) and secondary (RNCl2) chloramines (37, 38).

HOCI + RNH₂ → RNClH + H₂O + HOCl ≃ RNCl₂ + H₂O

**Reaction 2**

Studies with free α-amino acids have shown that they form unstable α-amino chloramines, which are deaminated and decarboxylated into aldehydes (39–41). In contrast, HOCI yields stable chloramines at the primary ε-amino group of lysine, which lacks an adjacent carboxylic acid (19). It has been suggested that N-centered radicals deriving from chloramines could contribute to protein fragmentation and tissue damage (42).

Both HOCI and chloramines react with aromatic and unsaturated compounds to form chlorinated products (7, 37, 43). HOCI is in equilibrium with molecular chlorine, and this potent electrophile has been proposed to mediate the chlorination of free tyrosine (28). It remains to be established whether this reaction pathway is relevant to the chlorination of protein-bound tyrosine residues. N-terminal chloramines have been proposed to be intermediates in the formation of 3-chlorotyrosine in synthetic peptides (27, 44). However, little is known about the factors that control the site-specific chlorination of tyrosine residues in proteins. In the current study, we use HDL, synthetic peptides, and tandem mass spectrometric analysis to investigate the role of HOCI and chloramines in tyrosine chlorination.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sodium hypochlorite (NaOCl), trifluoroacetic acid, and HPLC grade CH₃CN and methanol were obtained from Fisher. Phosphate-buffered saline (PBS), free and acetylated amino acids, and 100 mM NaCl in saline (PBS), free and acetylated amino acids, and 100 mM NaCl in PBS (10). Initiated by adding oxidant and were terminated by the addition of a stable chloramines at the primary (RNClH) and secondary (RNCl₂) chloramines (37, 38). Purity of the peptides was confirmed by HPLC and determined using buffers (100 mM) composed of phosphoric acid, monobasic sodium phosphate, dibasic sodium phosphate, and 100 mM NaCl. Protein was determined using the Lowry assay (Bio-Rad).

**HPLC Analysis of Peptides—** Peptides were separated at a flow rate of 1 ml/min on a reverse-phase column (Beckman ODS, 4.6 × 250 mm) using a Beckman HPLC system (Fullerton, CA) with UV detection at 215 nm. The peptides were eluted using a gradient of solvent A (0.1% trifluoroacetic acid in H₂O) and solvent B (0.1% trifluoroacetic acid in 90% CH₃CN, 10% H₂O). Solvent B was increased from 10 to 50% over 25 min.

**Isolation of Peptide-bound Chloramines—** Ac-GEYARKY (100 μM) was incubated with HOCI (100 μM) in PBS (pH 7.4) for 2 min at room temperature, and the reaction mixture was subjected immediately to HPLC without methionine addition. The methionine-sensitive oxidation product was collected and stored on ice for further analysis.

**Oxidation of Lipid-associated Peptides—** 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)-associated peptides were prepared using a protein/DPPC ratio of 1.2 (mol/mol). DPPC was dissolved in methanol and dried under nitrogen. PBS was added, and the mixture was vortexed vigorously and incubated for 15 min at 37 °C. Vesicles were aliquoted and incubated with 25 μM peptides for 20 min at 37 °C. Peptides were oxidized with HOCI in PBS at 37 °C (0.5 and 1 mol of oxidant/mol of peptide, lipid-free and DPPC-associated, respectively). The reaction was stopped with excess methionine after 30 min.

**Proteolytic Digestion of Proteins—** Native or HOCI-modified HDL was incubated overnight at 37 °C with sequencing grade modified trypsin (Promega, Madison, WI) at a ratio of 25:1 (w/w) protein/trypsin in 50 mM NH₄HCO₃, pH 8. Digestion was halted by acidification (pH 2–3) with trifluoroacetic acid.

**Electrospray Ionization Mass Spectrometry (ESI-MS)—** ESI-MS analyses were performed in the positive ion mode with a Finnigan Mat LCQ ion trap instrument (San Jose, CA) coupled to a Waters 2690 HPLC system (Milford, MA) (48). Synthetic peptides were separated at a flow rate of 0.2 ml/min on a reverse-phase column (Beckman ODS, 2.1 × 250 mm) using a gradient of solvent A (0.2% HOCl in H₂O) and solvent B (0.2% TFA in CH₃CN, 20% H₂O). Solvent B was increased from 10 to 50% over 25 min. Tryptic digests of HDL were separated at a flow rate of 0.2 ml/min on a reverse-phase column (Vydac C18 MS, 2.1 × 250 mm). The electrospray needle was held at 4500 V. Nitrogen, the sheath gas, was set at 80 units. The collision gas was helium. The temperature of the heated capillary was 220 °C.

**Electrospray Ionization Mass Spectrometry (ESI-MS)—** ESI-MS analyses were performed on a Voyager DE-STR (PerSeptive Biosystems, Foster City, CA) in the reflection mode using delayed ion extraction (49). The accelerating voltage was 25 kV, the delay time was 300 ns, the mirror to accelerating voltage ratio was 1.12, and the low mass gate was 400 Da. Spectra (100 shots) were acquired with a laser power of 1850, α-cyano-4-hydroxycinnamic acid (10 mg/ml) in 0.1% trifluoroacetic acid plus CH₃CN (1:1, v/v) as matrix.

**NMR Spectroscopy—** NMR spectra were obtained on the peptide and the chloramine intermediate (3.6 mM and 0.5 mM, respectively) in 0.5 ml of PBS (pH 7.4) at 4 °C using presaturation or hard pulse WATERGATE sequences for water suppression (50). Spectra were obtained under the following conditions: preconditioning delay = 0.5 s, acquisition time = 1.416 s (16,000 complex data points), pulse width = 7.8 μs, spectral width = 5650.1 Hz and 2 ms 15 G/cm field gradient pulses. Total correlation spectroscopy experiments for peptides and peptide-bound chloramines were recorded using an MELV-17 mixing sequence of 100 ms flanked by 2-ms trim pulses with 256 fired in 2048/2 data points. After two-dimensional Fourier transformation, the spectra resulted in 2048 × 2048 data points that were phase- and baseline-corrected in both dimensions.

**RESULTS**

**HOCI Preferentially Chlorinates a Single Tyrosine Residue in Apolipoprotein A-I**—The 10 amino acid residues in apolipoprotein A-I, the major protein of HDL, are thought to play essential roles in lipid binding, lipoprotein stability, and reverse cholesterol transport (51, 52). Five of the seven tyrosine residues in apolipoprotein A-I are located in amphipathic helices (Fig. 1A). It is noteworthy that three of those tyrosines reside in a YXXK motif (YXXKK, YXXKK, and YXXKK, X = an amino acid unreactive with HOCI). Importantly, the helical wheel representation of amphipathic helices predicts that tyrosine and lysine residues in this motif will lie next to each other...
other on the same face of the α-helix (Fig. 1B) (51, 52). We therefore used HDL to determine whether HOCl preferentially chlorinates specific tyrosine residues in proteins.

Liquid chromatography (LC)-ESI-MS analysis of the trypsin digest of apolipoprotein A-I detected peptides that collectively covered ~80% of the protein’s sequence and included all seven peptides predicted to contain tyrosine. To determine which tyrosine residues can be chlorinated, we oxidized HDL with HOCl and used reconstructed ion chromatograms to detect (i) each of the peptides that contained tyrosine and (ii) any tyrosine-containing peptides that had gained 34 atomic mass units (addition of 1 chlorine and loss of 1 hydrogen).

We exposed HDL to HOCl (80:1, mol/mol, oxidant/HDL particle) in a physiological buffer (138 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate) at neutral pH for 120 min at 37 °C and then terminated the reaction with a 20-fold molar excess (relative to oxidant) of methionine. Because the average HDL$_3$ particle contains 2 mol of apolipoprotein A-I (7 tyrosine residues, 243 amino acids) and 1 mol of apolipoprotein A-II (8 tyrosine residues, 154 amino acids), the ratio of oxidant to substrate (mol/mol) was ~30:1 for apolipoproteins A-I and A-II, 3:1 for tyrosine residues, and 1:8 for total amino acids.

After digesting unmodified and oxidized HDL with trypsin, we analyzed the resulting peptides. LC-ESI-MS and MS/MS analysis detected three tryptic peptides whose mass corresponded to the mass of the precursor peptide plus 34 atomic mass units, which suggests the formation of 3-chlorotyrosine (Table I). The three peptides came from residues 189–195 ([LAEYHAK + 34 atomic mass units + 2H]$^2+^+$, $m/z$ 433.2), residues 227–238 ([VSFLSALEYTK + 34 atomic mass units + 2H]$^2+^+$, $m/z$ 710.9), and residues 28–40 ([DYVSQFEGSALGK + 34 atomic mass units + 2H]$^2+^+$, $m/z$ 771.9). Using LC-ESI-MS/MS analysis, we confirmed each peptide’s sequence and showed that its tyrosine had been targeted for chlorination (data not shown). Quantification of the ion current of each precursor and product peptide using reconstructed ion chromatograms (Table I) indicated that the major tyrosine oxidation product was LAE(CLY)HAK (Fig. 2). Importantly, the tyrosine residue in this region of apolipoprotein A-I is located in a YXXX motif. The other chlorinated peptides were present at much lower levels. These findings indicate that HOCl chlorinates three of the seven tyrosines in apolipoprotein A-I (Table I) and that the major oxidation product (~50% 3-chlorotyrosine) was located in the YXXX motif of LAEYHAK. The other two tyrosines were chlorinated in much lower yield (<5% 3-chlorotyrosine); one of these tyrosine residues was also located in a YXXX motif (VSFLSALEYTK[K]), where [K] represents a lysine residue removed by trypsin. These observations suggest that the amino group of lysine might direct protein oxidation to specific sites.

**Lysine Residues Direct the Regiospecific Chlorination of Tyrosine in Peptides**—To explore the influence of lysine residues on the chlorination of nearby tyrosine residues, we investigated the reaction of HOCl with three model peptides: Ac-GYKRAVE,

![Table I](image)

**Table I**

| Residues | Sequences         | Precursor, $m/z$ | Product, $m/z$ | Yield$^a$ |
|----------|-------------------|------------------|----------------|-----------|
|          |                   | [M + H]$^+$     | [M + 2H]$^2+^+$ | [M + 34 + H]$^+$ | [M + 34 + 2H]$^2+^+$ |
| 13–23    | DLATYYVVLK        | 1235.7          | 618.3          | ND$^a$    | ND        |
| 28–40    | DYVSQFEGSALGK     | 1400.7          | 700.8          | 1434.7    | 717.8     | 2         |
| 97–106   | VQPYLDFQK         | 1252.6          | 626.8          | ND        | ND        |           |
| 108–116  | WQFEMEKLRY        | 1283.6          | 642.3          | ND        | ND        |           |
| 109–116  | WQFEMEKLRY        | 1299.6          | 650.3          | ND        | ND        |           |
| 161–171  | TALAPSYDLR        | 1301.6          | 651.3          | ND        | ND        |           |
| 189–195  | LAEYHAK           | 831.4           | 416.2          | 865.4     | 433.2     | 52        |
| 227–238  | VSFLSALEYTK       | 1386.7          | 693.9          | 1420.7    | 710.9     | 6         |

$^a$ Values are (mol of product peptide/mol of precursor peptide) × 100.

$^a$ ND, not detectable.

$^a$ HOCl oxidizes methionine to methionine sulfoxide (34).
Ac-GEYARKY, and Ac-GEYAREY (YKXX, YXXX, and YXYX). We included lysine in two of the peptides because it has a primary ε-amino group that forms a long lived chloramine when exposed to HOCl (19), and chloramines can react with phenolic groups (37) such as the one in tyrosine. The three peptides contained the same amino acids (acetyl-Gly, β-Tyr, β-Lys, Arg, Ala, and Glu), including glutamic acid, but the last replaced lysine in YXXX. Glutamic acid was included because its negative charge ensures aqueous solubility. Arginine was included because its positive charge promotes peptide ionization during mass spectrometric analysis. The acetyl group on the N terminus prevented the primary amine from forming a chloramine.

We exposed each peptide to HOCl (1:1, mol/mol) in a physiological buffer for 30 min at 37 °C, terminated the reaction with methionine, and analyzed the reaction products by HPLC with UV detection, LC-ESI-MS, and LC-ESI-MS/MS. When the substrate was YKXXY or YXXXY, HPLC with UV detection revealed a high yield (>50%, mol of product/mol of oxidant) of one major product and smaller yields of a minor product (Fig. 3, A and B, and Table II). In contrast, oxidation of YXXXY generated four minor products that individually accounted for only ~11% of the total oxidant (Fig. 3C, peaks 1–4, and Table II). LC-ESI-MS analysis of the peptides YKXXY and YXXXY incubated in buffer alone demonstrated doubly charged ions at a mass/charge ratio (m/z) of 464.8, the anticipated m/z of the doubly protonated unmodified peptide [peptide + 2H]^{2+}. LC-ESI-MS analysis of the two reaction products derived from YKXXY and the two derived from YXXXY revealed ions of m/z 481.8, corresponding to the masses of the doubly protonated peptides plus 34 atomic mass units [peptide + 34 atomic mass units + 2H]^{2+}. These observations suggest that each peptide loses a hydrogen atom and gains a chlorine atom when it is exposed to HOCl (~1 atomic mass unit, 35 atomic mass units), which is consistent with chlorination of the phenolic ring of one of each peptide’s 2 tyrosine residues. LC-ESI-MS analysis of YXXXY incubated in buffer alone demonstrated a singly charged ion of m/z 929.3, the anticipated m/z of the unmodified peptide [peptide + H]^{+}. Two of its oxidation products (Fig. 3C, peaks 1 and 2) exhibited ions at m/z 963.3 [peptide + 34 atomic mass units + H]^{+}, suggesting that each contained a single chlorine atom. In contrast, the other two (Fig. 3C, peaks 3 and 4) exhibited ions of m/z 997.3 [precursor peptide + 88 atomic mass units + H]^{+}, suggesting that HOCl oxidizes YXXXY into a mixture of peptides that have 3-chlorotyrosine at either the first or last position or into a peptide that

![Fig. 2. MS/MS identification of the major site of tyrosine chlorination in apolipoprotein A-1 exposed to HOCl. MS/MS analysis of [LAETHAK + H]^{+} (m/z 831.4) and [LAETHAK + H]^{+} (m/z 865.4) in HDL oxidized with HOCl. HDL was exposed to HOCl (80 μl/mL, oxidant/HDL particle) for 120 min at 37 °C in PBS (pH 7.4). After terminating the reaction with L-methionine, HDL proteins were digested with trypsin, and the tryptic digest peptides were subjected to analysis by LC-ESI-MS/MS.](image)

![Fig. 3. HPLC analysis of YKXXX (A), YXXXY (B), and YXXXY (C) exposed to HOCl. Peptide (100 μM) was incubated for 30 min at 37 °C in PBS (top) or PBS supplemented with 100 μM HOCl (bottom). Reactions were initiated by adding oxidant and terminated by adding L-methionine. The reaction mixture was analyzed by reverse-phase HPLC and UV detection at 215 nm.](image)

**Table II**

| Motif | Peptide | Product | Sequence | Product yield^{a} |
|-------|---------|---------|----------|------------------|
| YKXXX | Ac-GYKRAYE | Peak 1 | Ac-GGYKRAYE | 11 |
| YXXXY | Ac-GEYARKY | Peak 1 | Ac-GEYARYKYE | 60 |
| YXXXY | Ac-GEYAREY | Peak 1 | Ac-GEYAREY | 60 |
| YXXXY | Ac-GEYAREY | Peak 2 | Ac-GEYAREY | 49 |
| YXXXY | Ac-GEYAREY | Peak 2 | Ac-GEYAREY | 19 |
| YXXXY | Ac-GEYAREY | Peak 3 | Ac-GEYAREY | 8 |
| YXXXY | Ac-GEYAREY | Peak 4 | Ac-GEYAREY | 6 |

^{a} Values are (mol of product peptide/mol of HOCl) × 100.
contains 3,5-dichlorotyrosine residue at one position.

We used tandem mass spectrometry (MS/MS) to confirm the sequence of each precursor peptide and to identify the amino acid residue that was amenable to chlorination (Fig. 4). MS/MS analysis of YXXXY revealed a series of b-ions (b₂, m/z 228.8; b₃, m/z 219.2; b₄, m/z 216.9) and y-ions (y₁, m/z 181.9; y₄, m/z 537.2; y₅, m/z 700.3), consistent with the predicted sequence Ac-GEYARKY (Fig. 4A). MS/MS analysis of the major oxidation product of YXXXY (Fig. 3B, peak 1) demonstrated a series of b-ions and y-ions that had gained 34 atomic mass units (b₅ + 34, m/z 653.1; b₆ + 34, m/z 781.3; y₁ + 34, m/z 571.2; y₅ + 34, m/z 863.4). However, the masses of ions b₂ (m/z 228.9), y₁ (m/z 182.0), and y₄ (m/z 537.2) were unchanged (Fig. 4B). These observations indicate that exposing YXXXY to HOCl generates a major oxidation product that contains a chlorine atom on its first tyrosine residue (Ac-GEClYARKY; ClYXXXY). MS/MS analysis of peak 2 (Fig. 3B), the minor product of YXXXY oxidation, demonstrated y₁, y₄, and y₅ ions that had gained 34 atomic mass units (y₁ + 34, m/z 216.0; y₄ + 34, m/z 571.2; y₅ + 34, m/z 734.3) and ions that were unaffected (b₂, b₄, and b₆). These observations indicate that Ac-GEYARKCY (YXXCY) is the minor product when YXXXY is oxidized with HOCl. The sequences of the YXXXY peptide and its major and minor oxidation products were confirmed using MALDI-TOF-MS analysis with postsource decay (data not shown). These observations indicate that HOCl converts YXXXY into a single major oxidation product, ClYXXXY.

We used this analytical approach to confirm the sequences of YKXX and YXXY and to identify the residues that are chlorinated when these peptides are exposed to HOCl (data not shown). MS/MS analysis revealed that the major and minor products of YKXXY oxidation were Ac-GYKRCAY (YXXCY) and Ac-GClYKRAY (GYXXXY), respectively. Oxidation of YXXX produced a different pattern of products; peaks 1 and 2 were monochlorinated on a single tyrosine residue (Ac-GEClYARKY and Ac-GEYAREClY), whereas peaks 3 and 4 had two chlorine atoms on a single tyrosine residue (Ac-GECl₂YARKY and Ac-GEYARECl₂Y). Our results indicate that HOCl chlorinates tyrosine residues in peptides containing the motif KXXY or YKXX with high yield and that chlorination is regiospecific for the tyrosine residue 2 residues away from a lysine residue (Table II). Moreover, we found no evidence for chlorination of both tyrosines in YXXXY. Instead, each tyrosine residue was oxidized to either the monochlorinated or dichlorinated derivative (and the other was left unchlorinated). These findings suggest that lysine plays a regiospecific role in the chlorination of tyrosine in peptides.

YXXXY Reacts with HOCl to Produce a High Yield of Chlorotyrosine—To characterize the reaction of HOCl with peptides, we incubated YXXXY with HOCl (1:1, mol/mol) in a physiological buffer at neutral pH and 37 °C and identified the
Regiospecific Chlorination of Tyrosine Residues

Fig. 6. HPLC analysis of N-acetyltyrosine exposed to HOCl. N-acetyltyrosine (Ac-Tyr) alone or N'-acetylated amino acids (Ac- followed by amino acid one-letter code) mimicking the amino acid composition of the XXXXY and YXXXY or XXXXY peptides were incubated with 100 μM HOCl in PBS for 60 min at 37 °C. The reaction was terminated with l-methionine, and the mixture was subjected to HPLC analysis on a reverse-phase column with monitoring of products at 280 nm. A, Ac-Tyr alone (200 μM). B, amino acid composition of XXXXY (200 μM Ac-Tyr, 200 μM Ac-Glu, 100 μM Ac-Ala, 100 μM Ac-Gly, 100 μM Ac-Arg). C, amino acid composition of YXXXY and XXXXY (200 μM Ac-Tyr, 100 μM Ac-Ala, 100 μM Ac-Glu, 100 μM Ac-Gly, 100 μM Ac-Lys, 100 μM Arg).

Fig. 7. HPLC analysis of the methionine-sensitive reaction product formed in YXXXY exposed to HOCl A. YXXXY peptide was oxidized with HOCl as described in the legend to Fig. 3 without the addition of methionine and immediately subjected to HPLC analysis on a reverse-phase column. The oxidized peptide eluting at 20 min was collected and reanalyzed by reverse phase HPLC without further treatment (B) or was reduced with excess methionine and then reanalyzed (C).

YXXXY and YXXXY (Ac-Tyr, Ac-Gly, N'-Ac-Lys, Ac-Arg, Ac-Ala, and Ac-Glu) was exposed to HOCl, neither N-acetyl-3-chlorotyrosine nor N-acetyl-3,5-dichlorotyrosine were detected (Fig. 6C). These observations strongly suggest that lysine and tyrosine must be incorporated into a peptide for chlorination of the tyrosine to be efficient.

Chloramines Mediate the Regiospecific Chlorination of Peptide-bound Tyrosine—To determine whether the formation of lysine chloramine is a necessary step in the regiospecific chlorination of peptides, we incubated YXXXY with an equimolar amount of HOCl and immediately analyzed the reaction mixture. For these studies, we omitted methionine from the mixture because alkylated thiols rapidly reduce chloramines (37, 55). HPLC analysis revealed a single peak of material (Fig. 7A, retention time 20 min) that was absent in the reaction mixtures exposed to HOCl and then methionine (compare with Fig. 3B). This product could be collected and detected after it was again subjected to HPLC (Fig. 7B), indicating that it was reasonably stable under our analytical conditions. Moreover, methionine converted it back to its precursor peptide (Fig. 7C). These observations indicate that HOCl converts YXXXY into a stable intermediate that can be converted back to the precursor peptide by methionine.

To investigate the nature of the reaction intermediate, we subjected the unmodified peptide and the methionine-sensitive oxidation product to one- and two-dimensional NMR analyses (Fig. 8). High resolution 1H NMR analysis of the unmodified peptide revealed that the lysine ε NH2 and ε CH2 proton resonances were at 7.5 ppm and 2.85 ppm, respectively (Fig. 8A, left panel). The assignment of these protons was confirmed by total correlation spectroscopy (Fig. 8A, right panel). The methionine-sensitive oxidation product lacked the ε NH2 protons and exhibited a marked downfield shift of the ε CH2 resonance (3.55 ppm; Fig. 8B, right and left panels). These observations strongly suggest that the ε NH2 group in the methionine-sensitive oxidation product had been modified by the addition of a strongly electron-withdrawing group(s).

We used MALDI-TOF-MS analysis to confirm the identity of the methionine-sensitive intermediate derived from YXXXY. The HPLC-purified intermediate displayed three major ions of m/z 628.3, m/z 962.2, and m/z 996.2, corresponding to the masses of the singly protonated precursor peptide, the protonated peptide plus 34 atomic mass units, and the protonated peptide plus 68 atomic mass units (Fig. 9A). The peaks of material that had gained 34 atomic mass units and 68 atomic...
mass units demonstrated the isotopic clusters characteristic of monochlorinated and dichlorinated compounds, respectively. Postsource decay analysis of the protonated precursor peptide (m/z 628.3) showed an unmodified lysine residue (K in Fig. 9B). In contrast, postsource decay analysis of the ions at m/z 962.2 and 996.2 demonstrated that the lysine residue had gained 34 atomic mass units (K/H1100134 in Fig. 9C) and 68 atomic mass units (K/H1100168; Fig. 9D). These observations strongly suggest that HOCl converts the N*-amino group of YXXXK into a monochlorinated and/or dichlorinated intermediate. The mixture of chlorinated and nonchlorinated species detected by MALDI-TOF-MS probably reflects a loss and/or exchange of chlorine atoms during ionization.

To determine whether lysine N*-chloramine is an intermediate in the reaction pathway that generates peptide-bound 3-chlorotyrosine, we oxidized the YXXXK peptide with an equimolar amount of HOCl and used HPLC to monitor the progress curve of the reaction (Fig. 10). Immediately following the addition of oxidant, the reaction mixture contained approximately equal amounts of the unmodified peptide and its chloramine. As the reaction time increased, both the unmodified peptide and the lysine N*-chloramine disappeared in concert with the appearance of peptide-bound 3-chlorotyrosine. The stoichiometry of the reaction was ~1 mol of product peptide per 0.5 mol of chloramine intermediate. Collectively, these observations provide strong evidence that chloramines derived from the N*-amino group of lysine are crucial intermediates in the regioselective chlorination of peptide-bound tyrosine.

Lysine Residues in YXXK and YXXXXK Motifs Promote the Chlorination of Tyrosine Residues in Lipid-free and Lipid-associated Peptides—Molecular modeling indicates that tyrosine and lysine residues separated by 3 other amino acids (YXXXK) reside on the same side of an α-helix (51, 52), raising the possibility that the phenolic group of tyrosine and the N*-amino group of lysine could come close enough to interact chemically. Because apolipoprotein A-I contains only one tyrosine residue in a YXXXXK motif, it is difficult to assess whether lysine directs tyrosine chlorination by studying this molecule. We therefore...
synthesized the model peptide Ac-PYSDELRLQRAARLE-NH$_2$ (YXXXX), which duplicates helix 7 of apolipoprotein A-I. This helix contains tyrosine 166, which was not chlorinated by HOCl under our experimental conditions (Table I; peptide 161–171) and does not reside in a YXXK/KXXY motif (Fig. 1). Ac-PYSDELRLQRAARLE-NH$_2$ is predicted to adopt an α-helical conformation ~40% of the time (56), suggesting that it should approximate the secondary structure of the intact apolipoprotein.

Fig. 9. MALDI-TOF-MS and MS/MS analysis of the methionine-sensitive product formed in YXXKY exposed to HOCl. YXXKY peptide was oxidized with HOCl as described in the legend to Fig. 3, and its methionine-sensitive oxidation product was isolated by HPLC as described under “Methods.” A, the oxidation product was immediately analyzed by MALDI TOF-MS using α-cyano-4-hydroxycinnamic acid as the matrix. MS/MS spectrum of protonated precursor peptide (ion of m/z 928.3, M + H) (B), protonated and chlorinated peptide (ion of m/z 962.2, M + H + 34 atomic mass units) (C), or protonated and dichlorinated peptide (ion of m/z 996.2, M + H + 68 atomic mass units) (D). Note that MS/MS analysis demonstrates the addition of 34 and 68 atomic mass units to the lysine residue in the peptide.
This case, the lysine residue was separated from the tyrosine residue by reverse-phase HPLC and UV detection at 215 nm.

After acetylating the peptide’s N terminus to prevent the primary amine from forming a chloramine, we exposed it to HOCl and quantified its chlorination by LC-ESI-MS. In parallel studies, we attempted to mimic the lipid environment that envelopes apolipoprotein A-I in HDL by incubating the peptide with DPPC vesicles before exposing it to HOCl. When we exposed YXXXY to HOCl in a physiological buffer for 30 min at 37 °C, we detected very little chlorination of either the lipid-free or lipid-associated peptide (Fig. 11).

To explore the role of the YXXXK motif in promoting tyrosine chlorination, we prepared variants of the YXXXXY peptide. In this case, the lysine residue was separated from the tyrosine residue by 0, 1, 2, 3, or 4 amino acids (YXXXXK, YXKKX, YXXKX, YXKXX, and YXXXXK). After we exposed these peptides to HOCl, LC-ESI-MS analysis revealed that YXXXXK, YXKKX, and YXXKX yielded progressively greater amounts of chlorinated peptide (Fig. 11). The level of chlorination declined when the tyrosine and lysine residues were separated by 4 amino acids (YXXXXX). MS/MS analysis confirmed that tyrosine was always the site of chlorination. When the peptides were lipid-associated, those containing YXXXY motifs were most susceptible to chlorination, perhaps because of increased a-helical structure. These observations indicate that lysine residues permit the chlorination of tyrosine in synthetic peptides that mimic a region of apolipoprotein A-I that is normally resistant to chlorination. Chlorination of lipid-associated peptides was maximal when the lysine residue was 2 or 3 amino acid residues away from the tyrosine residue.

**DISCUSSION**

Our results provide strong evidence that lysine residues are involved in the regiospecific formation of 3-chlorotyrosine when peptides or proteins containing tyrosine are exposed to HOCl. They also indicate that chloramines are central to the reaction pathway. Thus, specific amino acid sequences (YXXXK and XXXXY motifs) directed the regioselective chlorination of synthetic peptides by HOCl in high yield. Moreover, the single major site of 3-chlorotyrosine formation in apolipoprotein A-I of HDL resided in a YXXXK motif. Remarkably, 50% of the tyrosine residues in this YXXXK motif were chlorinated when the molar ratio of HOCl to protein-bound amino acid residues in HDL was 0.125, indicating that this region of the protein was targeted selectively for oxidation. Collectively, these observations indicate that the proximity of tyrosine to lysine, which is able to form chloramines, is likely to be one important factor that determines the sites at which HOCl can chlorinate tyrosine residues in proteins.

We used synthetic peptides containing tyrosine to explore the mechanism by which specific sequence motifs direct chlorination by HOCl. Because of our results with apolipoprotein A-I and because primary amino groups form relatively stable chloramines, which can be potent chlorinating reagents (7, 37, 38), we also included lysine residues in some of the peptides. When we used the lysine-containing peptides YXXKY and YXKKX, the yield of 3-chlorotyrosine (relative to HOCl) exceeded 65%. Moreover, a single tyrosine residue was preferentially chlorinated with 50–80% yield. Significantly, a tyrosine residue located 2 amino acids away from a lysine residue was chlorinated in much higher yield than a tyrosine immediately adjacent to a lysine. In contrast, chlorination of tyrosine was not regiospecific in the peptide YXXXY, which lacked a lysine and therefore a primary Nα amino group. Moreover, the product yield for chlorination of each tyrosine residue in this peptide was <15%, demonstrating that YXXXY was a much poorer substrate for chlorination than the lysine-containing peptides. It is noteworthy that equal amounts of 3-chlorotyrosine and 3,5-dichlorotyrosine formed in YXXXY (CIYXXXK, C15YXXXK, YXXCYY, and YXXCYY) but that chlorination did not modify two different tyrosine residues in the same molecule (CIYXXXK). Also, we failed to detect tyrosine chlorination when we added HOCl to a mixture of N-acetylated amino acids that mimicked the composition of the lysine-containing YXXXY and YXKKK peptides. This strongly suggests that lysine must be an integral part of a peptide to direct the chlorination of tyrosine in high yield.

Primary amino groups react rapidly with HOCl to form chloramines (37, 38, 57). Chloramines located on carbon atoms that lack a carboxylic acid group are long lived and thus could be biologically relevant oxidizing intermediates (19, 27, 57). Indeed, our HPLC procedure isolated a relatively stable oxidation product from the reaction of HOCl with peptide YXXXY that was converted back to the precursor peptide by methionine. MALDI-TOF-MS analysis revealed the presence of three species in the methionine-sensitive oxidation product: precursor peptide, monochloramine, and dichloramine. This suggests
that molecular decomposition and rearrangement during ionization by MALDI allow chlorine to be exchanged among different species during the analysis (37, 44). NMR analysis confirmed that the oxidized intermediate was chlorinated on the N-terminal amino group of its lysine residue. Analysis of the progress curve of the reaction of YXXKY with HOCl revealed that the initial step in the reaction generated a high yield of peptide-bound chloramine, which disappeared as products containing chlorinated tyrosine appeared. These observations provide strong evidence that the reaction pathway for tyrosine chlorination involves the rapid initial formation of a chloramine on the ε-amino group of the lysine residue followed by a slower reaction in which the chloramine attacks the aromatic ring of tyrosine. It is noteworthy that the stoichiometry of peptide chlorination implicates a dichloramine as the intermediate in the reaction pathway and that our NMR and MS analyses detected dichlormamines in the methionine-sensitive oxidation product. These observations support the proposal that a dichloramine is the chlorinating intermediate in the reaction pathway (44). Because the rate constant for the reaction of HOCl with lysine is relatively high ($k_2 = 5 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$), the ε-amino group of lysine (and the free amino group at the N-terminus of protein) together with the thiol residues of cysteine and methionine ($k_2 = 3.0 \times 10^7$ and $3.8 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$, respectively) might be initial primary targets when HOCl oxidizes proteins (18, 42, 59).

It is noteworthy that 50% of the Tyr$^{192}$ residues in apolipoprotein A-I were chlorinated when the molar ratio of HOCl to protein-bound amino acid residues of HDL was 1 to 8. The reason why Tyr$^{192}$ is so much more amenable to chlorination than the two other tyrosine residues (Tyr$^{115}$ and Tyr$^{236}$) that also reside in a YXXK domain might relate to differences in spatial orientation, solvent accessibility, and the local amino acid environment. Importantly, Tyr$^{115}$ lies in close proximity to Met$^{112}$. The alkylated thiol of methionine is extremely reactive with lysine and the primary amino groups of lysine and protein-bound methionine residues have been proposed to act as local antioxidants (60). Thus, Met$^{112}$ might scavenge HOCl in this region of apolipoprotein A-I. Indeed, Met$^{112}$ is one of the primary targets for oxidation by HOCl in apolipoprotein A-I (34).

Another key factor controlling the reactivity of protein-bound amino acids is local secondary structure. Apolipoprotein A-I contains 10 antiparallel amphipathic α-helices that promote lipid association and are critical for removing cholesterol from cells (51, 52). The helical wheel representation of amphipathic helices predicts that tyrosine and lysine residues in the YXXK (and KXXX) motif will lie next to each other on the same face of the α-helix (51). Both the helical wheel representation (Fig. 1B) and the crystal structure of lipid-free apolipoprotein A-I (61) indicate that LAEYHAK, which includes the single tyrosine residue in apolipoprotein A-I that was chlorinated in high yield (50%), contains the tyrosine and lysine residues in close proximity. Importantly, the α-carbons of the 2 amino acid residues are separated by ~4.5 Å, which suggests that the phenolic ring and primary amino groups of the tyrosine and lysine residues in the YXXK/KXXX motif can interact chemically. The crystal structure of lipid-free apolipoprotein A-I reveals that Tyr$^{192}$ lies in the middle of an amphipathic α-helix, whereas Tyr$^{236}$ is located at the end of an amphipathic α-helix (61), where a less orderly structure might hamper the interaction of the lysine and tyrosine residues and the production of 3-chlorotyrosine.

Molecular modeling indicates that tyrosine and lysine residues that are separated by 3 other amino acids also lie next to one another on the same face of an α-helix. Because their α-carbons are separated by ~6 Å, their side chains might be able to interact chemically in YXXK/KXXX motifs. However, only one tyrosine in apolipoprotein A-I residues in such a motif. Therefore, it is difficult to use the intact molecule to explore the role of YXXK/KXXX in tyrosine chlorination. Instead, we synthesized the model peptide Ac-FYSDLQRLAARLE-NH$_2$ (YXXKY) and exposed it to HOCl. In parallel studies, we determined whether lipid association makes this peptide more amenable to chlorination. We selected this particular sequence because it mimics a region of apolipoprotein A-I that contains a tyrosine residue that is resistant to chlorination. When we exposed the peptide to HOCl, it was chlorinated in low yield.

When we introduced a lysine residue into the peptide at a position that was 2 or 3 amino acids away from the tyrosine residue (YXXKX and YXXXX), the lipid-free and lipid-associated peptides were chlorinated in high yield. These observations suggest that the YXXK/KXXX motif might also promote the chlorination of tyrosine residues in α-helices. We also observed a significant level of chlorination when the lysine and tyrosine residues were separated by 4 amino acids (YXXXXK) and therefore would be expected to lie on opposite sides of an α-helix. It is possible that chlorination was feasible because the side chains were long enough to permit the phenolic ring of tyrosine and the primary amino groups of lysine to interact. Alternatively, a conformational change might have brought the two groups together; synthetic peptides are very flexible, and the ones we studied are predicted to reside in an α-helix only ~40% of the time (56). In future studies, it will be important to determine whether lysine residues promote the chlorination of tyrosine residues when the two are separated by 3 or 4 amino acids in a well-defined α-helical structure.

Primary sequence and secondary structure alone do not determine the local structure of a protein, however, and many other factors could correctly orient the Nε chloride of lysine (or an ε-carboxylic chloride of the N-terminus of a protein) to the phenolic ring of tyrosine. For example, the tertiary structure might juxtapose residues that lie far apart in the primary sequence or secondary structure. It is even possible that lysine residues in one protein could facilitate the chlorination of tyrosine residues in a different but closely associated protein. Features of tertiary structure might also explain why Tyr$^{29}$ of apolipoprotein A-I was chlorinated although it does not reside in a YXXK motif and why Tyr$^{115}$, which does lie in a YXXK motif, was not chlorinated. For example, Tyr$^{29}$ lies in a globular region of apolipoprotein A-I and might interact with a primary amino group in another region of the protein. In addition, there might be a difference in solvent accessibility or an association of the side chains of Tyr$^{115}$ or its adjacent lysine with lipid.

Tyrosine 192 and 236 are located in amphipathic helices 8 and 10 of the C terminus of apolipoprotein A-I; these helices are essential for interactions with lipid (52, 62). It will be interesting to determine whether chlorination of these tyrosines alters the biochemical or biophysical properties of apolipoprotein A-I and its ability to mobilize cholesterol from cells. It also will be important to determine whether chlorination of specific protein residues by phagocytes is important for killing bacteria or damaging host tissue although only ~1 in 1,000–5,000 tyrosine residues is modified (13, 14, 63, 64). Presumably, loss of specific tyrosine residues that are critical to protein integrity or function could be deleterious to bacteria or host tissues despite the small absolute level of tyrosine chlorination.

Based on our findings, we propose that the primary ε-amino group of lysine facilitates the regioselective chlorination of tyrosine residues in proteins by a pathway involving a chloramine intermediate. Modeling and structural studies indicate that tyrosine and lysine residues separated by two amino acids are adjacent on the same face of an α-helix, suggesting that the YXXK motif could direct protein chlorination if it resided in an
α-helix. Consistent with this proposal, we found that a single tyrosine residue in the eighth amphipathic α-helix of apolipoprotein A-I was the major site of chlorination by HOCl and that this tyrosine resided in the YXXX motif. Moreover, additional structural features might also juxtapose primary amino groups and tyrosine residues in proteins to permit site-specific chlorination of tyrosine residues. It will be of great interest to determine whether regiospecific chlorination can affect protein function and to investigate the possible role of this reaction mechanism in the chlorination of proteins in vivo.

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