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Evaluation of Histidine Reactivity and Byproduct Formation during Peptide Chlorination

Jong Kwon Choe,[#] Lap-Cuong Hua,[#] Yukako Komaki, Adam M.-A. Simpson, Daniel L. McCurry, and William A. Mitch*

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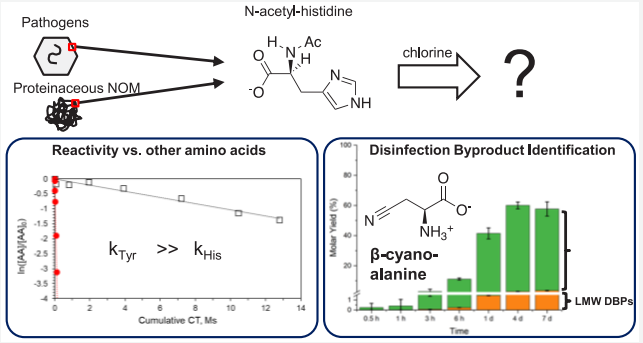
ABSTRACT: The covalent modifications resulting from chlorine reactions with peptide-bound amino acids contribute to pathogen inactivation and disinfection byproduct (DBP) formation. Previous research suggested that histidine is the third most reactive of the seven chlorine-reactive amino acids, leading to the formation of 2-chlorohistidine, 2-oxohistidine, or low-molecular-weight byproducts such as trihalomethanes. This study demonstrates that histidine is less reactive toward formation of chlorine transformation products (transformation time scale of hours to days) than five of the seven chlorine-reactive amino acids, including tyrosine (transformation time scale of minutes). Chlorine targeted tyrosine in preference to histidine within peptides, indicating that chlorine reactions with tyrosine and other more reactive amino acids could contribute more to the structural modifications to proteins over the short time scales relevant to pathogen inactivation. Over the longer time scales relevant to disinfection byproduct formation in treatment plants or distribution systems, this study identified β-cyanoalanine as the dominant transformation product of chlorine reactions with peptide-bound histidine, with molar yields of ∼50% after 1 day. While a chlorinated histidine intermediate was observed at lower yields (maximum ∼5%), the cumulative concentration of the conventional low-molecular-weight DBPs (e.g., trihalomethanes) was ≤7%. These findings support the need to identify the high-yield initial transformation products of chlorine reactions with important precursor structures to facilitate the identification of unknown DBPs.



INTRODUCTION

Chlorine reactions with peptides and proteins are important for both pathogen inactivation and disinfection byproduct (DBP) formation. Chlorine reactions with the viral capsid proteins in MS2 bacteriophage accounted for ∼45% of the MS2 inactivation.[1] The covalent modifications to amino acid side chains alter their interactions within proteins, disrupting protein structure and function.[2,3] The dissolved organic matter (DOM) serving as the precursor for DBPs in drinking water supplies ultimately derives from biomolecules. Recent DBP research has focused on nitrogen-based DBPs (N-DBPs) as important contributors to the DBP-associated toxicity of disinfected waters, based upon their higher toxic potency-weighted concentrations relative to carbon-based DBPs, including regulated trihalomethanes (THMs) and haloacetic acids.[4−8] Among biomolecules contributing to DOM, protein is an important N-DBP precursor, since protein accounts for the majority of organic nitrogen in cell mass (e.g., ∼55% in *Escherichia coli*[9]).

Microbial biomass and DOM are complex mixtures of components that compete to react with chlorine. A better characterization of which amino acid constituents of peptides are most reactive with chlorine would facilitate a mechanistic understanding of microbial inactivation and the identification of important DBPs. While the α-amino group of free amino acids is highly reactive with chlorine,[10−12] the peptide bond in peptides and proteins deactivates the α-amino group toward reaction with chlorine.[11,13] Most amino acids in both biomass and DOM (e.g., ∼90% in drinking water supplies[14]) are combined within peptides and proteins. Using *N*-acetylated amino acids to mimic peptide bonds, previous research identified seven amino acids whose side chains were more reactive with chlorine than peptide bonds: *N*-acetyl-methionine (3.8×10^7 M^{-1} s^{-1}) > *N*-acetyl-cysteine (3.0×10^7 M^{-1} s^{-1}) > *N*-acetyl-histidine (1.0×10^5 M^{-1} s^{-1}) > *N*-acetyl-tryptophan (1.1×10^4 M^{-1} s^{-1}) > *N*-acetyl-lysine (5.0×10^3 M^{-1} s^{-1}) > *N*-acetyl-tyrosine (44 M^{-1} s^{-1}) > *N*-acetyl-arginine

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($26\ M^{-1}\ s^{-1}$); the rate constants with peptide bonds and other *N*-acetylated amino acids were $<10\ M^{-1}\ s^{-1}$.[11,13] While this research would suggest that histidine should be more reactive than tyrosine toward forming transformation products, it was based upon changes in UV spectra after mixing the *N*-acetylated amino acids with HOCl in a stopped-flow apparatus; the changes were attributed to the formation of chloramines, but specific products were not identified. Other research involving HOCl treatment of proteins, such as lysozyme,[15,16] adenylate kinase,[16] and ribose binding protein,[16] quenching of the total chlorine residual, and characterization of the residual amino acids, has suggested that tyrosine degradation precedes histidine degradation.

The covalent modifications to amino acids resulting from chlorination alter their interactions within proteins, contributing to the loss of structure and function,[2,3] and pathogen inactivation. These same transformation products are disinfection byproducts. In both contexts, it is important to identify the predominant products. DBP research remains focused on low-molecular-weight DBPs (e.g., THMs, haloacetonitriles). Despite identifying more than 600 DBPs,[17] these DBPs account for only ∼30% of the total organic halogen (TOX) in chlorinated waters.[18] Molar yields of these low-molecular-weight DBPs per amino acid residue during chlorination typically are <1%.[19−23] Liberation of one- or two-carbon products from these larger structures would require substantial cleavage of carbon−carbon bonds. Carbon−carbon bond cleavage reactions typically feature low yields. For example, we found that ozonation, followed by chlorination converts the side chain of lysine into dichloronitrolysine, only liberating trichloronitromethane (chloropicrin) when high hydroxyl radical production during ozonation favors carbon−carbon bond cleavage.[24] While DBP yields from free amino acids are higher in some cases (e.g., ∼3% dichloroacetic acid[19] and ∼3% dichloroacetonitrile[23] from free histidine), yields typically are lower for *N*-acetyl-amino acids due to deactivation of the α-amino group.[25] Of the seven most reactive amino acids, the high-yield (>10%) chlorine transformation products, typically representing initial transformation products, have been identified for methionine (methionine sulfoxide), cysteine (cysteic acid), tyrosine (3-chlorotyrosine and 3,5-dichlorotyrosine), and lysine (lysine nitrile).[2,25−27] Recently, we characterized seven initial transformation products of *N*-acetyl-tryptophan, including two chlorinated products, at combined yields much higher (∼25−100%) than the combined yields of low-molecular-weight DBPs (∼1−20%).[28] While previous researchers have hypothesized that 2-oxohistidine and 2-chlorohistidine are significant products of histidine chlorination,[26,29,30] the dominant chlorine transformation products of histidine and arginine have not been identified.

Given the conflicting results between the high reactivity of chlorine with histidine as measured by changes in UV spectra[11,13] and the low transformation of histidine within three proteins,[15,16] the first objective was to re-evaluate histidine reactivity both by directly measuring the degradation rate constant of *N*-acetyl-histidine after a reaction with HOCl and by comparing the degradation of histidine to tyrosine within peptides. The limited histidine degradation observed in chlorinated proteins may have resulted from either innate resistance of histidine to chlorine-mediated transformation or from protein-specific differences in the geometrical arrangement of amino acids, resulting in reduced access of chlorine to histidine. We focused on short peptides to avoid these geometry-associated access restrictions. The second objective of this study was to determine the high-yield reaction products of HOCl reactions with *N*-acetyl-histidine as a model for peptide-bound histidines. The third objective was to compare the yields of newly identified products with those of the low-molecular-weight DBPs of current research interest.

MATERIALS AND METHODS

Reagents. Sodium hypochlorite (∼6%, w/v; Fisher Scientific), *N*-acetyl-histidine (*N*-Ac-His, 99%; Acros), β-cyano-L-alanine (Chem-Impex International, Wood Dale, IL) (98%), 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC, 95%; Chemodex Ltd., Switzerland), 3,5-dichloro-L-tyrosine (98%; Ark Pharm, Inc., Arlington Heights, IL), and *N*-acetyl-L-tyrosine (*N*-Ac-Tyr; 99%), *N*-acetyl-phenylalanine (*N*-Ac-Phe; 99%), *N*-acetyl-methionine (*N*-Ac-Met; 98.5%), 3-chlorotyrosine (97%), an amino acid standard solution (consisting of 2.5 ± 0.1 mM each of L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine), sodium phosphate monobasic dihydrate (99%), sodium phosphate dibasic (99%), sodium thiosulfate (99%), and sodium hydroxide (98%) (Sigma-Aldrich) were used without further purification. Deionized water (electrical resistivity > 18.0 MΩ cm; Milli-Q purification system; Millipore) was used in all experiments. Concentrations of HOCl stock solutions were standardized using UV−vis spectrophotometry at 292 nm ($365\ M^{-1}\ cm^{-1}$[31]).

N-Acetyl-β-cyanoalanine was synthesized according to the procedure of Kuvaeva et al.[32] Briefly, 870 μL (9.2 mmol) of acetic anhydride was added dropwise to 0.7 g (6.1 mmol) of β-cyanoalanine (6.1 mmol) in 4 mL of water at pH 8 and 15 °C. After 1 h, hydrochloric acid was added to reduce the pH to ∼2. The solution was extracted into 4 mL of methyl *tert*-butyl ether (MtBE) by manual shaking for 2 min. The MtBE was evaporated under nitrogen blowdown and then lyophilization, forming white crystals. Figures S1 and S2 provide a liquid chromatography−mass spectrometry (LC−MS) chromatogram, and LC−MS and LC−MS/MS mass spectra for the purified product.

N-Acetylated peptides were synthesized and prepared using the Symphony/Multiplex TM automated peptide synthesizer at the Protein and Nucleic Acid Facility at Stanford University. These peptides featured histidine (H) and tyrosine (Y) separated by different numbers of other amino acid residues (x) whose side chains are poorly reactive with chlorine (i.e., glycine (G), glutamic acid (E), and alanine (A)), including *N*-Ac-GYHEAE (xYHxxx), *N*-Ac-GYEHAE (xYxHxx), *N*-Ac-GYEAHE (xYxxHx), and *N*-Ac-GYEAEH (xYxxxH).

Chlorination Experiments. Experiments to determine the HOCl reaction rate constants with *N*-Ac-Tyr and *N*-Ac-His were conducted in duplicate in a 5 mM phosphate-buffered aqueous solution at pH 7.4 at room temperature in the dark. HOCl (0−200 μM) was added to 10 μM *N*-Ac-Tyr or *N*-Ac-His (HOCl/amino acid molar ratios of 0−20) in 100 mL solutions in borosilicate glass reagent bottles. Aliquots (5 mL) were withdrawn over the course of 96 h for measurement of the total chlorine residual by the DPD colorimetric method.[33] Additional aliquots (1 mL) were collected for analysis of the residual *N*-Ac-Tyr or *N*-Ac-His after quenching the total chlorine residual by addition of *N*-Ac-Met at 1.2 times the

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molar concentration of oxidants applied. While ascorbic acid is frequently used to quench residual chlorine for analysis of DBPs,[5−7] its co-elution with *N*-Ac-His prevented analysis of residual *N*-Ac-His during analysis by LC−MS. *N*-Ac-Met and its oxidation product (*N*-Ac-Met sulfoxide) did not interfere with LC−MS analysis and methionine has been used previously to quench residual chlorine for analysis of amino acids.[34]

For peptide chlorination, a 300 μM peptide was treated with 0−600 μM HOCl (HOCl/peptide molar ratios of 0−2) in 20 mL solutions buffered at pH 7.4 with 10 mM phosphate buffer for 15 min in duplicate. Because byproducts were analyzed during these experiments, the residual chlorine was quenched with ascorbic acid. One half of each solution was subjected to acid-catalyzed hydrolysis to liberate and analyze the individual amino acid residues after derivatization (see below). The other half of each solution was analyzed by LC−MS without digestion to measure residual parent peptides. The parent peptides and the liberated, derivatized amino acids featured greater separation via liquid chromatography, avoiding interference by ascorbic acid. For the *N*-Ac-xYxxHx motif peptide, 1700 μM peptide was also treated with equimolar HOCl for 1−60 min in duplicate for analysis by LC−MS/MS; the higher concentrations facilitated analysis of the product fragments produced during tandem mass spectrometry. The residual chlorine was not quenched, but the samples were injected into the LC-MS/MS within 1 min of sample collection. This halted reactions via chromatographic separation of the chlorine and the peptide while enabling the observation of chloramine intermediates.

Experiments evaluating byproduct yields from the chlorination of *N*-Ac-His were conducted under headspace-free conditions in 60 mL borosilicate glass vials capped with Teflon-lined septa in triplicate. HOCl (50−500 μM) was added to 50 μM *N*-Ac-His in 5 mM phosphate buffer adjusted to pH 5.5−8.5 with hydrochloric acid or sodium hydroxide. Solutions were held in the dark at room temperature (22 °C). Samples were sacrificed after various reaction times (30 min to 7 days). Aliquots (5 mL) were withdrawn for measurement of the total chlorine residual. The remaining sample volume was treated with ascorbic acid (equimolar relative to the initial chlorine concentration) to quench the total chlorine residual. While ascorbic acid inhibited the detection of residual *N*-Ac-His, it did not interfere with the ability to detect *N*-Ac-His byproducts, including *N*-acetyl-β-cyanoalanine and low-molecular-weight byproducts. Aliquots (1 mL) were spiked with *N*-Ac-Tyr as an internal standard for the evaluation of nonvolatile byproducts (e.g., *N*-acetyl-β-cyanoalanine) by liquid chromatography mass spectrometry (LC−MS). The remaining sample volume was extracted into MtBE following modified US EPA Methods 551.1 to evaluate low-molecular-weight byproduct formation by gas chromatography mass spectrometry (GC-MS) with ∼0.2 μg/L reporting limits.[35,36] Because experiments were conducted in deionized water without bromide, only chlorinated DBPs were measured, including one THM (chloroform), two haloacetonitriles (dichloroacetonitrile and trichloroacetonitrile), one haloacetaldehyde (trichloroacetaldehyde), two haloketones (1,1-dichloropropanone and 1,1,1-trichloropropanone), and one halonitromethane (chloropicrin).

Peptide Digestion. To quantify the oxidation of histidine and tyrosine residues in peptides, amino acids were liberated from the peptides by acid-catalyzed hydrolysis.[37] Briefly, 0.5 mL aliquots of peptide-containing samples were mixed with 0.5 mL of 8 N methanesulfonic acid in a 2 mL scintillation tube. After adding 0.2 wt % tryptamine to the 1 mL solution, the scintillation tube was placed on a heating block for 24 h at 115 °C within an anaerobic glovebox. Following hydrolysis, 1 mL of 4 N NaOH was added to neutralize the solution. The digested amino acids were then derivatized using AQC for analysis by LC−MS.[38] The digested peptide solution (20 μL) was mixed with 20 μL of 10 mM AQC-containing acetonitrile solution and 60 μL of 0.6 M borate buffer solution (pH 8.8) and heated at 55 °C for 10 min before the analysis.

Analyses. Amino acids and peptides were analyzed using an Agilent 1260 HPLC system coupled with a 6460 triple quadrupole mass spectrometer and equipped with an Agilent Poroshell 120 EC-C18 column (3 mm × 50 mm, 2.7 μm). *N*-Ac-His and *N*-Ac-Tyr were quantified via LC−MS in the negative ion mode using selected ion monitoring with *N*-Ac-Phe as the internal standard for quantification of *N*-Ac-His and *N*-Ac-Tyr loss or full-scan mode with *N*-Ac-Tyr as the internal standard for the identification and quantification of *N*-Ac-His byproduct formation. The AQC-derivatized amino acids liberated from peptides were quantified via liquid chromatography tandem mass spectrometry (LC−MS/MS) using electrospray ionization in the negative ion mode with *N*-Ac-Phe as an internal standard. Whole peptides were characterized by LC−MS/MS in the positive ion mode, which featured higher sensitivity. First, the full-scan mode was used (mass range 100−1000 m/z) to detect product peptides formed by chlorination. Three major peaks were observed (747, 781, and 815 m/z). These major peaks were further analyzed using the product ion scan mode, wherein the m/z of each of the three major peaks served as a precursor ion, and the product fragments were detected by scanning from 50-M m/z, where M corresponds to the m/z of the precursor ion. The fragmentor voltage was set at 84 V and the collision energy was set to 30 eV. High-performance liquid chromatography (HPLC) eluents and gradient profiles are provided in Text S1.

RESULTS AND DISCUSSION

***N*-Ac-His vs *N*-Ac-Tyr Degradation Kinetics.** The loss of 10 μM *N*-Ac-His or *N*-Ac-Tyr after treatment with HOCl (0, 10, 30, 50, 100, and 200 μM) was measured at pH 7.4 over 4 days. Figures S3 and S4 provide examples for the loss of the total chlorine residual and total *N*-Ac-Tyr and *N*-Ac-His (i.e., after quenching the chlorine residual to revert any *N*-Ac-Tyr or *N*-Ac-His chloramines back to the parent compounds) over time for a 5:1 molar ratio of chlorine to the *N*-acetylated amino acid. For *N*-Ac-Tyr, both the total chlorine residual and *N*-Ac-Tyr declined over a 1-h time scale. However, for *N*-Ac-His, both *N*-Ac-His and the total chlorine residual declined over a 4-days time scale. Since HOCl concentrations declined over time, the integrated exposure to chlorine ($\sum[\mathrm{HOCl}]\Delta t$) was used to determine the second-order rate constants ($k_{2,\mathrm{HOCl}}$) for *N*-Ac-His and *N*-Ac-Tyr transformation by HOCl according to eq 1, where $[AA]_0$ and $[AA]_t$ are the concentrations of the *N*-acetylated amino acids at time 0 and time t, respectively (Figures S3 and S4). Using results from 1, 3, 5, 10, and 20 chlorine to *N*-acetylated amino acid molar ratios (each conducted in duplicate; Table S1), the second-order rate constants for amino acid degradation were 0.12 (± 0.026 standard deviation) M^{-1} s^{-1} for *N*-Ac-His and 27.3 (± 1.7) M^{-1} s^{-1} for *N*-Ac-Tyr. The $k_{2,\mathrm{HOCl}}$ measured in this study for *N*-Ac-Tyr degradation is comparable to the value (44 M^{-1} s^{-1})

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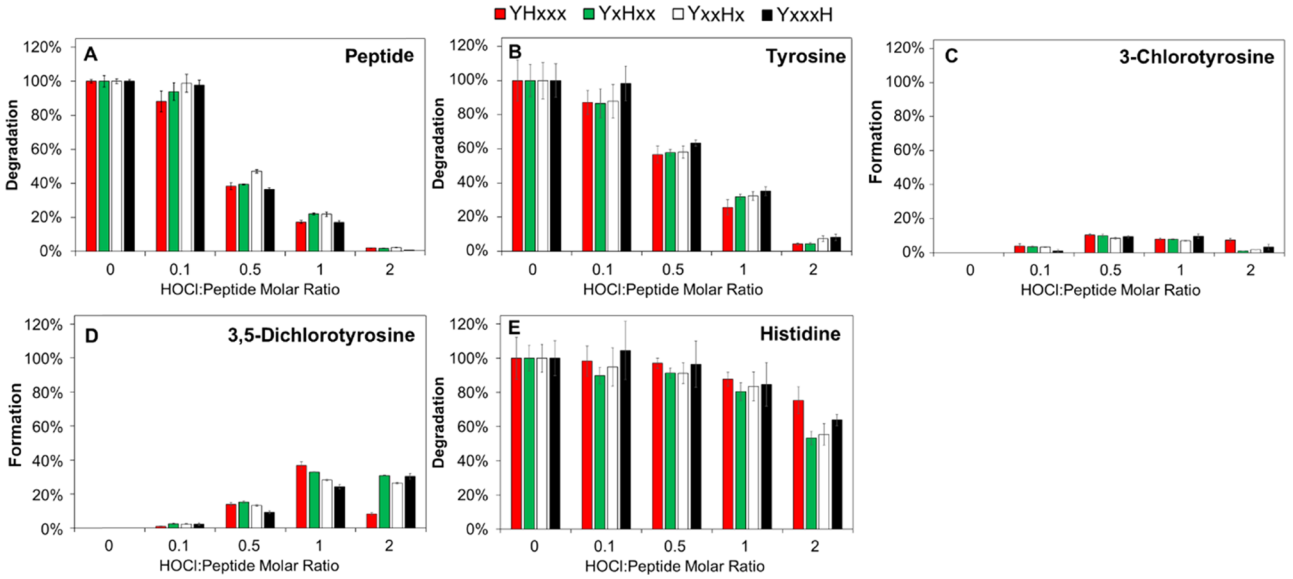

Figure 1. Degradation of (A) parent peptides and (B) tyrosine, formation of (C) 3-chlorotyrosine and (D) 3,5-dichlorotyrosine, and (E) degradation of histidine during treatment of 300 μM peptides with different HOCl/peptide molar ratios for 15 min at pH 7.4 and then quenching the residual chlorine. In each case, measured peptide or amino acid concentrations are expressed as a percent of the molar concentrations of the respective peptides, tyrosines, or histidines in the parent peptides. Tyrosine, chlorotyrosines, and histidine were measured after liberation from peptides by acid digestion. Error bars represent the range of duplicate experiments.

reported based upon changes in UV spectra,[11] suggesting that the initial reaction involves chlorination of the tyrosine side chain, to form the known products 3-chlorotyrosine and 3,5-dichlorotyrosine.[2,16,26] In contrast, the $k_{2,\mathrm{HOCl}}$ determined for *N*-Ac-His degradation is 6 orders of magnitude lower than the value (1.0×10^5 M^{-1} s^{-1}) reported based on changes in UV spectra.

$$\ln\left(\frac{[AA]_t}{[AA]_0}\right) = -k_{2,\,\mathrm{HOCl}} \sum_i [\mathrm{HOCl}]_i \Delta t_i \quad (1)$$

This difference suggests the initial rapid formation of a UV-absorbing chloramine associated with the imidazole ring in the histidine side chain. Indeed, the UV spectrum that we obtained 10 min after mixing 0.65 mM HOCl with 1.26 mM *N*-Ac-His (without quenching the chlorine residual) was significantly different from that expected by superimposing the two spectra of the separate reagents (Figure S5), supporting a rapid reaction between HOCl and *N*-Ac-His to form a chloramine. The application of *N*-Ac-Met to quench total chlorine residuals in our *N*-Ac-His degradation experiments would quench the chloramines, such that they would revert to the parent *N*-Ac-His and no rapid transformation of *N*-Ac-His would be observed. Thus, while previous research suggested that histidine is the third most reactive amino acid with chlorine based upon changes in UV spectra, our results indicate that this reaction involves reversible histidine chloramine formation. In contrast, histidine is poorly reactive (i.e., 2 orders of magnitude less reactive than tyrosine) with respect to the permanent, covalent modifications leading to DBP formation.

Indeed, there have been some suggestions that the histidine chloramine intermediates promote the degradation of other amino acid residues (e.g., tyrosines) by serving as chlorine-transfer agents, regenerating the parent histidine rather than proceeding to histidine degradation.[13,39,40] This suggestion is based on other research demonstrating that lysine chloramines promote the formation of chlorotyrosines by serving as chlorine-transfer agents.[2,34] Based on changes in UV spectra, previous research measured a first-order rate constant for *N*-Ac-His chloramine decay (k_1) of 6.4×10^{-5} s^{-1},[40] indicating a characteristic time (i.e., $\frac{1}{k_1}$) of 4.3 h. Similarly, previous research measured second-order reaction rate constants (k_2) for *N*-Ac-His chloramine with *N*-Ac-Tyr of 9 M^{-1} s^{-1} and of 2.8×10^3 M^{-1} s^{-1} with an indole mimic of the tryptophan side chain.[13] For 10 μM *N*-Ac-Tyr, the characteristic time for chlorine transfer (i.e., $\frac{1}{k_2[\mathrm{N-Ac-Tyr}]}$) would be 3.1 h, comparable to the time scale of *N*-Ac-His chloramine decay. After rapid *N*-Ac-His formation, these results would suggest that significant degradation of *N*-Ac-His should be observed even when the presence of *N*-Ac-Tyr promotes chlorine transfer and regeneration of the parent *N*-Ac-His. In contrast, the 0.12 M^{-1} s^{-1} rate constant that we measured between *N*-Ac-His and HOCl leading to *N*-Ac-His decay indicates a characteristic time (i.e., $\frac{1}{k_2[\mathrm{HOCl}]}$) for 50 μM HOCl of 46 h. Since this characteristic time is greater than that for chlorine transfer from *N*-Ac-His chloramine to *N*-Ac-Tyr, *N*-Ac-His degradation would be expected to be minor in the presence of *N*-Ac-Tyr, even if *N*-Ac-His chloramine rapidly formed. Our analyses involved direct measurement of total *N*-Ac-His by mass spectrometry after quenching *N*-Ac-His chloramine, which reverts *N*-Ac-His to the parent *N*-Ac-His. We take these measurements to be more accurate than previous research based on changes in UV spectra, which are difficult to attribute to specific species.

However, other research has indicated that the loss of histidine residues occurred more readily within the proteins lysozyme and insulin than within mixtures of *N*-acetylated amino acids featuring the same amino acid concentrations,[40] suggesting that histidine degradation competes with chlorine transfer. Resolving this conflict using whole proteins is difficult due to the constraints imposed by the protein-specific differences in the three-dimensional arrangement of amino

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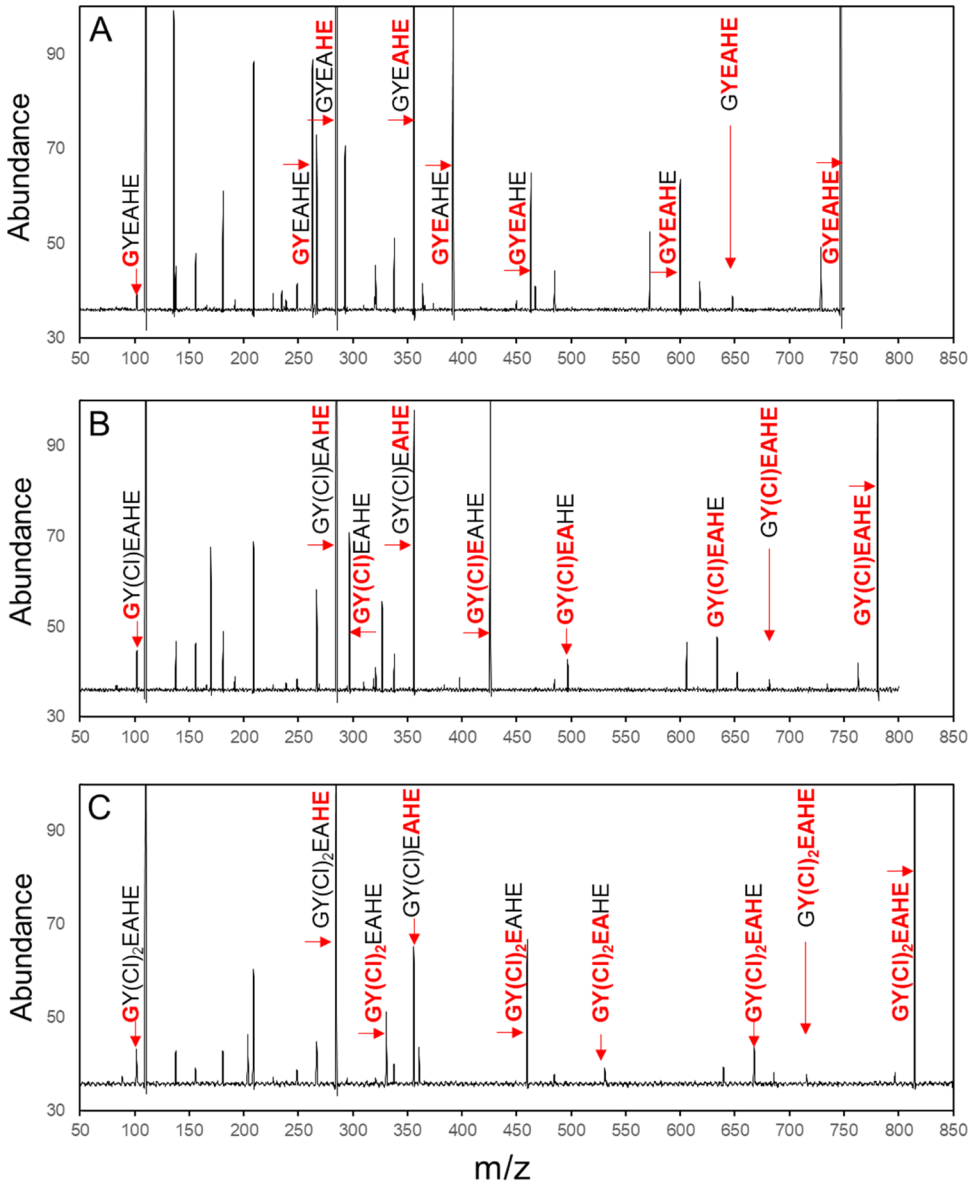

Figure 2. MS/MS spectra from fragmentation of parent ions at (A) m/z 747 (*N*-Ac-GYEAHE), and the two chlorinated products at (B) m/z 781 (*N*-Ac-GY(Cl)EAHE) and (C) m/z 815 (*N*-Ac-GY$(Cl)_2$EAHE), formed after 15 min of treatment of 1700 μM *N*-Ac-GYEAHE at a 1.0 HOCl/peptide molar ratio at pH 7.4. Samples were analyzed at 15 min immediately without quenching the chlorine residual. The red text indicates the portion of the peptide corresponding to the fragment.

acid residues. For example, chlorine transfer from histidine to tyrosine within proteins relies upon the presence of a vicinal tyrosine. In the two proteins examined, it is possible that their three-dimensional structures inhibited contact between tyrosines and histidine chloramines, thereby favoring histidine degradation. Moreover, the microenvironments within proteins created by adjacent amino acid residues may differ from the bulk solution in terms of hydrophobicity and pH, the latter potentially affecting the reactivity of histidine, given that the pK_a of the conjugate acid of the side chain is 6.0.

To avoid protein-specific differences in structure, we conducted additional experiments involving chlorination of four small (six amino acid) peptides, each featuring an *N*-acetylated α-amino terminus and a histidine (H) and a tyrosine (Y) separated by different numbers of other amino acid residues (x) whose side chains are poorly reactive with chlorine, including *N*-Ac-xYHxxx, *N*-Ac-xYxHxx, *N*-Ac-xYxxHx, and *N*-Ac-xYxxxH. The small peptides incorporate differences in the primary structure while avoiding the complexities associated with secondary and tertiary protein structures. The loss of peptides, constituent tyrosines, and histidines, and formation of 3-chlorotyrosine and 3,5-dichlorotyrosine were measured 15 min after application of chlorine to 300 μM peptides at 0.1, 0.5, 1.0, and 2.0 HOCl/peptide molar ratios at pH 7.4 and then quenching residual chlorine with ascorbic acid (Figure 1); individual amino acids and chlorotyrosines were measured after liberation from peptides by acid digestion. Losses of parent peptides were 53−61% at the 0.5 HOCl/peptide molar ratio and ≥98% for the 2.0 HOCl/peptide molar ratio. The losses of tyrosine residues were similar in pattern, although somewhat lower than the losses of parent peptides. For example, tyrosine degradation was ∼40% at the 0.5 HOCl/peptide molar ratio, but still >92% at the 2.0 HOCl/peptide molar ratio. The formation of 3-chlorotyrosine (Cl-Tyr) and

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3,5-dichlorotyrosine (Cl_2-Tyr) increased with the HOCl/peptide molar ratio, with Cl-Tyr formation declining in favor of Cl_2-Tyr as the HOCl/peptide molar ratio increased to 2 (Figure 1). At a 1.0 HOCl/peptide molar ratio, the total chlorotyrosine yield represented ∼40% relative to the initial peptide concentrations and ∼55% relative to the tyrosine degraded, comparable to the ∼50% maximum yield of chlorotyrosines relative to tyrosine degraded observed previously during chlorination of whole proteins.[16] Previous research has demonstrated that chlorotyrosines degrade with increasing chlorine exposure,[16] as additional reactions of chlorine with tyrosine's phenol ring result in ring fragmentation.[41,42]

Histidine degradation was lower, yet still observable, reaching 25−47% at the 2.0 HOCl/peptide molar ratio. The degradation of histidine residues within the peptides was faster than that observed with isolated *N*-Ac-His, but this is partially related to the higher HOCl and peptide concentrations used in these experiments. For example, at the 2.0 HOCl/peptide molar ratio, the characteristic times for degradation of histidine and tyrosine calculated using the rate constants that we determined using *N*-Ac-His and *N*-Ac-Tyr would be 3.9 h and 1 min, respectively. While some differences were observed between peptides that would be consistent with chlorine transfer from histidine chloramine to tyrosine (e.g., lowest histidine and greatest tyrosine degradation at a 2.0 HOCl/peptide ratio for the peptide with histidine adjacent to tyrosine), these differences were relatively minor. Overall, the results confirm that tyrosine is degraded in preference to histidine within peptides.

To further confirm the preferential degradation of tyrosine relative to histidine, 300 μM *N*-Ac-xYxxHx (i.e., *N*-Ac-GYEAHE; *m*/*z* 747) was treated with equimolar HOCl for direct and immediate analysis of amino acid transformations using LC−MS without quenching the chlorine residual. After 1 min of chlorine contact, the dominant product peaks by LC−MS had *m*/*z* ratios of 781 and 815, consistent with mass increases of 34 and 68 amu, respectively, relative to the original peptide. These mass increases correspond to the replacement of one or two hydrogen atoms with corresponding numbers of chlorine atoms, suggesting that the dominant transformation mechanism is chlorine substitution. The mass spectrum for product *m*/*z* 781 also exhibited a peak at *m*/*z* 783 in the ∼3:1 isotopic ratio expected for a compound with one chlorine (Figure S6). Similarly, the mass spectrum for product *m*/*z* 815 also exhibited peaks at *m*/*z* 817 and 819 in the ∼9:6:1 isotopic ratio expected for a compound with two chlorines (Figure S7). The area counts for these products indicated no further increase over the following 30 min, illustrating their rapid formation.

To identify which amino acids were chlorinated, LC−MS/MS (in positive ion mode) was applied to fragment the unchlorinated peptide (*m*/*z* 747) and the products (*m*/*z* 781 and *m*/*z* 815; Figure 2) formed 15 min after HOCl application to the 1700 μM *N*-Ac-xYxxHx peptide at a 1:1 molar ratio. The collision-induced fragmentation occurring during MS/MS analysis results in the dissociation of the peptide bond between amino acids.[34,43] For example, the MS/MS spectrum for the unchlorinated peptide shows peaks at *m*/*z* 102, 263, 392, 463, 600, and 747, corresponding to the following underlined fragments: N-Ac-GYEAHE, N-Ac-GYEAHE, N-Ac-GYEAHE, N-Ac-GYEAHE, N-Ac-GYEAHE, and N-Ac-GYEAHE (Figure 2A). Similarly, the peaks at *m*/*z* 285 and 356 correspond to *N*-Ac-GYEAHE and *N*-Ac-GYEAHE.

MS/MS analysis of the mono-chlorinated peptide (*m*/*z* 781) shows no change in the peak at *m*/*z* 102 (N-Ac-GYEAHE), but the peak at *m*/*z* 263 shifts to *m*/*z* 297 (+34 amu), indicating chlorination ([+^{35}Cl-^{1}H]) of the tyrosine residue (N-Ac-GY(Cl)EAHE; Figure 2B). Note that the lack of a product fragment at *m*/*z* 299 associated with the characteristic chlorine isotope ratio (i.e., 3:1 for 35 and 37 amu) arises from the selection of a specific precursor ion (*m*/*z* 781) containing the 35 amu isotope. All of the remaining fragments retaining the *N*-Ac-GY motif within the original peptide are also shifted by +34 amu, while the peaks at *m*/*z* 285 (*N*-Ac-GYEAHE) and 356 (*N*-Ac-GYEAHE) did not change. Similarly, MS/MS analysis of the di-chlorinated peptide (*m*/*z* 815) shows no change in the peak at *m*/*z* 102 (N-Ac-GYEAHE), while the peak at *m*/*z* 331 represents N-Ac-GY(Cl)$_2$EAHE, a +68 amu shift in the parent N-Ac-GYEAHE fragment at *m*/*z* 263. Again, all other fragments retaining the *N*-Ac-GY motif are shifted by +68 amu, while the fragments representing *N*-Ac-GYEAHE and *N*-Ac-GYEAHE were unchanged (Figure 2C). These results indicate that the dominant products feature one or two chlorines on the tyrosine residue.

Overall, the results indicate that histidine is 2 orders of magnitude less reactive toward degradation by chlorine than tyrosine, which is one of the least reactive of the chlorine-reactive amino acids (as observed by changes in UV spectra[11,13]). Therefore, within peptides, degradation of tyrosines (and other more chlorine-reactive amino acids such as methionine) should precede histidine degradation. Although there was evidence for the formation of histidine chloramine, any conversion of histidine chloramine to degradation products occurred over longer time scales. While it is possible that histidine chloramine transfers chlorine to tyrosines and other chlorine-reactive amino acids, there was no strong evidence that chlorine transfer was a dominant process. Tyrosine degradation was similar for peptides differing in primary structure, where differences in the number of amino acids separating the tyrosine and histidine would be expected to impact the extent of their interactions. Since the reported rate constant for tyrosine chlorination by histidine chloramine (9 M^{-1} s^{-1}[13]) is only somewhat slower than that for the direct reaction of tyrosine with HOCl (27−44 M^{-1} s^{-1}[11]), distinguishing the importance of these two pathways would be difficult. Previous research with whole proteins, including soybean trypsin inhibitor,[15] lysozyme,[15,16] ribose binding protein,[16] adenylate kinase,[16] and bacteriophage MS2 coat protein,[16] consistently demonstrated tyrosine degradation in preference to histidine. The current results with peptides differing in primary structure demonstrate that this preference is general, and not the result of protein-specific interactions between amino acids associated with their geometrical arrangement within proteins.

Histidine Byproducts. HOCl was applied to 50 μM *N*-Ac-His at pH 7.4 at HOCl/*N*-Ac-His molar ratios ranging from 1 to 10 for different contact times, and the chlorine residual was quenched with ascorbic acid. Two products were observed by LC−MS in the full-scan, negative ion mode at *m*/*z* 155 and 230, respectively. The peak at *m*/*z* 155 was identified as *N*-acetyl-β-cyanoalanine (2-acetamido-3-cyanopropanoic acid) by comparison of its retention time and MS and MS/MS mass spectra against a synthesized standard (Figure S2). For each HOCl/*N*-Ac-His molar ratio, molar yields relative to the initial

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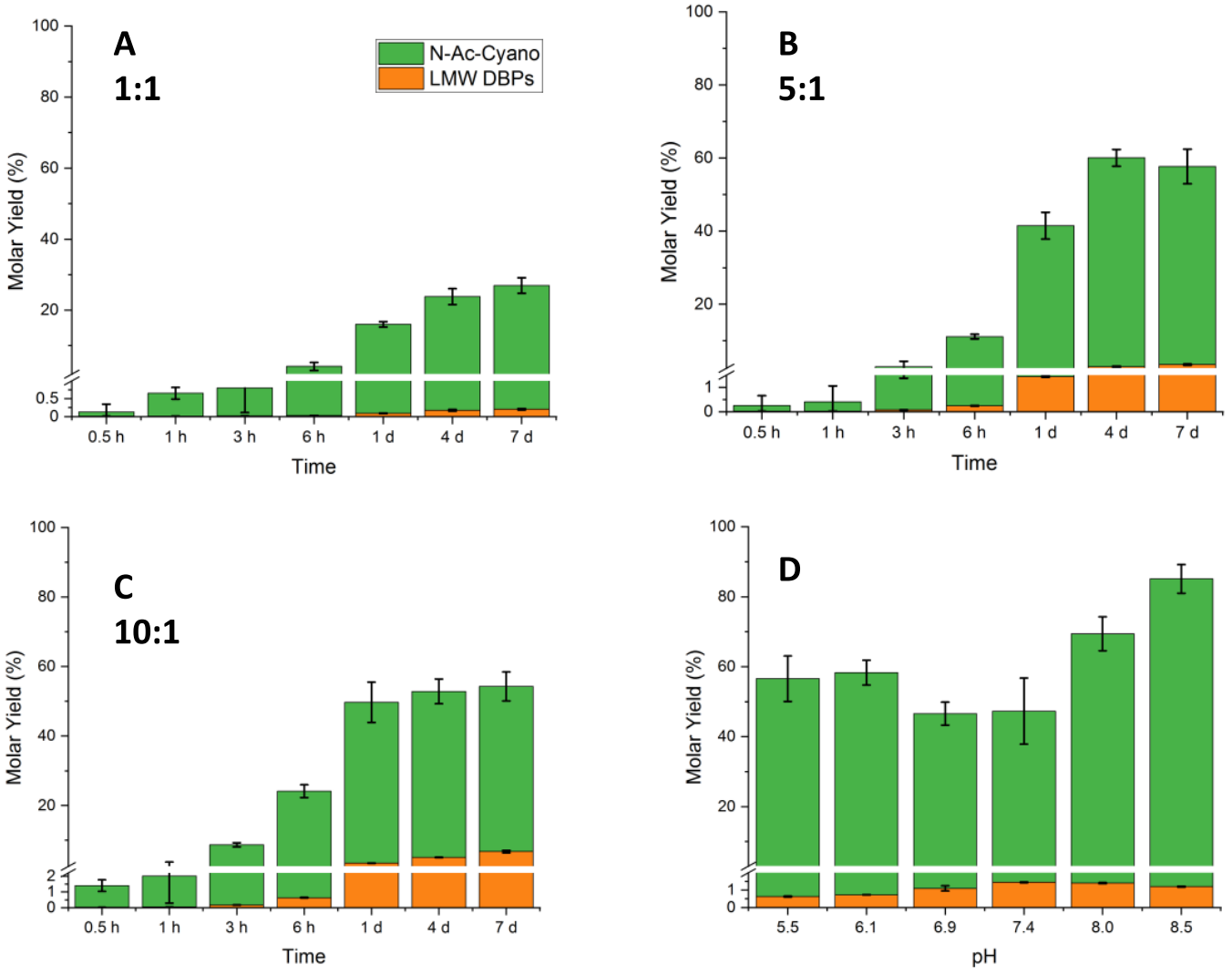

Figure 3. Molar yields of *N*-acetyl-*β*-cyanoalanine (N-Ac-Cyano) and cumulative yield of low-molecular-weight DBPs (LMW DBPs) during chlorination of 50 *μ*M *N*-Ac-His as a function of time at (A) 1:1, (B) 5:1, or (C) 10:1 HOCl/*N*-Ac-His molar ratios at pH 7.4 or (D) as a function of pH 1 day after chlorinating at a 4:1 HOCl/*N*-Ac-His molar ratio. The chlorine residual was quenched with ascorbic acid. Error bars represent the standard deviations of experimental triplicates.

N-Ac-His increased with time, reaching maxima typically after 1 or 4 days (Figure 3A–C for 1, 5, and 10 HOCl/*N*-Ac-His molar ratios and Figure S8 for other molar ratios), leveling out thereafter in concert with the decline in the total chlorine residuals. The total chlorine residuals declined by ∼70% after 1 day and by ≥94% after 4 day, except at the highest (10:1) HOCl/peptide molar ratio, where the total chlorine residual remained at 20% of its initial value, even after 7 day (Figure S9). Yields also increased with HOCl/*N*-Ac-His molar ratios up to 4:1, but leveled out at higher molar ratios at ∼50% after 1 day. *N*-Acetyl-*β*-cyanoalanine yields measured after 1 day were ∼50% for pH 5.5–7.4, but increased to 84% as the pH increased to 8.5 (Figure 3D).

The mass spectrum for the other product peak at *m*/*z* 230 (+34 amu relative to *N*-Ac-His) also featured a smaller peak at *m*/*z* 232 (relative abundance ∼4:1 in Figure S10; Figure S2 provides the MS/MS mass spectrum), characteristic of replacement of a proton with a chlorine atom. The fact that this peak was observed after quenching the total chlorine residual with ascorbic acid indicates that the product was not an *N*-chloramine, but that the chlorine was attached to a carbon. The peak for this product increased over 7 day for HOCl/*N*-Ac-His molar ratios of 1 or 2, but reached maxima after 6 h or 1 day and then declined for the higher HOCl/*N*-Ac-His molar ratios. While a standard for this product was not available, this product was minor relative to *N*-acetyl-*β*-cyanoalanine; using the standard curve for *N*-acetyl-*β*-cyanoalanine to estimate the concentration of the chlorinated product, the maximum molar yield was ∼4% at pH 7.4, observed after 6 h for the 10:1 HOCl/*N*-Ac-His molar ratio. For treatment at the 4:1 HOCl/*N*-Ac-His molar ratio for 1 day at pH 5.5–8.5, the estimated molar yield reached a maximum of ∼5% at pH 6.1 (see Table S2 for estimated concentrations at each sampling point).

Chloroform, chloral hydrate, and dichloroacetonitrile were the only three low-molecular-weight DBPs that were measurable after chlorination of *N*-Ac-His. Their concentrations increased with time and with the HOCl/*N*-Ac-His molar ratio. However, the cumulative molar yield of these low-molecular-weight products reached a maximum of only 6.8% (1.5% chloroform, 4.6% chloral hydrate, and 0.7% dichloroacetonitrile) after 7 day for treatment at a 10:1 HOCl/*N*-Ac-

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His molar ratio at pH 7.4 (Figure 3 and Table S2). These low molar yields are consistent with those observed previously with chlorinated amino acids,[19−23] but well below the yields for *N*-acetyl-*β*-cyanoalanine.

Although the fact that the peak at *m*/*z* 230 increased and then declined over time indicates that it is an intermediate, it is not clear whether it is an intermediate in the pathway leading to *N*-acetyl-*β*-cyanoalanine formation, or toward other products. The lack of other identified intermediates limits the ability to characterize a formation pathway. However, Figure S11 and Text S2 provide a plausible pathway consistent with our results.

Environmental Implications. Proteins are an important target for chlorine reactions during disinfection, with reactions with capsid proteins accounting for ∼45% of the inactivation of MS2 bacteriophage.[1] Identifying which amino acids react with chlorine and the nature of the resulting chemical transformations is key to a mechanistic understanding of how chlorine reactions with proteins lead to inactivation. Previous research involving measurement of changes in UV spectra suggested that histidine was the third most reactive of the seven chlorine-reactive amino acids, and ∼4 orders of magnitude more reactive than tyrosine.[11,13] In contrast, our results indicate that, although histidine may react rapidly with chlorine to form a reversible chloramine, histidine is ∼2 orders of magnitude less reactive than tyrosine in terms of permanent covalent transformations. The high reactivity observed by changes in UV spectra is associated with formation of histidine chloramine that may transfer chlorine to other residues; however, our experiments involving chlorination of peptides differing in the primary structure did not provide strong evidence that such chlorine-transfer reactions are important for altering the order in which amino acids degrade during chlorination. These results suggest that researchers seeking to understand the sequence of events leading to inactivation should focus on chlorine reactions with the more reactive amino acids (i.e., methionine, cysteine, tryptophan, lysine, and tyrosine), and how the resulting covalent modifications to these residues alter their interactions within proteins, leading to loss of protein structure and function.

Regarding DBP formation, chlorine contact times within treatment plants and distribution systems are sufficiently long (hours to days) that covalent modifications to peptide-bound histidines within DOM would occur. While most previous research with amino acids and peptides has focused on low-molecular-weight DBPs,[19−23] recent studies are demonstrating that these typically are low-yield (<5%) byproducts forming at the end of a long series of reactions needed to liberate these 1−2-carbon structures from larger molecules.[28,44−49] The initial products of chlorine reactions with amino acids form at much higher yields, and so may account for a greater fraction of the DBP pool. Of the seven chlorine-reactive amino acids, the dominant initial transformation products were previously known for methionine (methionine sulfoxide), cysteine (cysteic acid), tyrosine (3-chlorotyrosine and 3,5-dichlorotyrosine), lysine (lysine nitrile), and tryptophan (five products, including two indole ring chlorination products).[2,11,13,16,27,28] This study identified *β*-cyanoalanine as a dominant chlorine transformation product of peptide-bound histidines. A chlorinated transient intermediate product was observed at maximum yields of ∼5%, but there was no significant evidence for 2-oxohistidine, hypothesized as an important transformation product in previous research.[26,29,30] 3-Chlorotyrosine and 3,5-dichlorotyrosine exhibited higher cytotoxicity in Chinese hamster ovary cells than that for any of the regulated THMs.[50] The toxicity of the other dominant amino acid transformation products is unclear, although the halogenated transformation products are more likely to exhibit higher toxicity than the nonhalogenated transformation products. However, although *β*-cyanoalanine is not halogenated, it has been identified as a neurotoxin resulting in death in rats at dosages of 20 mg/100 g and has been linked to neurotoxicity upon human consumption of certain types of peas.[51,52] Overall, given that the >600 DBPs identified to date account for only ∼30% of the byproduct pool,[17] focusing on the high-yield transformation products of important DOM constituents provides an efficient pathway for future research.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c07408.

Analytical details; example kinetic plots; UV spectrum of chlorinated *N*-acetyl-histidine; mass spectra for chlorinated peptides and *N*-acetyl-histidine chlorination products; and proposed reaction pathway (PDF)

■ AUTHOR INFORMATION

Corresponding Author

William A. Mitch − *Department of Civil and Environmental Engineering, Stanford University, Stanford, California 94305, United States;* orcid.org/0000-0002-4917-0938; Phone: (650) 725-9298; Email: wamitch@stanford.edu

Authors

Jong Kwon Choe − *Department of Civil and Environmental Engineering and Institute of Construction and Environmental Engineering, Seoul National University, Seoul 08826, Republic of Korea*

Lap-Cuong Hua − *Institute of Environmental Engineering, National Chiao Tung University, Hsinchu 30010, Taiwan*

Yukako Komaki − *Graduate Division of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka 422-8529, Japan;* orcid.org/0000-0003-3339-2939

Adam M.-A. Simpson − *Department of Civil and Environmental Engineering, Stanford University, Stanford, California 94305, United States;* orcid.org/0000-0002-4850-4828

Daniel L. McCurry − *Department of Civil and Environmental Engineering, University of Southern California, Los Angeles, California 90089, United States;* orcid.org/0000-0002-5599-2540

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.0c07408

Author Contributions

#J.K.C. and L.-C.H. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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