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Lysine and Arginine Reactivity and Transformation Products during Peptide Chlorination

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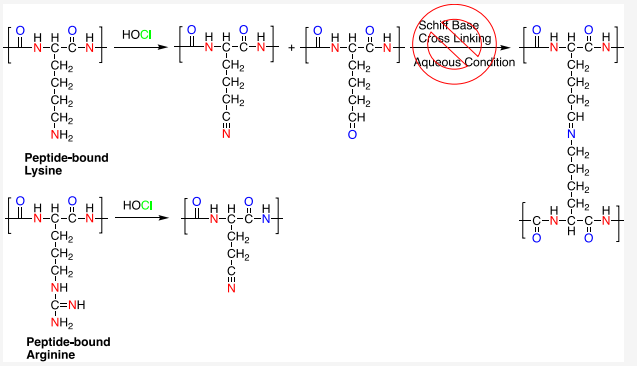
ABSTRACT: Chlorine reactions with peptide-bound amino acids form disinfection byproducts and contribute to pathogen inactivation by degrading protein structure and function. Peptide-bound lysine and arginine are two of the seven chlorine-reactive amino acids, but their reactions with chlorine are poorly characterized. Using *N*-acetylated lysine and arginine as models for peptide-bound amino acids and authentic small peptides, this study demonstrated conversion of the lysine side chain to mono- and dichloramines and the arginine side chain to mono-, di-, and trichloramines in ≤0.5 h. The lysine chloramines formed lysine nitrile and lysine aldehyde at ∼6% yield over ∼1 week. The arginine chloramines formed ornithine nitrile at ∼3% yield over ∼1 week but not the corresponding aldehyde. While researchers hypothesized that the protein aggregation observed during chlorination arises from covalent Schiff base cross-links between lysine aldehyde and lysine on different proteins, no evidence for Schiff base formation was observed. The rapid formation of chloramines and their slow decay indicate that they are more relevant than the aldehydes and nitriles to byproduct formation and pathogen inactivation over timescales relevant to drinking water distribution. Previous research has indicated that lysine chloramines are cytotoxic and genotoxic to human cells. The conversion of lysine and arginine cationic side chains to neutral chloramines should alter protein structure and function and enhance protein aggregation by hydrophobic interactions, contributing to pathogen inactivation.



KEYWORDS: *peptides, chlorination, disinfection byproducts, lysine, arginine*

INTRODUCTION

Chlorine reactions with peptide-bound amino acids are important for understanding pathogen inactivation and for characterizing disinfection byproduct (DBP) formation. Amino acid side chains mediate catalytic reactions within enzyme active sites, and their non-covalent interactions control protein structure.[1] Chemical transformations to these side chains resulting from chlorine reactions ultimately are responsible for degradation of protein structure and function,[1] but the nature of these transformations remains poorly characterized. The fact that these transformations are important for pathogen inactivation is indicated by the finding that chlorine oxidation of the bacteriophage MS2 protein capsid disrupted RNA injection into the *Escherichia coli* host, accounting for ∼42% of MS2 inactivation.[2] Similarly, the decreased infectivity of human astrovirus and norovirus during chlorination was correlated with an increase in carbonyl oxidation products within the viral capsid protein.[3]

Whether formed from chlorine reactions with proteins in pathogens or with peptides in source waters, the chemical transformation products represent DBPs. Most previous DBP research on amino acid chlorination has focused on the formation of 1−2 carbon halogenated byproducts (e.g., trihalomethanes) from free amino acids.[4−6] Yields typically are <1% due to the multiple reaction steps associated with the carbon−carbon bond cleavage needed to liberate 1−2 carbon products from these larger structures. Chlorine rapidly reacts with the α-amino group of free amino acids, forming organic mono- or dichloramines (Scheme S1).[4,7,8] Decarboxylation coupled with chloride elimination (i.e., concerted decarboxylation) from the organic monochloramine forms an aldehyde, while hydrochloric acid elimination followed by concerted decarboxylation from the dichloramine forms a nitrile at yields >1% over hour timescales.[4,7,9] However, the importance of DBPs formed by chlorination of free amino acids is unclear, since other research has indicated that free amino acid concentrations in water supplies are ∼2% of those of peptide-bound amino acids.[10]

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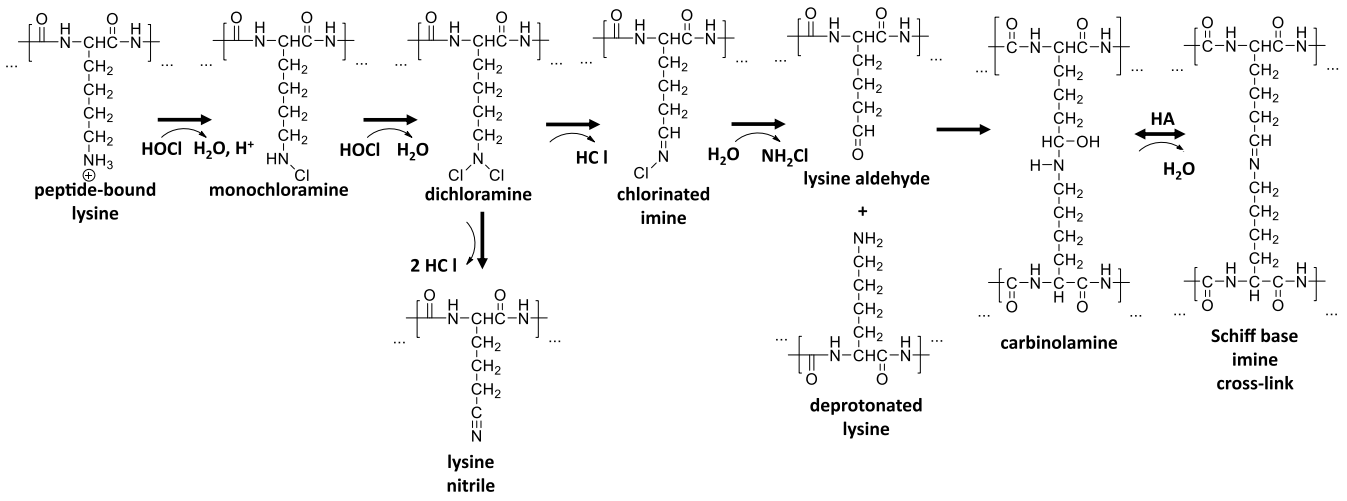
Scheme 1. Proposed Reaction Mechanisms for *N*-Acetyl Lysine Chlorination and Schiff Base Formation



More limited research with peptide-bound amino acids has indicated that peptide bonds deactivate the α-amino nitrogen, directing chlorine toward reaction with the side chains.[11] Only a subset of the 20 common amino acids has chlorine-reactive side chains with reactivity in the order of methionine (3.8×10^7 M^{-1} s^{-1}) > cysteine (3.0×10^7 M^{-1} s^{-1}) > histidine (1.0×10^5 M^{-1} s^{-1}) ~ α-amino groups (1.0×10^5 M^{-1} s^{-1}) > tryptophan (1.1×10^4 M^{-1} s^{-1}) > lysine (5.0×10^3 M^{-1} s^{-1}) > tyrosine (44 M^{-1} s^{-1}) ~ arginine (26 M^{-1} s^{-1}) > peptide bonds (10^1–10^{-3} M^{-1} s^{-1}).[11] Determination of these rate constants involved UV spectroscopy to monitor the initial reaction of chlorine with these amino acids, without characterizing final products. Other research characterized high-yield (>10%) transformation products of chlorine reactions with peptide-bound amino acids, including methionine sulfoxide and sulfone from methionine,[11,12] cysteic acid from cysteine,[11] β-cyanoalanine from histidine,[13] five different products from tryptophan (including two halogenated indole derivatives),[14] and 3-chlorotyrosine and 3,5-dichlorotyrosine from tyrosine.[11,12,15] While the rate constants mentioned above suggest rapid initial reactions with chlorine (e.g., ~15 min timescale for arginine reaction with 3 mg/L free chlorine), product formation may involve several steps and occur over significantly longer timescales. For example, although the initial chlorine reaction with histidine is faster than with tyrosine, peptide-bound histidine conversion to β-cyanoalanine occurred over days, much more slowly than the conversion of peptide-bound tyrosine to chlorotyrosines.[13]

Along with histidine, lysine and arginine are positively charged near neutral pH,[18] with important implications for protein structure and behavior. These cationic residues frequently decorate protein exteriors, resulting in electrostatic repulsion between proteins that inhibits aggregation.[19−21] Chlorination of proteins promotes aggregation,[16,22−24] but the underlying mechanism remains controversial. One hypothesis is that chlorination converts the cationic primary amine in the lysine side chain to neutral chloramines, promoting aggregation by non-covalent hydrophobic interactions;[22] chlorination has been demonstrated to increase protein sorption to hydrophobic surfaces.[25] Other researchers have hypothesized that aggregation results from the formation of a covalent imine (Schiff base) cross-linkage between lysine and aldehydes on adjacent proteins formed by chlorine oxidation of lysine side chains (Scheme 1).[16,26]

However, the chemical transformations resulting from chlorination of peptide-bound lysine and arginine remain poorly characterized. Research has indicated that chlorination of the primary amine group in the lysine side chain forms organic mono- and dichloramines (Scheme 1).[1] Lysine nitrile formed during chlorination of proteins[12] and was measured at 105 ng/L in chlorinated tap water.[17] However, the effect of reaction conditions and the timescale on peptide-bound lysine nitrile formation remains unclear. Direct evidence for lysine oxidation to an aldehyde and the importance of the subsequent formation of covalent cross-links is lacking. Similarly, the timescale and products of peptide-bound arginine chlorination have not been described.

The objectives of this study were to (1) identify transformation products of chlorine reactions with models for peptide-bound lysine and arginine; (2) quantify the yields and timescales for product formation to delineate reaction pathways; (3) demonstrate that these products form in authentic chlorine-treated peptides; and (4) evaluate the importance of Schiff base covalent cross-links between peptide-bound lysines during chlorination. The results clarify the nature of the chemical transformations occurring within proteins during chlorine inactivation of pathogens. Moreover, recent research has suggested that the larger (>1−2 carbon) DBPs formed during chlorination of drinking waters and potable reuse waters contribute more to the toxicity of disinfected waters than the 1−2 carbon DBPs of current research interest.[27] Since this higher molecular weight DBP fraction remains poorly characterized, the identification of chlorination products of these important reactive amino acids could facilitate the characterization of this fraction in disinfected waters.

METHODS AND MATERIALS

Text S1 provides material sources and procedures to synthesize nitrile standards.

Chlorination Experiments. All free chlorine stock solutions were prepared by diluting 5% by weight sodium hypochlorite into deionized water. Stock solutions were stored at 4 °C and standardized daily before use by UV spectroscopy at 292 nm (ε = 365 M^{-1} cm^{-1}).[28] Nac-Lys or Nac-Arg (25

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μM) was treated with 0−250 μM sodium hypochlorite in 20 mL of deionized water buffered with 5 mM acetate (pH 4.0 and 5.0), phosphate (pH 7.0, 7.4, 8.0, and 11.0), or carbonate (pH 9.0) at room temperature; control experiments indicated that chlorine residuals after 3 d were similar between acetate and phosphate buffers at pH 5. Samples (1 mL) were withdrawn for analysis of residual parent compounds and products. Treatment of 10 μM peptides (Nac-Lys-Ala-Ala and Nac-Leu-Leu-Arg) was conducted under similar conditions at pH 9.0. Chlorine was added at a 2:1 chlorine/peptide molar ratio for Nac-Lys-Ala-Ala and a 10:1 molar ratio for Nac-Leu-Leu-Arg. Nac-Lys, Nac-Arg, peptides, and their transformation products were analyzed by high-performance liquid chromatography (HPLC) with triple quadrupole mass spectrometry (LC/MS/MS) or ultraviolet detection (HPLC/UV); Text S2 provides details.

Evaluation of Mulliken Charge Distribution. The Mulliken charge distributions for the organic chloramines of Nac-Arg were calculated using density functional theory within the Gaussian 16 program with the B3LYP method, the 6-311G (++, d,p) basis set, and the SMD aqueous solvation model.

RESULTS

Chlorination of *N*-Acetyl Lysine. Previous research involving chlorination of the primary amines monomethylamine and propylamine demonstrated rapid formation of their organic monochloramines at a 1:1 chlorine/amine molar ratio and the formation of their organic dichloramines at higher molar ratios.[29] Monomethylamine's dichloramine decayed 4-fold faster than its monochloramine. Dichloramine degradation rates and product yields depended strongly on the alkyl chain length of the primary amines. Propylamine's dichloramine (half-life 3.5 d) decayed 3.5-fold faster than monomethylamine's dichloramine (half-life 12 d). While degradation of both dichloramines formed their corresponding aldehydes and nitriles, maximum yields were much higher from propylamine's dichloramine (~28% propionaldehyde and ~38% propionitrile) than from monomethylamine's dichloramine (~1.3% formaldehyde and ~1.6% cyanogen chloride).

Given that the primary amine in the lysine side chain is longer (butylamine), we expected that degradation of lysine's dichloramine would be faster and form the corresponding aldehyde and nitrile at higher yields. We used *N*-acetyl-lysine (Nac-Lys) as a model for protein-bound lysine, since the *N*-acetyl linkage mimics the peptide bond (Scheme 1). We focused on peptide-bound lysine, since peptide-bound amino acids are 10-fold more prevalent in water supplies than free amino acids.[10] Mimicking the peptide bond is important because previous research using UV spectrophotometry to measure organic chloramine formation has indicated that the α-amino group of free amino acids is much more reactive with chlorine (10^5 M^{-1} s^{-1}) than with the lysine side chain (5×10^3 M^{-1} s^{-1}) or peptide bonds ($\leq 10^3$ M^{-1} s^{-1}).[11] Thus, chlorine targets the α-amino group in free amino acids but the lysine side chain in peptide-bound lysine.

The addition of 25 μM chlorine to 25 μM Nac-Lys at pH 7.4 resulted in the disappearance of the parent Nac-Lys peak (retention time 0.95 min and m/z 187) within the ~10 min needed for LC/MS analysis. Based on the 5.0×10^3 M^{-1} s^{-1} rate constant for lysine reaction with chlorine[11] and the 25 μM chlorine concentration, the reaction timescale would be ~10 s. Two products were detected by HPLC/UV using the same analytical column and elution protocol used with LC/MS, one with a retention time of 6.2 min and a UV absorption peak at 265 nm and one with a retention time of 10.5 min and a UV absorption peak at 305 nm (Figure S4). These two peaks were not observed after the addition of 50 μM thiosulfate, a quenching agent for total chlorine residual, indicating that these products were organic chloramines. When the same samples were analyzed by LC/MS in the negative-ion mode, the first peak was not observed, likely because the compound is thermally unstable under the electrospray ionization conditions. The m/z 255, 257, and 259 in the mass spectrum of the second peak occurred in the 10:6:1 ratio indicating two chlorines (Figure S5), while the m/z 255 matched the m/z expected for Nac-Lys dichloramine; evaluation of the mass spectrum indicated no co-eluting compounds. Only the second peak was observed at chlorine/Nac-Lys molar ratios ≥2. Based upon its UV absorbance, its disappearance after thiosulfate addition, and its lack of occurrence at chlorine/Nac-Lys molar ratios ≥2, the first peak is suggested to be Nac-Lys monochloramine. Nac-Lys dichloramine featured a substantial UV absorbance peak area (~500 vs ~1000 for the proposed Nac-Lys monochloramine; Figure S4D,E) at the 1:1 chlorine/Nac-Lys molar ratio, suggesting that the disproportionation reaction between two Nac-Lys monochloramines to form the dichloramine (eq 1) is important.[30]

$$2\ \text{R-NHCl} + \text{H}^+ \rightarrow \text{R-NCl}_2\text{-} + \text{R-NH}_3^+ \quad (1)$$

Samples collected over 1 week after chlorine addition to 25 μM Nac-Lys at pH 7.4 and 0.5−5.0 chlorine/Nac-Lys molar ratios were treated with thiosulfate to quench the residual chlorine, halting the reaction and converting the chloramines back to Nac-Lys. Unlike the chloramines, this Nac-Lys can be readily measured and quantified. The decrease in the concentrations of this Nac-Lys after thiosulfate addition with increasing chlorine contact time reflects the conversion of chloramines to other products that do not revert to Nac-Lys after thiosulfate addition. This decrease in Nac-Lys concentration fits first-order kinetics (Figure S6). The observed first-order decay rate constant (k_{obs}) was 5.10×10^{-3} h^{-1} at the 1:1 chlorine/Nac-Lys molar ratio, corresponding to ~5 d half-life. The k_{obs} values increased as the chlorine/Nac-Lys molar ratio increased to 2:1 but then leveled out up through the 5:1 molar ratio (Figure S7), exhibiting an ~4 d half-life. Since this half-life was observed for chlorine/Nac-Lys molar ratios ≥2, the half-life reflects Nac-Lys dichloramine degradation. For treatment at a 4:1 chlorine/Nac-Lys molar ratio, the k_{obs} values increased with pH (Figure S8). Nac-Lys dichloramine decay rates and half-lives were very similar to those observed for the dichloramine of propylamine.[29]

At chlorine/Nac-Lys molar ratios ≥2:1, two products were observed by LC/MS. These products were observed with or without thiosulfate addition, indicating that these were not chloramines. One product (retention time 2 min) with m/z 183.1 was confirmed as Nac-Lys-nitrile (Scheme 1) against an authentic standard (Figure S9). The second product (retention time 1.8 min) was proposed as Nac-Lys-aldehyde (Scheme 1) based on two factors. First, the mass spectrum featured a parent ion with m/z 186.0 (Figure S10), consistent with the $C_8H_{12}NO_4$ elemental formula of Nac-Lys-aldehyde, and daughter ions at m/z 144.1, corresponding to Lys-aldehyde ($C_6H_{10}NO_3$), and at m/z 57.9, corresponding to the acetamide anion (C_2H_4NO). Second, the retention time was shorter than that of Nac-Lys-nitrile, consistent with the greater polarity expected for an aldehyde compared to a nitrile. Unfortunately,

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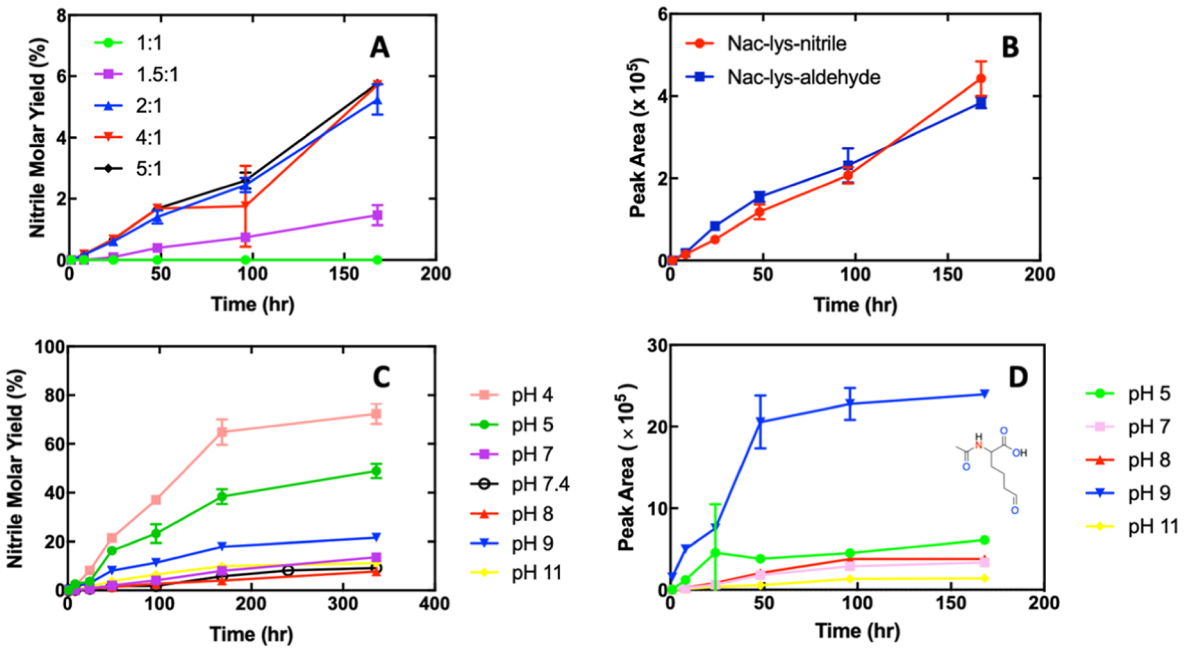

Figure 1. (A) Molar yields of Nac-Lys-nitrile over time during treatment of 25 μM Nac-Lys with different chlorine/Nac-Lys molar ratios in deionized water buffered at pH 7.4 with 5 mM phosphate buffer. (B) LC/MS peak areas corresponding to Nac-Lys-nitrile and Nac-Lys-aldehyde during treatment of 25 μM Nac-Lys with 50 μM chlorine in deionized water buffered at pH 7.4 with 5 mM phosphate. (C) Molar yields of Nac-Lys-nitrile at different pH values during the addition of 100 μM chlorine in deionized water buffered with 5 mM phosphate (pH 4.0 and 5.0), 5 mM phosphate (pH 7.0, 7.4, 8.0, and 11.0), or 5 mM carbonate (pH 9.0). (D) LC/MS peak areas corresponding to Nac-Lys-aldehyde during treatment of 25 μM Nac-Lys with 100 μM chlorine at different pH values. Error bars represent the standard deviation of experimental triplicates.

we were unable to synthesize a purified standard of Nac-Lys-aldehyde.

At pH 7.4, Nac-Lys-nitrile yields were negligible for a 1:1 chlorine/Nac-Lys molar ratio. Nac-Lys-nitrile yields reached ~1.5% after 1 week at the 1.5:1 molar ratio and ~5.5% for the 2:1 molar ratio (Figure 1A). Yields did not change at higher molar ratios up through 5:1. Although Nac-Lys-aldehyde yields could not be quantified without a standard, the peak areas over the course of 1 week were similar for Nac-Lys-nitrile and Nac-Lys-aldehyde for all molar ratios (e.g., Figures 1B and S11), suggesting comparable yields. Propionitrile and propionaldehyde yields observed during propylamine chlorination also were similar, but their maximum yields were much higher (~30−40%) and yields declined as the chlorine/propylamine molar ratio increased above 2:1.[29] A previous study evaluated chlorination of 40-fold higher Nac-Lys concentrations relevant to amino acid reactions with chlorine produced in vivo as a part of the inflammatory response.[30] That study found that Nac-Lys-nitrile was the dominant product at both 1:1 and 2:1 chlorine/Nac-Lys molar ratios; Nac-Lys-aldehyde was only observed as a transient intermediate at a very low (1:1000) chlorine/Nac-Lys molar ratio.[30] The difference in our results likely reflects the different Nac-Lys concentrations, with the lower, drinking water-relevant Nac-Lys concentrations we used inhibiting disproportionation reactions that promote Nac-Lys dichloramine and hence nitrile formation.

At the 4:1 chlorine/Nac-Lys molar ratio, Nac-Lys-nitrile yields were the highest at pH 4 (~70% after 1 week) and then declined with pH through pH 8.0 (Figure 1C). Yields increased at pH 9.0 (~10% after 1 week) but then declined at pH 11.0. This pH dependence is the opposite of that observed for propylamine, where propionitrile and propionaldehyde yields were maximized at pH 7.[29] The yield of the proposed Nac-Lys-aldehyde was maximized at pH 9.0 (Figure 1D). Comparing the LC/MS peak areas between Nac-Lys-nitrile and Nac-Lys-aldehyde at pH 5.0, 7.4 and 9.0 indicated that yields of Nac-Lys-nitrile declined in favor of Nac-Lys-aldehyde with increasing pH (Figures 1B and S12A,B). The peak areas of Nac-Lys-nitrile were higher than those of Nac-Lys-aldehyde at pH 5.0, comparable at pH 7.4, and lower at pH 9.0.

Scheme 1 presents reaction pathways proposed for Nac-Lys-aldehyde and Nac-Lys-nitrile formation during Nac-Lys chlorination. Elimination of hydrochloric acid (HCl) from Nac-Lys dichloramine would form a chlorinated imine. Hydrolysis of the chlorinated imine would form Nac-Lys-aldehyde, releasing NH_2Cl, as demonstrated previously for other chlorinated imines.[31] The chlorinated imine intermediate was not detected by LC/MS in either the positive- or negative-ion mode, suggesting that it is likely an unstable intermediate. Alternatively, elimination of a second HCl would form Nac-Lys-nitrile. The comparable peak areas of Nac-Lys-aldehyde and Nac-Lys-nitrile at pH 7.4 suggest that hydrolysis of the chlorinated imine competes with a second HCl elimination.

Regarding the pH dependence, the deprotonated lysine side chain (pK_a = 10.4)[32] should form chloramines more readily than the protonated side chain. However, the comparable peak areas for the Nac-Lys dichloramine observed 3 h after chlorine addition at a 4:1 chlorine/Nac-Lys molar ratio across pH 4−9 (Figure S13) indicate that dichloramine formation is rapid, relative to its decay to Nac-Lys-nitrile and other products. The increasing ratio of Nac-Lys-aldehyde/Nac-Lys-nitrile peak areas with increasing pH concurs with expectations that increasing pH should promote hydrolysis of the chlorinated imine intermediate. However, previous research has demonstrated that HCl elimination from organic chloramines and chlorinated imines is general base-catalyzed.[33,34] Indeed, the higher Nac-Lys-nitrile yields at pH 9.0 vs 7.4 (Figure 1C)

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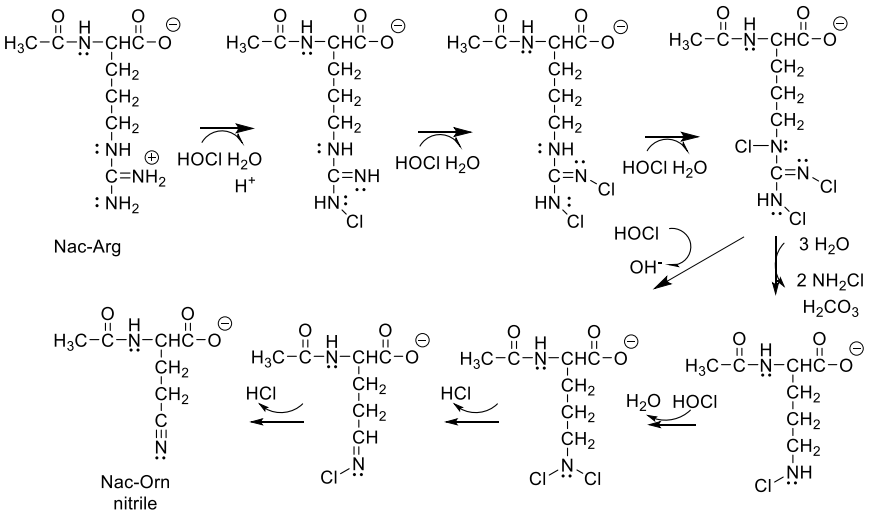
Scheme 2. Proposed Reaction Mechanisms for *N*-Acetyl Arginine Chlorination



concur with base catalysis of HCl elimination from the chlorinated imine, while the simultaneous increase in Nac-Lys-aldehyde peak areas indicates that chlorinated imine formation via HCl elimination from the dichloramine is also enhanced with increasing pH. The decrease in Nac-Lys-nitrile yields at pH 11 (Figure 1C) suggests faster hydrolysis of Nac-Lys-nitrile at basic pH, as observed for other nitriles and aldehydes.[35−37] In a separate experiment, 10 μM Nac-Lys-nitrile decreased by 10 and 70% when held for 48 h at pH 7.0 and 11.0, respectively (Figure S14).

However, the significant increase in Nac-Lys-nitrile yields observed as pH decreased below pH 7.0 suggests that HCl elimination from the chlorinated imine is acid-catalyzed (Scheme S2) in addition to being base-catalyzed. Indeed, Nac-Lys was treated with chlorine at a 4:1 molar ratio at pH 7.0 for 8 h to form the dichloramine and then adjusted to pH 3.0, 7.0, or 9.0 for 48 h. The peak areas for Nac-Lys dichloramine were the lowest at pH 3.0, while those for Nac-Lys-nitrile and, to a lesser degree, Nac-Lys-aldehyde, were the highest at pH 3.0 (Figure S15). These results suggest that HCl elimination from the dichloramine and the chlorinated imine are acid-catalyzed, while hydrolysis of the chlorinated imine is acid-catalyzed to a lesser extent.

Chlorination of *N*-Acetyl Arginine. When 100 μM chlorine was added to 25 μM Nac-Arg in deionized water at pH 7, LC/MS analysis in the negative-ion mode indicated 80% loss of the parent compound (m/z 215; retention time 1.0 min) within 10 min. Based on the 26 M^{-1} s^{-1} rate constant reported for chlorine reaction with arginine[11] and the 100 μM chlorine concentration, the reaction timescale would be 6.4 min. Three product peaks were observed after 10 min (Figure S16A). After 35 min, the first peak decreased in size, while the other two increased (Figure S16B). All three peaks were absent after treatment with 300 μM sodium thiosulfate, indicating that these products were organic chloramines. The mass spectrum of the first peak (retention time 1.5 min) featured m/z 249 and 251 at the 3:1 abundance ratio, indicating a product containing one chlorine (Figure S16C) and corresponding to the m/z expected for Nac-Arg monochloramine. The mass spectrum of the second peak (retention time 9.2 min) exhibited m/z 283, 285, and 287 in the ∼10:6:1 ratio expected for a compound containing two chlorines (Figure S16D) and aligning with the m/z expected for Nac-Arg dichloramine. The mass spectrum of the third peak (retention time 10.2 min) featured m/z 169.1 as the predominant ion, along with m/z 262.9, 264.9, and 266.8 in the ∼10:6:1 ratio, indicative of a fragment containing two chlorines (Figure S16E). Based on the 4:1 chlorine/Nac-Arg molar ratio and the greater retention time relative to the proposed Nac-Arg dichloramine, we suspected that the product was Nac-Arg trichloramine but that it was less stable than the monochloramine and dichloramine, fragmenting under the electrospray conditions to yield a dichlorinated fragment.

Mulliken charge analysis calculated using Gaussian was used to indicate which of the three nitrogens within the guanidinium functional group would be the most chlorine-reactive. The guanidinium functional group within the Nac-Arg side chain is protonated at pH 7 (pK_a = 12.5).[18] The Mulliken charge analysis of protonated Nac-Arg (Figure S17A) indicates that the two terminal nitrogens feature equivalent electron charge densities that are higher than the charge density associated with the nitrogen linking the guanidinium functional group with Nac-Arg. These results suggest that the monochloramine results from chlorine addition to one of these terminal nitrogens (Scheme 2). The very short retention time for the monochloramine peak relative to the other chloramines (i.e., 1.5 min vs 9.2 min and 10.2 min) suggests that the monochlorinated guanidinium functional group remains protonated under the acidic conditions of the HPLC eluent (0.1% formic acid). Similarly, Mulliken charge analysis of the monochloramine formed by chlorine addition to a terminal nitrogen (Figure S17B) indicates that the dichloramine would form via chlorine addition to the other terminal nitrogen; the much greater HPLC retention time suggests that the guanidinium group is no longer charged, even under the acidic HPLC eluent conditions. The Mulliken charge analysis of the dichloramine (Figure S17C) suggests that the electronic charge density is similar to one of the terminal nitrogens and to the nitrogen connecting the guanidine group with the arginine backbone. However, Mulliken charge analysis indicates that chlorine addition to the latter nitrogen (Scheme 2) forms a weak *N*−Cl bond (Figure S17D). The instability of this weak bond within the harsh conditions of electrospray ionization during LC/MS analysis could explain the observation of a dichlorinated fragment, but no trichlorinated parent ion, within the mass spectrum.

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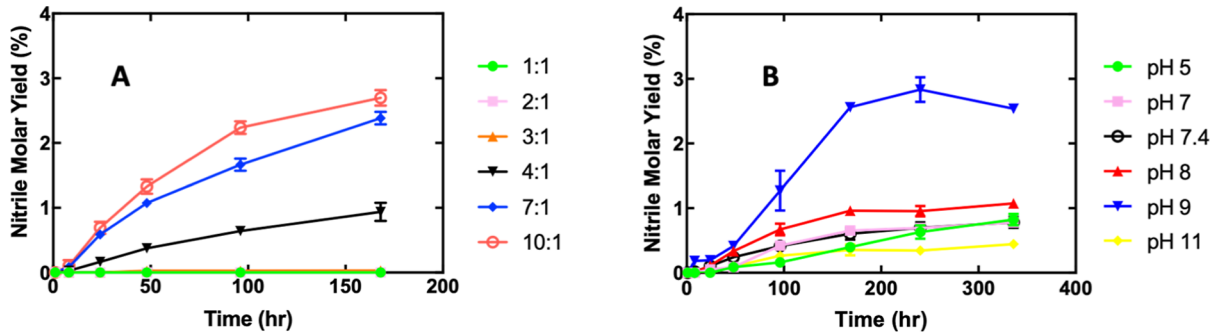

Figure 2. Molar yields of Nac-Orn-nitrile formed over time by treating 25 μM Nac-Arg with (A) different chlorine/Nac-Arg molar ratios in deionized water buffered at pH 7.4 with 5 mM phosphate or (B) with 100 μM chlorine in deionized water buffered at pH 5.0 (5 mM acetate), 7.0, 7.4, 8.0, 11.0 (5 mM phosphate), or 9.0 (5 mM carbonate). Error bars represent the standard deviation of experimental triplicates.

When 100 μM chlorine was added to 25 μM Nac-Arg at pH 7.0, the LC/MS peak areas associated with the chloramines after ~2.5 h were in the order monochloramine < dichloramine < trichloramine (Figure S18B). However, after 24 h, the monochloramine and dichloramine peaks were no longer observed, while the trichloramine peak area nearly tripled, suggesting conversion of the monochloramine and dichloramine to the trichloramine. After 48 h, the trichloramine peak area declined by ~40%, suggesting conversion to other products. At pH 5.0, the monochloramine peak remained the dominant peak over 48 h, while the dichloramine and trichloramine peaks slowly formed, reaching 15% of the monochloramine peak area (Figure S18A). At pH 9.0, the trichloramine peak areas were similar to those observed at pH 7, but the monochloramine and dichloramine peaks were never observed (Figure S18C). These results suggest that chlorine addition to Nac-Arg and its mono- and dichloramines increases with pH.

Chloramine formation at pH 7.4 also was characterized as a function of chlorine/Nac-Arg molar ratio. When 25 μM chlorine was added to 25 μM Nac-Arg at pH 7.4, only a small monochloramine peak (~10% of that observed at the 4:1 molar ratio at pH 5.0; Figure S18A) was observed, declining by ~40% over 1 week (Figure S19A). At the 2:1 chlorine/Nac-Arg molar ratio, the monochloramine peak was ~50% smaller, while the trichloramine peak increased rapidly to exceed that of the monochloramine after ~1.5 h (Figure S19B). Interestingly, the dichloramine peak was not observed. These results suggest that the monochloramine and dichloramine tend to react to form the trichloramine, in the same fashion as observed for Nac-Lys monochloramine (Figure S4D,E and eq 1). At the 3:1 molar ratio, the trichloramine peak was always dominant, reaching a maximum after 1 d and then declining by 69% over 1 week (Figure S19C). The monochloramine peak declined over 1 d, while the dichloramine peak was not observed. At the 4:1 molar ratio, the same pattern was observed, but the trichloramine peak area was 3.4 times larger after 1 d and then declined by 80% over 1 week (Figure S19D). Overall, the results concur with the expected increase in the trichloramine peak area at the expense of monochloramine with increasing chlorine/Nac-Arg molar ratio, but the limited detection of the dichloramine (only under low pH conditions) suggests that the monochloramine and dichloramine rapidly convert to the trichloramine.

For chlorine/Nac-Arg molar ratios ≥4:1 at pH 7.4, a new product (retention time 1.2 min) featuring m/z 169.1 in its mass spectrum was observed without or with thiosulfate addition. The product was identified as Nac-Orn-nitrile by comparison against an authentic standard (Figure S20). Figure 2A provides Nac-Orn-nitrile molar yields over 170 h (1 week) when chlorine was applied to 25 μM Nac-Arg at pH 7.4 at molar ratios ranging from 1:1 to 10:1. Nac-Orn-nitrile formation was not observed at molar ratios ≤3:1. At a 4:1 molar ratio, Nac-Orn-nitrile yields increased over time to ~1% after 1 week. Yields increased with increasing molar ratio, reaching ~2.8% after 1 week for a 10:1 molar ratio. At a 4:1 chlorine/Nac-Arg molar ratio, Nac-Orn-nitrile yields also increased as pH increased from 5.0 to 9.0, reaching ~3% after 10 d (Figure 2B); the increase in nitrile yield with increasing pH suggests that base-catalyzed elimination of hydrochloric acid may be involved in the same fashion observed with lysine nitrile formation. However, Nac-Orn-nitrile yields were much lower at pH 11.0 (Figure 2B), suggesting that hydrolysis of Nac-Orn-nitrile becomes significant at this pH, as observed for Nac-Lys-nitrile (Figure S14).

No other product peak was observed, including any peak corresponding to Nac-Orn-aldehyde (m/z 172). To further characterize the potential for Nac-Orn-aldehyde formation, 50 μM chlorine was added to 25 μM Nac-Orn at pH 7.4. After 4 days, Nac-Orn-nitrile was detected at ~3.5% molar yield (Figure S21), similar to Nac-Lys-nitrile yields (Figure 1A). However, Nac-Orn-aldehyde formation was not observed, in contrast to the similar LC/MS peak areas of Nac-Lys-aldehyde and Nac-Lys-nitrile from Nac-Lys (Figure S12C). These results suggest significant differences in the behavior of Nac-Lys and Nac-Orn during chlorination, despite Nac-Orn exhibiting only one less methylene group in its primary amine side chain than Nac-Lys. The results further concur with the high variability observed in the yields of nitriles and aldehydes with the primary amine chain length (e.g., ~1% each from monomethylamine vs ~30% each from propylamine[29]).

Scheme 2 presents a reaction pathway consistent with the experimental observations. While the 3:1 chlorine/Nac-Arg molar ratio produces Nac-Arg trichloramine as a stable product, higher molar ratios initiate trichloramine decay, forming Nac-Orn-nitrile. The electron-withdrawing nature of the three chlorine substituents on the guanidine group renders it susceptible to hydrolysis, forming Nac-Orn monochloramine and releasing carbonic acid and NH_2Cl.[38,39] Previous research demonstrated NH_2Cl formation during chlorination of free L-arginine. We confirmed NH_2Cl formation from Nac-Arg, with yields increasing from 1% at chlorine/Nac-Arg molar ratios of 1:1 or 2:1 to 3.2% at a 7:1 chlorine/Nac-Arg molar ratio

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(Table S1). Since chlorine is in excess at these molar ratios, breakpoint chlorination reactions would prevent NH_2Cl accumulation.[40] For chlorine/Nac-Arg molar ratios ≥4:1, rapid reaction of chlorine with Nac-Orn monochloramine would form Nac-Orn dichloramine. Nac-Orn-nitrile formation by elimination of two hydrochloric acids would increase with pH due to base catalysis of HCl elimination.

Chlorination of Peptides Containing Lysine and Arginine Residues. Chlorine was applied to N_α-acetyl-lysine-alanine-alanine (Nac-Lys-Ala-Ala) and N_α-acetyl-leucine-leucine-argininal (Nac-Leu-Leu-Arg) to demonstrate that the same transformation products are observed when lysine and arginine are contained within peptides; the two model peptides were selected because they contained either lysine or arginine without other chlorine-reactive amino acids, thereby simplifying the characterization of their products by mass spectrometry. Initial experiments involved the addition of 20 μM chlorine to 10 μM Nac-Lys-Ala-Ala at pH 9 for 7 d, conditions designed to maximize the conversion of lysine to the aldehyde and nitrile and facilitate LC/MS detection. LC/MS analysis in the full-scan negative-ion mode revealed that the parent peptide (m/z 329.2, retention time 1.2 min) was converted into two products, one (retention time 3.2 min) with m/z 327.8 and one (retention time 3.9 min) with m/z 325.2. When the samples were re-analyzed using tandem mass spectrometry to fragment these parent ions, the daughter ions formed from the product with m/z 327.8 indicated that the product represented conversion of the lysine to lysine-aldehyde. This product featured fragments at m/z 88.0, corresponding to alanine, m/z 158.8, corresponding to alanyl-alanine, and m/z 224.4, corresponding to the dipeptide containing *N*-acetyl-lysine aldehyde and alanine (Figure S22). Similarly, the fragments formed from the m/z 325.2 product indicated that the lysine residue had been converted to lysine nitrile. While the fragments observed at m/z 88.1 and m/z 159.3 corresponded to the same unmodified alanine and alanyl-alanine fragments observed in the aldehyde product, the fragment at m/z 239.2 corresponded to the dipeptide containing *N*-acetyl-lysine nitrile and alanine (Figure S23).

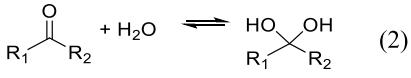
When this experiment was repeated at pH 7.4, the abundance of the two product peaks was monitored over time using LC/MS analysis in the full-scan negative-ion mode (Figure S24). The results were similar to those observed with Nac-Lys chlorination. While the abundance of the aldehyde-containing product exceeded that of the nitrile product throughout the experiment, both products were important.

Chlorine (50 μM) was applied to 10 μM Nac-Leu-Leu-Arg at pH 9 for 14 d; pH 9 was selected to maximize conversion of the arginine to Orn-nitrile. LC/MS analysis in the full-scan positive-ion mode demonstrated that the parent peptide (m/z 427.3, retention time 11.5 min) was converted into a product with m/z 381.1 at a 13 min retention time (Figure S25). The m/z 381.1 product parent mass reflects a $C_{19}H_{32}N_4O_4$ elemental formula, consistent with arginine conversion to Orn-nitrile. Tandem mass spectrometry indicated a fragment at m/z 240.1 ($C_{13}H_{24}N_2O_2$), corresponding to Leu−Leu, resulting from removal of the Nac group and cleavage between the Leu and Arg residues. A second fragment at m/z 327.7 ($C_{16}H_{29}N_3O_4$) corresponds to cleavage to remove the Arg side chain. A third fragment at m/z 55 (C_3H_5N) reflects the side chain of the Arg residue after its conversion to the nitrile.

Covalent Cross-Links by Schiff Base Formation between Lysines. No evidence of a Schiff base (Scheme 1) cross-linkage between chlorine-treated Nac-Lys residues (m/z 356.2 for the monoanion or m/z 177.6 for the dianion by LC/MS) was observed when 50 μM free chlorine was added to 25 μM Nac-Lys at pH 7.4 over 1−10 d. To ensure the presence of unchlorinated Nac-Lys to promote Schiff base formation via reaction with Nac-Lys-aldehyde, 30 μM free chlorine was mixed with 50 μM Nac-Lys at pH 7.4 with 5 mM phosphate buffer; again, no evidence of Schiff base formation was observed by LC/MS. To further favor Schiff base formation, Nac-Lys-aldehyde was reacted with an unchlorinated amine as follows. Nac-Lys (25 μM) was treated with 100 μM free chlorine at pH 7.4 and the chlorine residual was quenched with thiosulfate after 2 d. Nac-Lys-aldehyde formation was verified by LC/MS analysis. After 25 μM methylamine was added, no evidence of the associated Schiff base (m/z 199.1) was observed after 24 h. To evaluate the effect of pH, 25 μM Nac-Lys was reacted with 50 μM chlorine at pH 5, 7, or 9 for 5 d and the formation of Nac-Lys-aldehyde was validated. The residual chlorine was quenched by the addition of 50 μM thiosulfate, which represents excess relative to the residual chlorine, due to partial consumption of the chlorine during aldehyde formation. No Schiff base formation or loss of Nac-Lys-aldehyde was observed 24 h after the addition of 25 μM Nac-Lys, even when the LC/MS method used an isocratic method with 0.1% formic acid in deionized water.

Additional experiments with model precursors demonstrated that Schiff base formation occurs in organic solvents but is disfavored in water. These experiments employed the aromatic primary amines, 2-aminophenol (pK_a 4.8) and aniline (pK_a 4.6), because their low pK_a values favor the deprotonated amine species involved in Schiff base formation (Scheme 1). When 5 mg/L (46 μM) 2-aminophenol was mixed with 5 mg/L (114 μM) acetaldehyde or 5 mg/L (86 μM) propionaldehyde at pH 5 with 5 mM acetate buffer, no decrease in 2-aminophenol concentration (Figure S26) or evidence for the formation of Schiff base products (m/z 136 for acetaldehyde and m/z 150 for propionaldehyde) was observed by LC/MS in the full-scan positive-ion mode over 75 h. Schiff base formation between aniline and acetone was observed within 24 h when 5 μM aniline was treated with 0.1% by volume formic acid in acetone (Figure S27); the formic acid was added because Schiff base formation is generally acid-catalyzed (Scheme 1).[41] However, when 5 μM aniline was treated with 5 μM acetone and 0.1% by volume formic acid in water, no Schiff base formation or decrease in aniline concentration was observed over 24 h.

Schiff base formation between Nac-lys-aldehyde and Nac-Lys is disfavored in aqueous solution for three reasons. First, Schiff base formation would involve the deprotonated species of Nac-Lys (Scheme 1), but the primary amine in the Nac-Lys side chain is predominantly protonated at pH 7.4. Second, aldehydes occur in equilibrium with their geminal diols in aqueous solution (eq 2), and the geminal diol form is not



(2)

involved in Schiff base formation (Scheme 1). The fraction occurring in the geminal diol form decreases with increasing chain length of aldehydes (e.g., 57% for acetaldehyde and 42% for propionaldehyde)[42] but is still expected to be significant for Nac-Lys-aldehyde. Third, imine formation via dehydration of the carbinolamine intermediate is reversible (Scheme 1) and is

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disfavored in aqueous solution;[31] indeed, removing water from reaction solutions is needed to obtain high yields during synthesis of imines.[43] Together, these results indicate that chlorine-induced protein aggregation is not driven by the formation of covalent imine cross-linkages between lysine aldehyde and lysine.

Environmental Implications. The nitrogen-containing side chains of lysine and arginine are positively charged at neutral pH and tend to decorate protein exteriors.[19−21] Our results indicate that chlorine reacts with these cationic nitrogen functional groups to form neutral organic chloramines over timescales <0.5 h. Interestingly, substantial dichloramine formation was observed at a 1:1 chlorine/Nac-Lys molar ratio, and trichloramine formation was observed at a 2:1 chlorine/Nac-Arg molar ratio, indicating a tendency for the formation of higher order chloramines by disproportionation reactions between lower order chloramines (e.g., eq 1). These neutral chloramines were relatively stable, degrading over timescales of ∼1 week, comparable to residence times in drinking water distribution systems.

Using both *N*-acetylated models for peptide-bound lysine and arginine and small peptides containing these residues, nitriles were substantial products of organic chloramine degradation. While lysine formed lysine nitrile, the guanidine residue in the arginine side chain was converted to ornithine nitrile. Nitrile yields were ∼3−6% after ∼5 d, higher than those observed in previous research[29] for monomethylamine (∼1%) but lower than those observed for propylamine (∼30%). Although yields were <10%, they were substantially higher than the <1% yields of 1−2 carbon byproducts that have been measured previously.[4−6] Furthermore, LC/MS area counts indicated that lysine aldehyde formed at yields comparable to lysine nitrile, even at chlorine/lysine molar ratios ≥2. This is in contrast to the behavior observed during chlorination of the α-amino groups of free amino acids, where aldehyde formation is favored at a 1:1 chlorine/amino acid molar ratio, but nitrile formation is favored at higher molar ratios (Scheme S1).[4,7,9] Moreover, aldehyde formation from arginine and ornithine was not observed. These results indicate that, while nitriles and aldehydes are important products formed during the degradation of chlorinated primary amines, their yields vary substantially with the primary amine structure in a fashion that we cannot yet predict.

Given the rapid formation of chloramines and their rather slow conversion to aldehydes and nitriles, the chloramines are likely of greater relevance both for DBP formation and for pathogen inactivation. With respect to DBPs, the slow (∼1 week) conversion of these chloramines to nitrile and aldehyde products is in contrast to the rapid (hours) formation of terminal products from methionine and tyrosine[12,13] and even the 1−2 d timescale for the conversion of the organic chloramines of histidine[13] and tryptophan[14] to terminal products. However, previous research has indicated that the chloramines formed during chlorination of the lysine side chain are cytotoxic and genotoxic to human lymphoblastoid cells;[44] the toxicity of the chloramine products of arginine has not been assessed. Regarding pathogen inactivation, the chloramine, nitrile, and aldehyde products all are neutral. Thus, within ∼0.5 h of chlorine contact, the cationic side chains of lysine and arginine decorating protein exteriors are transformed into neutral functional groups. This conversion fundamentally alters interactions with neighboring amino acids and thereby could contribute to the loss of the protein structure and enzymatic activity observed during protein chlorination[1,12,45] and ultimately pathogen inactivation.[2] The conversion should also enhance protein hydrophobicity, contributing to the observed increase in their tendency to adhere to hydrophobic surfaces after chlorination.[25] The limited formation of aldehydes and the lack of evidence for the formation of covalent Schiff base cross-links between proteins indicate that the protein aggregation that occurs during chlorination results instead from non-covalent, hydrophobic interactions.[22,46]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c09556.

Reaction scheme for chlorination of free amino acids, materials, synthesis of nitrile standards, Nac-Lys chloramine mass spectra and decay rate constants, Nac-Lys nitrile and aldehyde formation under different conditions, Nac-Arg chloramine formation and Mulliken charge distributions, Nac-Orn nitrile formation, mass spectra of chlorinated peptides, and Schiff base formation results (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Sivey, J. D.; Howell, S. C.; Bean, D. J.; McCurry, D. L.; Mitch, W. A.; Wilson, C. J. Role of lysine during protein modification by HOCl and HOBr: halogen-transfer agent or sacrificial antioxidant? *Biochemistry* **2013**, *52*, 1260−1271.

(2) Wigginton, K. R.; Pecson, B. M.; Sigstam, T.; Bosshard, F.; Kohn, T. Virus inactivation mechanisms: impact of disinfectants on virus function and structural integrity. *Environ. Sci. Technol.* **2012**, *46*, 12069−12078.

(3) Sano, D.; Pintó, R. M.; Omura, T.; Bosch, A. Detection of oxidative damages on viral capsid protein for evaluating structural integrity and infectivity of human norovirus. *Environ. Sci. Technol.* **2010**, *44*, 808−812.

(4) Shah, A. D.; Mitch, W. A. Halonitroalkanes, halonitriles, haloamides, and N-nitrosamines: a critical review of nitrogenous disinfection byproduct formation pathways. *Environ. Sci. Technol.* **2012**, *46*, 119−131.

(5) Li, X.-F.; Mitch, W. A. Drinking water disinfection byproducts (DBPs) and human health effects: multidisciplinary challenges and opportunities. *Environ. Sci. Technol.* **2018**, *52*, 1681−1689.
(6) Scully, F. E.; Howell, G. D.; Kravitz, R.; Jewell, J. T.; Hahn, V.; Speed, M. Proteins in natural waters and their relation to the formation of chlorinated organics during water disinfection. *Environ. Sci. Technol.* **1988**, *22*, 537−542.
(7) Conyers, B.; Scully, F. E. V. Products and implications of the chlorination of lysine in municipal wastewaters. *Environ. Sci. Technol.* **1997**, *31*, 1680−1685.
(8) How, Z. T.; Linge, K. L.; Busetti, F.; Joll, C. A. Organic chloramines in drinking water: an assessment of formation, stability, reactivity and risk. *Water Res.* **2016**, *93*, 65−73.
(9) How, Z. T.; Linge, K. L.; Busetti, F.; Joll, C. A. Chlorination of amino acids: reaction pathways and reaction rates. *Environ. Sci. Technol.* **2017**, *51*, 4870−4876.
(10) Dotson, A.; Westerhoff, P. Occurrence and removal of amino acids during drinking water treatment. *J. Amer. Water Works Assoc.* **2009**, *101*, 101−115.
(11) Pattison, D. I.; Davies, M. J. Absolute rate constants for the reaction of hypochlorous acid with protein side chains and peptide bonds. *Chem. Res. Toxicol.* **2001**, *14*, 1453−1464.
(12) Choe, J. K.; Richards, D. H.; Wilson, C. J.; Mitch, W. A. Degradation of amino acids and structure in model proteins and bacteriophage MS2 by chlorine, bromine, and ozone. *Environ. Sci. Technol.* **2015**, *49*, 13331−13339.
(13) Choe, J. K.; Hua, L.-C.; Komaki, Y.; Simpson, A. M.-A.; McCurry, D. L.; Mitch, W. A. Evaluation of histidine reactivity and byproduct formation during peptide chlorination. *Environ. Sci. Technol.* **2021**, *55*, 1790−1799.
(14) Hua, L.-C.; Kim, E.; McCurry, D. L.; Huang, C.; Mitch, W. A. Novel chlorination byproducts of tryptophan: initial high-yield transformation products versus small molecule disinfection byproducts. *Environ. Sci. Technol. Lett.* **2020**, *7*, 149−155.
(15) Komaki, Y.; Simpson, A. M.-A.; Choe, J. K.; Plewa, M. J.; Mitch, W. A. Chlorotyrosines versus volatile byproducts from chlorine disinfection during washing of spinach and lettuce. *Environ. Sci. Technol.* **2018**, *52*, 9361−9369.
(16) Hazell, L.; van den Berg, J.; Stocker, R. Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation. *Biochem. J.* **1994**, *302*, 297−304.
(17) Walse, S. S.; Plewa, M. J.; Mitch, W. A. Exploring amino acid side chain decomposition using enzymatic digestion and HPLC-MS: combined lysine transformations in chlorinated waters. *Anal. Chem.* **2009**, *81*, 7650−7659.
(18) Lehninger, A. L.; Nelson, D. L.; Cox, M. M.; Cox, M. M. *Principles of Biochemistry*, 2nd Edition; Worth Publishers, 1993.
(19) Baynes, B. M.; Wang, D. I.; Trout, B. L. Role of arginine in the stabilization of proteins against aggregation. *Biochemistry* **2005**, *44*, 4919−4925.
(20) Pednekar, D.; Tendulkar, A.; Durani, S. Electrostatics-defying interaction between arginine termini as a thermodynamic driving force in protein−protein interaction. *Proteins: Struct., Funct., Bioinf.* **2009**, *74*, 155−163.
(21) Ishibashi, M.; Tsumoto, K.; Tokunaga, M.; Ejima, D.; Kita, Y.; Arakawa, T. Is arginine a protein-denaturant? *Protein Expr. Purif* **2005**, *42*, 1−6.
(22) Winter, J.; Ilbert, M.; Graf, P.; Özcelik, D.; Jakob, U. Bleach activates a redox-regulated chaperone by oxidative protein unfolding. *Cell* **2008**, *135*, 691−701.
(23) Vissers, M. C.; Winterbourn, C. C. Oxidative damage to fibronectin: I. The effects of the neutrophil myeloperoxidase system and HOCl. *Arch. Biochem. Biophys.* **1991**, *285*, 53−59.
(24) Malle, E.; Marsche, G.; Arnhold, J.; Davies, M. J. Modification of low-density lipoprotein by myeloperoxidase-derived oxidants and reagent hypochlorous acid. *Biochim. Biophys. Acta* **2006**, *1761*, 392−415.
(25) Zeng, T.; Wilson, C. J.; Mitch, W. A. Effect of chemical oxidation on the sorption tendency of dissolved organic matter to a model hydrophobic surface. *Environ. Sci. Technol.* **2014**, *48*, 5118−5126.
(26) Matoba, T.; Shiono, T.; Kito, M. Cross-Linking of αs-1 Casein by Sodium Hypochlorite. *J. Food Sci.* **1985**, *50*, 1738−1741.
(27) Lau, S. S.; Bokenkamp, K.; Tecza, A.; Wagner, E. D.; Plewa, M. J.; Mitch, W. A. Toxicological assessment of potable reuse and conventional drinking waters. *Nature Sustainability* **2023**, *6*, 39−46.
(28) Feng, Y.; Smith, D. W.; Bolton, J. R. Photolysis of aqueous free chlorine species (HOCl and OCl) with 254 nm ultraviolet light. *J. Environ. Engineer. Sci.* **2007**, *6*, 277−284.
(29) Joo, S. H.; Mitch, W. A. Nitrile, aldehyde, and halonitroalkane formation during chlorination/chloramination of primary amines. *Environ. Sci. Technol.* **2007**, *41*, 1288−1296.
(30) Lin, H.; Levison, B. S.; Buffa, J. A.; Huang, Y.; Fu, X.; Wang, Z.; Gogonea, V.; DiDonato, J. A.; Hazen, S. L. Myeloperoxidase-mediated protein lysine oxidation generates 2-aminoadipic acid and lysine nitrile in vivo. *Free Rad. Biol. Med.* **2017**, *104*, 20−31.
(31) Hine, J.; Craig, J. C.; Underwood, J. G.; Via, F. A. Kinetics and mechanism of the hydrolysis of N-isobutylidenemethylamine in aqueous solution. *J. Amer. Chem. Soc.* **1970**, *92*, 5194−5199.
(32) Xu, K. Y. Acid dissociation constant and apparent nucleophilicity of lysine-501 of the. alpha.-polypeptide of sodium and potassium ion-activated adenosinetriphosphatase. *Biochemistry* **1989**, *28*, 6894−6899.
(33) Hauser, C. R.; LeMaistre, J. W.; Rainsford, A. The Removal of Hydrogen and Acid Radicals from Organic Compounds by Means of Bases. I. The Removal of Hydrogen Chloride from Ald-chlorimines by Sodium Hydroxide. Rates of Reaction in Alcoholic Solution. *J. Amer. Chem. Soc.* **1935**, *57*, 1056−1059.
(34) Jordan, W.; Dyas, H.; Hill, D. The Activated Complex Theory Applied to Fast Reactions. The Reaction of Aldchlorimines with Bases. *J. Amer. Chem. Soc.* **1941**, *63*, 2383−2387.
(35) Buckingham, D.; Keene, F.; Sargeson, A. Base hydrolysis of coordinated acetonitrile. *J. Amer. Chem. Soc.* **1973**, *95*, 5649−5652.
(36) Scott, W.; Bover, W.; Bratin, K.; Zuman, P. Nucleophilic additions to aldehydes and ketones. 2. Reactions to heterocyclic aldehydes with hydroxide ions. *J. Org. Chem.* **1976**, *41*, 1952−1957.
(37) Izzo, B.; Harrell, C. L.; Klein, M. T. Nitrile reaction in high-temperature water: Kinetics and mechanism. *AIChE J.* **1997**, *43*, 2048−2058.
(38) Zhang, C.; Reiter, C.; Eiserich, J. P.; Boersma, B.; Parks, D. A.; Beckman, J. S.; Barnes, S.; Kirk, M.; Baldus, S.; Darley-Usmar, V. M.; White, C. R. L-Arginine chlorination products inhibit endothelial nitric oxide production. *J. Biol. Chem.* **2001**, *276*, 27159−27165.
(39) Weng, S.; Blatchley, E. R. Ultraviolet-induced effects on chloramine and cyanogen chloride formation from chlorination of amino acids. *Environ. Sci. Technol.* **2013**, *47*, 4269−4276.
(40) Jafvert, C. T.; Valentine, R. L. Reaction scheme for the chlorination of ammoniacal water. *Environ. Sci. Technol.* **1992**, *26*, 577−586.
(41) Cordes, E.; Jencks, W. General acid catalysis of semicarbazone formation. *J. Amer. Chem. Soc.* **1962**, *84*, 4319−4328.
(42) Urbansky, E. T. Carbinolamines and geminal diols in aqueous environmental organic chemistry. *J. Chem. Educ.* **2000**, *77*, 1644.
(43) Love, B. E.; Ren, J. Synthesis of sterically hindered imines. *J. Org. Chem.* **1993**, *58*, 5556−5557.
(44) Laingam, S.; Froscio, S. M.; Bull, R. J.; Humpage, A. R. In vitro toxicity and genotoxicity assessment of disinfection by-products, organic N-chloramines. *Environ. Molec. Mutagen.* **2012**, *53*, 83−93.
(45) Howell, S. C.; Richards, D. H.; Mitch, W. A.; Wilson, C. J. Leveraging the Mechanism of Oxidative Decay for Adenylate Kinase to Design Structural and Functional Resistances. *ACS Chem. Biol.* **2015**, *10*, 2393−2404.
(46) Chapman, A. L.; Winterbourn, C. C.; Brennan, S. O.; Jordan, T. W.; Kettle, A. J. Characterization of non-covalent oligomers of proteins treated with hypochlorous acid. *Biochem. J.* **2003**, *375*, 33−40.

https://doi.org/10.1021/acs.est.2c09556
Environ. Sci. Technol. 2023, 57, 5852−5860