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Relative Reactivity of Amino Acids with Chlorine in Mixtures

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The relative reactivity of chlorine with amino acids is an important determinant of the resulting chlorination products in systems where chlorine is the limiting reagent, for example, in the human gastrointestinal tract after consumption of chlorine-containing water, or during food preparation with chlorinated water. Since few direct determinations of the initial reactivity of chlorine with amino acids have been made, 17 amino acids were compared in this study using competitive kinetic principles. The experimental results showed that (1) most amino acids have similar initial reactivities at neutral pH; (2) amino acids with thiol groups such as methionine and cysteine are exceptionally reactive and produce sulfoxides; (3) amino acids without thiol groups primarily undergo monochlorination of the amino nitrogen; and (4) glycine and proline are the least reactive. Dichlorination was estimated to occur with approximately 26% of the amino acid groups when the total amino acid: chlorine concentrations were equal.

Introduction

Amino acids have been recognized as important precursors of undesirable disinfection byproducts (DBP) formed during drinking water treatment. For example, glycine, one of the most common amino acids in natural water (*1*), was found to react with disinfectant chlorine (i.e., hypochlorous acid and hypochlorite) to produce toxic cyanogen chloride (CNCl) (*2*). This reaction has been demonstrated to account for over 40% of CNCl formed in a river water sample (*3*).

Reactions of amino acids with chlorine are also of interest at points of drinking water use. Such reactions can conceivably occur in the human gastrointestinal (GI) tract through direct consumption of water containing residual chlorine or, inadvertently, through food preparation, where amino acids, peptides, and proteins are abundant (*4*). The chemistry of amino acid reactions with chlorine is also relevant to physiological processes, for example, during in vivo generation of HOCl by phagocyte cells to kill foreign cells (*5*, *6*). While chlorine-to-amino acid ratios are greater than one under drinking water disinfection conditions, amino acids can be in excess relative to chlorine after drinking water is consumed or used. When chlorine is limiting, an assessment of whether amino acids are important precursors of hazardous chlorinated byproducts requires first a determination of their relative reactivity and ability to compete for chlorine.

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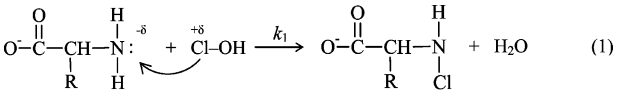
Direct measurements of the reaction rates between individual amino acids and chlorine have been performed for a few free amino acids. The pH dependence of the reaction rates suggested that the reaction occurred as the electrophilic attack of HOCl to carboxylate amino acids (*7*, *8*):



(1)

R = side-chain functional group

The side-chain functional groups also have the potential to react with chlorine; they are much less reactive, however, with the exception of the thiol groups in methionine and cysteine and the imine group in histidine (*9*).

Estimates of the second-order rate constant k_1 from the literature are provided in Table 1. A true comparison of the relative reactivity of amino acids, however, must also account for differences in their acid−base speciation. At a fixed pH, a more useful measure of reactivity would be the apparent second-order rate constant, k_{app}:

$$v = -k_{app}[HOCl][RCH(CO_2H)NH_2]_T \quad (2)$$

where $[RCH(CO_2H)NH_2]_T$ is the total concentration of unreacted amino acid. If K_1 and K_2 are the acid dissociation constants of the amino acid, the pH-dependent relationship between k_{app} and k_1 is

$$k_{app} = \frac{k_1 K_1 K_2}{[H^+]^2 + K_1[H^+] + K_1 K_2} \quad (3)$$

Values of k_{app} are also compared in Table 1 at pH 7. Within this limited data set, proline had the slowest reaction rate with HOCl. There is also a 2-fold difference in the estimated values of the rate constants for the reaction of HOCl with glycine and alanine. In view of the small subset of amino acid chlorination rate constants available in the literature and their uncertainty, additional studies are needed to definitively assess which amino acids are likely to react most rapidly with chlorine.

Although slower than a diffusion-controlled reaction, chlorine transfer from the HOCl oxygen to the amino group nitrogen is a relatively rapid reaction, with half-lives of at most a few milliseconds at neutral pH and typical reagent concentrations. Direct analytical measurements of amino acid chlorination rates require rapid data acquisition capabilities, such as the stopped-flow techniques used by the groups referenced in Table 1. Rather than assessing the reactivity of each amino acid individually, a qualitative assessment of their relative reactivity with chlorine was performed in this study using mixtures of amino acids and competitive kinetic principles. Such comparisons are helpful in determining which amino acids would be most reactive when chlorine is limiting. This approach has the added advantage of considering the effects of chlorine reactivity with chlorination products that compete with amino acids for chlorine.

Materials and Methods

Reagents. Seventeen α-amino acids were used in this study. Glycine (Gly) was purchased from Alfa Aesar. Alanine (Ala), aspartic acid (Asp), cysteine (Cys), glutamic acid (Glu), isoleucine (Ile), leucine (Leu), methionine (Met), phenylalaine (Phe), proline (Pro), serine (Ser), threonine (Thr), and valine

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TABLE 1. Comparison of Apparent Amino Acid Reactivity with Chlorine at pH 7, 25 °C

amino acid	k_1 (10^7 $M^{-1}s^{-1}$)	reference for k_1	pK_1[a]	pK_2[a]	k_{app}[b] (10^5 $M^{-1}s^{-1}$)
glycine	5	*8*	2.34	9.60	3.9
	11.3	*7*			8.9
alanine	5.4	*8*	2.34	9.69	3.5
	3.5	*7*			2.2
isoleucine	6.4	*7*	2.36	9.60	5.0
proline	2.0	*7*	1.99	10.60	1.6

[a] From Lide et al. (2004) (*10*). [b] Calculated from k_1, K_1, K_2, and [H^+] according to eq 3.

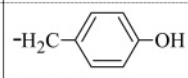
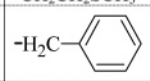
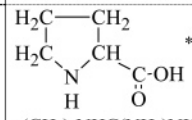
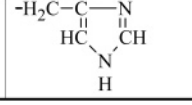
(Val) were obtained from Sigma Aldrich. Arginine (Arg), histidine (His), and lysine (Lys) were received from Acros Organics. Tyrosine (Tyr) was purchased from ICN Biomedicals Inc. Solutions of single amino acid or amino acid mixtures were made with deionized (DI) water (Nanopure D4744, Barnstead International) and stored at 4 °C for no more than 2 weeks. Sodium hypochlorite (4−6%) used as a source of chlorine was obtained from Fisher Scientific. Working solutions were made fresh daily by diluting the stock solution with DI water and then standardizing it by *N, N*-diethyl-*p*-phenylenediamine (DPD) titration (*11*) using agents purchased from LabChem, Inc. Reagents for amino acid analyses were obtained from several sources, including standards of amino acid mixtures (2.5 mM each, from Pierce), methionine sulfoxide, and methionine sulfone (both from Sigma Aldrich), derivatization agents, eluent concentrate (both from Waters), and acetonitrile (Fisher Scientific).

HPLC Analysis of Amino Acids. The concentration of each amino acid, before and after reacting with chlorine, was measured by a precolumn derivatization fluorescence high-performance liquid chromatography (HPLC) method (*12*). The HPLC (HP model 1590) was equipped with a fluorescence detector (Agilent 1100 series) and an Accq·Tag column (Millipore Corp.) The derivatization was conducted using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, acetonitrile and borate buffer. The mobile phase consisted of DI water, a 1:10 dilution of Accq·Tag eluent A concentrate (sodium acetate trihydrate, phosphoric acid, triethylamine and sodium azide) and HPLC-grade acetonitrile. The gradient of the three components was programmed as 100% eluent A at 0 min, 99% eluent A and 1% acetonitrile at 0.5 min, 95% eluent A and 5% acetonitrile at 18 min, 91% eluent A and 9% acetonitrile at 19 min, 83% eluent A and 9% acetonitrile at 32 min, 0% eluent A and 60% acetonitrile at 36 min, and 100% eluent A at 39 min until 45 min.

Experimental Procedures. The competitive chlorination experiments were conducted by reacting amino acid mixtures with free chlorine over a range of initial chlorine-to-total-amino acid molar ratio ($[Cl_2]_o$:$[TAA]_o$) from 0 to 2 in a 1.8 mL headspace-free vial. In each of the mixture subsets, individual amino acids were present in equal concentrations of approximately 50 μM. After 3 h of reaction in darkness, which ensured the decomposition of unstable *N, N*-dichloroamino acids that might cause analytical interferences, the concentrations of residual amino acid were measured by HPLC (*12*). Similar experiments were also conducted with single amino acids to explore chlorination mechanisms. The reaction solutions were not buffered because common buffers such as phosphate could accelerate the decomposition of *N*-chloroamino acids (*13*) and promote undesirable secondary reactions. Even without buffer, the reaction solutions remained near the desired circumneutral pH range.

To avoid known analytical interferences of some of the chlorination products with the amino acids (common peak elution times), the 16 amino acids listed in Table 2 were strategically grouped into three mixture sets. Cysteine (Cys) was not included in these experiments because a preliminary study with all 17 amino acids provided strong evidence that cysteine had a similar reactivity to methionine (*9*) (data not included because of analytical interferences among the other

TABLE 2. Composition of Amino Acid Mixtures Used in Competitive Chlorination Experiments

Amino Acid	Abbreviation	Structure of R Functional Group*	Mixture Group I	Mixture Group II	Mixture Group III	pK_1†	pK_2†	k_1/k_{app}‡ at pH 7
Glycine	Gly	-H	↑			2.34	9.60	399
Valine	Val	$-CH(CH_3)_2$				2.32	9.62	418
Alanine	Ala	$-CH_3$				2.34	9.69	491
Glutamic Acid	Glu	$-CH_2CH_2COOH$				2.19	9.67	469
Aspartic Acid	Asp	$-CH_2COOH$	↓	↑		1.88	9.60	399
Threonine	Thr					2.09	9.10	127
Leucine	Leu	$-CH_2CH(CH_3)_2$				2.36	9.60	399
Serine	Ser	$-CH_2OH$				2.21	9.15	142
Tyrosine	Tyr	$-CH(CH_3)OH$				2.20	9.11	130
Methionine	Met	$-CH_2CH_2SCH_3$				2.28	9.21	163
Phenolalanine	Phe			↓	↕	1.83	9.13	136
Proline	Pro	 **				1.99	10.60	3982
Arginine	Arg	$-(CH_2)_3NHC(NH_2)NH$				2.17	9.04	111
Lysine	Lys	$-CH_2(CH_2)_3NH_2$				2.18	8.95	90
Histidine	His				↓	1.82	9.17	149

* The backbone structure is depicted in Equation (1). ** Entire structure. † From Lide et al. (2004) (*10*). Data are for 25 °C. ‡ Calculated from K_1, K_2 and [H^+] = 1-^{-7} M according to Equation (3).

amino acids). Both cysteine and methionine contain highly reactive thiol groups. Aspartic acid and phenylalanine were included as "benchmark" amino acids in more than one mixture to facilitate cross-comparison of the resulting data sets. These benchmarks are indicated with the set numbers to which they are common in Table 2.

Results and Discussion

Relative Reactivity of Amino Acids with Chlorine. The relative reactivity of amino acids with chlorine was compared by measuring the residual amino acid concentrations after adding varying amounts of chlorine to amino acid mixtures. Because the total amino acid concentration was in excess, free chlorine was completely consumed by the end of the reaction (i.e., after 3 h).

The pH of the amino acid mixtures was initially around 6.5 (±0.2). Adding sodium hypochlorite solution slightly increased the solution pH. The final pH depended on the amount of free chlorine used. The highest pH, about 7.5 (±0.2), was obtained when the chlorine-to-total amino acid molar ratio ($[Cl_2]_o/[TAA]_o$) was approximately 2. A pH change from 6.5 to 7.5, however, would not be expected to result in a different ranking of relative reactivity of chlorine among the amino acids. Given the pH dependence of k_{app} described in eq 3, and the typical values of K_1 and K_2 of the amino acids, the pH dependence of k_{app} over the pH range of our experiments (i.e., 6.5 < pH < 7.5) is approximately

$$k_{app} \approx k_1 \frac{K_2}{[H^+]} \tag{4}$$

Thus the ratio of k_{app} for any two amino acids in a mixture that undergoes a pH change in this range is

$$\frac{k_{app,\ amino\ acid\ 1}}{k_{app,\ amino\ acid\ 2}} = \frac{(k_1K_2)_{amino\ acid\ 1}}{(k_1K_2)_{amino\ acid\ 2}} \tag{5}$$

which is independent of pH.

Immediately after the addition of concentrated stock NaOCl, the reaction between amino acids and chlorine might have occurred locally at higher pH due to the incomplete mixing. Chlorine in these local alkaline regions, however, would be much less reactive, since hypochlorite, a much weaker electrophile, is the predominant chlorine species at pH > 7.5.

Results of the competitive chlorination experiments after a 3-hour reaction period are provided in Figure 1. The amino acid content of the reacted mixtures are reported in terms of residual percentage (RP), which is the residual concentration (×100) divided by the initial concentration, to eliminate the small differences among initial amino acid concentrations. In each mixture, the residual percentages of all amino acids decreased with increases in the chlorine-to-total amino acid molar ratio as expected. Moreover, most of the amino acids had very similar residual percentages at each $[Cl_2]_o/[TAA]_o$ dose, which suggests that they had similar relative reactivities with chlorine. The similarity of the relative reactivity among most amino acids is consistent with the limited kinetic data available in the literature (*7*, *8*). The residual percentages of these amino acids approached zero at $1 < [Cl_2]_o/[TAA]_o < 1.5$. This chlorine-to-amino acid ratio is greater than unity, which would be expected from the 1:1 stoichiometry of *N*-monochloroamino acid formation, indicating that reactions of chlorine with chlorination products were significant in the competition for free chlorine, although monochlorination was probably the predominant reaction. The competition between HOCl and chlorination products for amino acids was, however, expected to be negligible. Chlorination reactions with HOCl were found to be hundreds times faster than with *N*-monochloroglycine (*14*).

The relative reativities of the amino acids in each mixture may be summarized as follows:

mixture I: Asp ≈ Ala ≈ Val ≈ Ile ≈ Glu > Gly

mixture II: Met > Asp ≈ Thr ≈ Ser ≈ Leu ≈ Tyr ≈ Phe

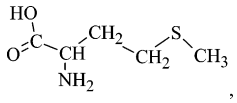
mixture III: Phe ≈ Lys ≈ Arg ≈ His > Pro

Using the "benchmarks" in each mixture, i.e., $Asp_I = Asp_{II}$ and $Phe_{II} = Phe_{III}$, and Cys ≈ Met (*9*), the relative apparent reactivity with chlorine was obtained for the 17 amino acids of interest. Three groupings of fast, medium, and slow reactions were observed as follows:

$$\begin{matrix}\text{Met} \\ \text{Cys}\end{matrix} > \text{Ala Arg Asp His Glu Ile Leu} > \begin{matrix}\text{Pro} \\ \text{Gly}\end{matrix}$$
$$\text{Lys Phe Ser Thr Tyr Val}$$

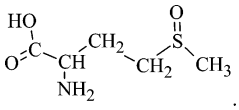
This order of relative reactivity may be interpreted as the order of the values of the apparent rate constant, k_{app}, around pH 7 for each amino acid. According to eq 3, this order is subject to the influence of amino acid speciation. However, given the large differences between the fast, medium, and slow groups, a comparison based on intrinsic k_1 values is likely to yield similar groups.

Chlorination Kinetics of Methionine. Methionine was completely consumed in mixture II (Figure 1b), at the lowest $[Cl_2]_o/[TAA]_o = 0.3$, suggesting it has superior reactivity with chlorine. This is consistent with a previous report by Pattison and Davies (*9*), in which the k_{app} value of methionine thiol was estimated to be over 2 orders of magnitude higher than that of the α-amino groups in aliphatic amino acids. Methionine, with a thiol functional group,



is known to be very reactive with oxidants. The initial oxidation likely occurs at the thiol function group rather than the amine nitrogen as depicted by reaction 1.

The chlorination of methionine has been reported to produce methionine sulfoxide (MSD) (*15*):



Indeed, the formation of methionine sulfoxide was demonstrated in this study during the chlorination of methionine alone. As illustrated in Figure 2, with the decrease of the peak representing methionine at 26.6 min, a new peak appeared with a retention time of 22.2 min. This new peak was identified as methionine sulfoxide by comparing it to the peaks obtained with MSD standards. As shown in Figure 3, as the residual concentration of methionine decreased with the increase of initial chlorine-to-methionine ratio ($[Cl_2]_o/[Met]_o$), the MSD concentration increased up to $[Cl_2]_T/[Met]_o = 1.2$. Further increases in the chlorine concentration caused methionine sulfoxide to diminish, while at the same time, the ammonia concentration increased, suggesting ammonia was likely a product of methionine sulfoxide decay. The sum of [Met], [MSD], and $[NH_3]$ gradually decreased with increasing chlorine dose, indicating the formation of other chlorination products.

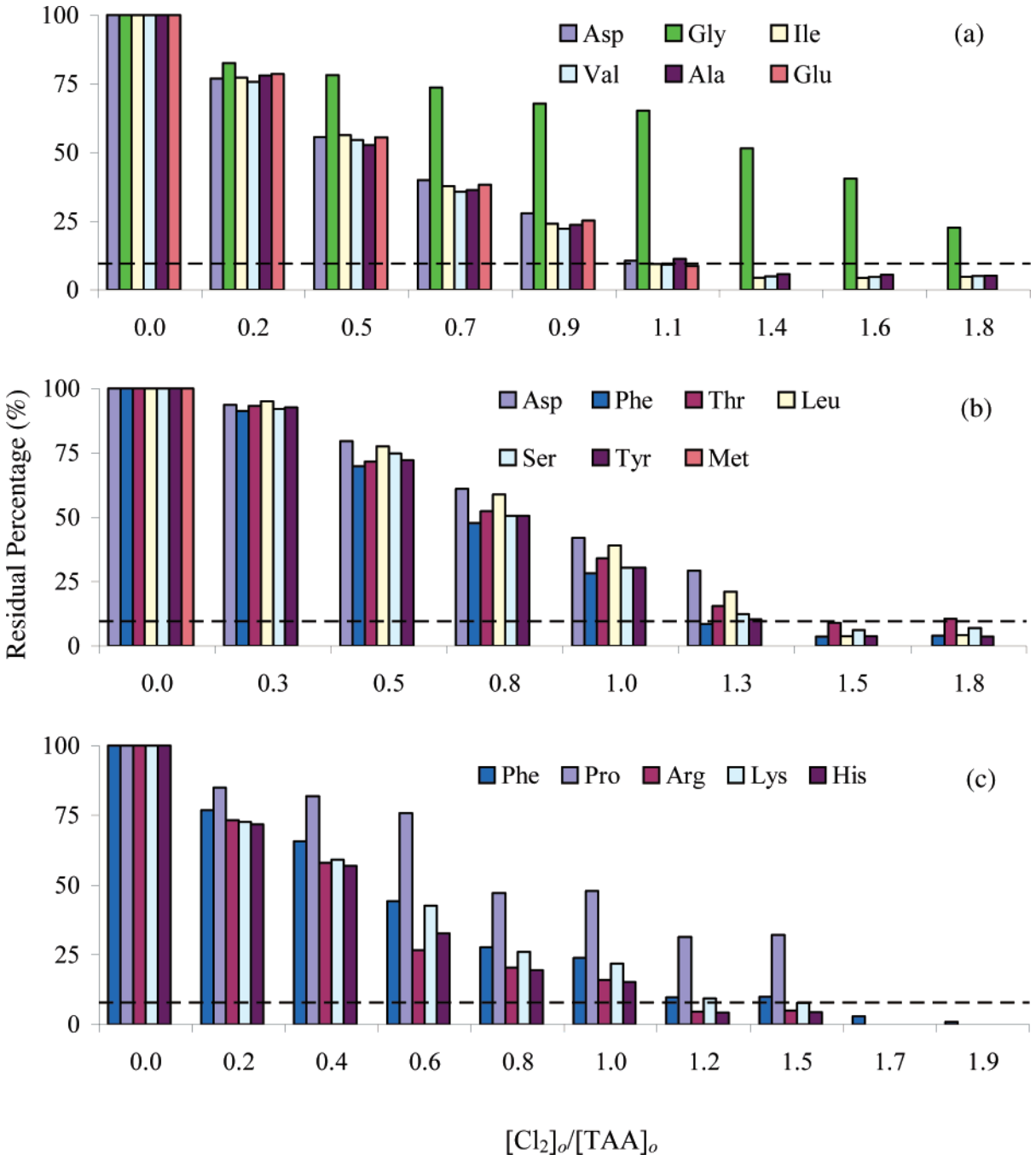
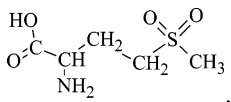


FIGURE 1. Relative reactivity of amino acids with chlorine. Asp and Phe were used as "benchmarks" for cross-comparisons. Initial concentrations of individual amino acid averaged 45(±4) μM. (a) Mixture I contained Asp (41 μM), Gly (43 μM), Ile (44 μM), Val (51 μM), Ala (45 μM), and Glu (42 μM); (b) mixture II contained Asp (36 μM), Phe (37 μM), Thr (40 μM), Leu (39 μM), Ser (44 μM), Tyr (38 μM), and Met (39 μM); and (c) mixture III contained Phe (50 μM), Pro (48 μM), Lys (48 μM), His (46 μM), and Arg (49 μM). Dashed lines are the average detection limits for each mixture calculated from the HPLC calibration curves at 95% confidence level.

One possible product was methionine sulfone (MSN),



which is a known product of the complete oxidation of methionine thiol (*16*). A comparison between the chromatograms of chlorinated methionine solutions and MSN standards, however, showed that methionine sulfone was not detected (Figure 2). This is consistent with the results obtained by Scully et al. (*17*) that methionine sulfone was only observed at $[Cl_2]_T/[Met]_o > 2$ whereas in our system $[Cl_2]_T/[Met]_o < 2$. At $[Cl_2]_T/[Met]_o < 2$, the decrease of methionine sulfoxide concentration together with the absence of methionine sulfone suggest that one or more additional intermediates were involved in the oxidation of methionine sulfoxide to methionine sulfone by chlorine.

Slow Kinetics of Glycine and Proline. Glycine was conspicuously less able to compete for chlorine than most of the other amino acids. As illustrated by Figure 1a, glycine had much higher residual percentages than other amino acids in mixture I. This suggests that glycine was much less reactive than other amino acids. An alternative explanation for glycine's higher residual percentages could be due to an analytical interference of chlorinated glycine. It has been

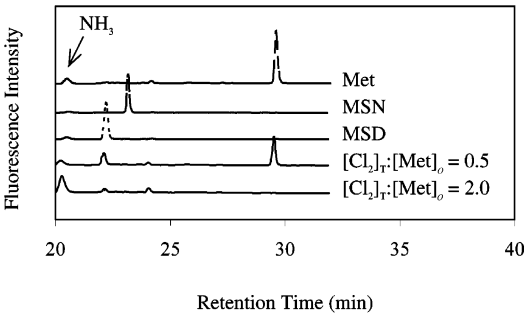


FIGURE 2. HPLC chromatograms of methionine (Met), methionine sulfone (MSN), methionine sulfoxide (MSD) standards, and chlorinated methionine. [Met] ≈ [MSN] ≈ [MSD] = 50 μM.

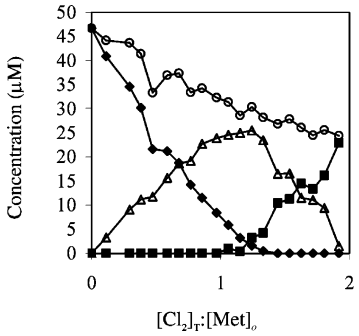


FIGURE 3. Formation of methionine sulfoxide (MSD) from the chlorination of methionine as a function of the initial chlorine: methionine ratio after 3 h. ◆ = [Met]. △ = [MSD]. ■ = [NH_3]. ○ = [Met] + [MSD] + [NH_3].

previously reported that some derivatization reagents could react with both amino acids and monochlorinated amino acids and give HPLC peaks with similar retention times (*18*). However, it was experimentally (data not shown) determined that the derivatization reagent used in this analysis (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) was not subject to such interference.

Proline was the least reactive in mixture III (Figure 1c). As was shown for glycine, chlorination experiments of proline alone demonstrated that the higher residual percentages of proline in the chlorination experiments of mixture III were not due to interference from chlorinated proline. By considering the "benchmark" amino acids, it appears that the relative reactivities of glycine and proline are similar.

The slow reactivities of glycine and proline may be explained by their unique structures. Most of the amino acids are primary amines with an alkyl group bonded to the α-carbon (see Table 2). In the case of glycine, the substituent is a hydrogen atom, instead of an alkyl group, whereas for proline it is a secondary amine. In general, the inductive or electron donating effect is greater for alkyl groups than hydrogen atoms. Consequently, for glycine, the lack of an alkyl functional group on the α-carbon makes the carbon more electron positive, which in turn makes the glycine nitrogen less electron negative and thus less nucleophilic. The reaction between amino acids and chlorine, as shown in reaction 1, occurs as the nucleophilic attack of amino nitrogen on the hypochlorous acid chlorine atom. The lesser nucleophilicity of the amino nitrogen in glycine explains its lesser reactivity with chlorine. For proline, the substitution of the amine hydrogen by an alkyl group renders more electron density on the amine nitrogen; thus it actually becomes more nucleophilic. However, steric hindrance effects are also more important in secondary amines, especially for proline whose alkyl substitutent forms a cyclic structure with the α-carbon. Acid−base speciation effects would, in addition, contribute to the apparent lower reactivity of proline. Proline has the highest pK_2 value among the amino acids tested, and hence, the fully deprotonated reactive form of proline would be smaller in concentration relative to the other amino acids. We propose that the slower reactivity of proline was due to these steric and acid−base speciation effects.

Kinetics of Other Amino Acids. Amino acids other than methionine, cysteine, glycine, and proline, had similar relative reactivities at pH 7 and, therefore, similar apparent rate constants, k_{app}. According to eq 3, the amino acids that have higher pK_1 and pK_2 values should have higher k_1 values. The largest difference in k_1 values among this group, therefore, would be between alanine (pK_2 = 9.69) and lysine (pK_2 = 8.95) (*10*). Based on eq 3, the k_1 value of alanine should be about 5.4 times that of lysine around pH 7 (Table 2). This is also consistent with the nucleophilicity theory used to explain the slow reactivities of glycine and proline. For the amino acids with higher pK_2 values, their amino nitrogens have a higher affinity for protons, which can be interpreted as higher nucleophilicity. Greater nucleophilicity of the amino nitrogen promotes its reaction with hypochlorous acid.

Implications for the Ingestion of Chlorinated Water. The superior reactivity of methionine and cysteine suggests that they may act as some of the first "quenching" agents for chlorine. Wherever they are present, chlorine will first consume nearly all methionine and cysteine before reacting with other amino acids. More research is needed, therefore, to determine whether the chlorination products of methionine, cysteine, and other thiol-containing compounds are of human health concern.

Our research suggests that further chlorination of the remaining amino acids in a mixture, when chlorine is limiting, is likely to produce a variety of mainly monochlorinated amino acids due to their similar reactivity. Monochlorinated amino acids are known to be relatively stable at neutral pH and would be expected to persist. *N*-monochloroglycine, for example, has a half-life of over 45 h at pH 7 (*19*). Although few examples exist in which monochlorinated amino acids have been measured in natural amino acid rich samples, an in-vitro chlorination study of rat stomach fluid is available. In this study Scully et al. (*20*) measured the formation of several *N*-chloroamino acids, including *N*-monochloroglycine, *N*-monochloroalanine, *N*-monochloroleucine, *N*-monochlorophenylalanine, *N*-monochloroserine, and *N*-monochlorovaline. Their findings appear to corroborate the expected persistence and wide variety of monochlorinated amino acids that is suggested by our research.

The eventual slow decomposition of *N*-chloroamino acids and the more rapid decay of *N, N*-dichloroamino acids are known to yield nitriles, aldehydes, and chloraldimines (*21*−*24*). In previous chlorination studies with glycine, we found that nitrile formation required *N, N*-dichloroglycine formation, however (*2*). Aldehydes and chloraldimines, on the other hand, are known products of *N*-monochloroamino acids. By virtue of the similar reactivities of amino acids, the tendency to form nitriles may be small when chlorine is limiting. The slow kinetics of monochloroamino acid decay, however, may inhibit the formation of aldehydes and chloraldimines. Although these are factors which would limit nitrile, aldehyde, and chloroaldimine formation, more research is needed to conclusively determine their formation potential in amino acid rich systems.

A wide range of pH conditions are possible in the human gastrointestinal system, but it is not clear whether amino acid chlorination would occur throughout this system as a result of drinking chlorinated water. In saliva, for example, the pH ranges from 5.45 to 7.4, while in the stomach, the pH may be as low as 1.2 (*4*). Given the rapid kinetics of amino acid reactions with chlorine, it is possible that chlorine extensively reacts before reaching this acidic environment.

Additional studies of amino acid chlorination over a wider range of pH, however, would be helpful to determine whether the relative reactivity of amino acids with chlorine changes as a function of pH.

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