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The formation of *N*-chloramines with proteinogenic amino acids

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ABSTRACT

In this study, the formation of 17 *N*-chloramines from proteinogenic amino acids and HOCl was studied by direct kinetic method in the pH = 3–13 range. Thus, the uncertainties associated with the indirect methods used in some of the previous studies were eliminated. Each reaction proceeds according to an overall second order kinetics: $v = -k\,[HOCl][R{-}NH_2]$ and the rate constants are several times $10^7\ M^{-1}s^{-1}$. A very slight correlation was found between the lg*k* and the pK_{AA} of the amino acids. The results make possible to predict the reactivity order of the amino acids toward HOCl under various conditions. A comparison of the parameters of activation indicates that the presence of a bulky substituent on the side chain close to the α-carbon atom decreases the strength of bonding between the reactants and make the structure more diffuse in the transition state. The chlorination of histidine proceeds via two pH dependent paths presumably leading to the formation of *N*-chloramine and a side chain chlorinated product. The latter compound may be involved in fast subsequent trans-chlorination reactions. The results presented here resolve earlier discrepancies in the literature and are relevant in chlorination water treatment technologies as well as in the interpretation of *in vivo* processes involving the formation of *N*-chloro amino acids in a wide pH range.

1. Introduction

Chlorination of organic amines frequently occurs in hypochlorous acid/chlorine based water treatment technologies(White, 1992). These processes typically generate *N*-chloro or *N,N*-dichloro compounds (eqs (1), (2)) depending on the dosage of the oxidant(Conyers and Scully, 1993, 1997; Fox et al., 1997; Fox and Scully, 1997; Keefe et al., 1997; McCormick et al., 1993; Scully et al., 1988). In the case of ammonia (R = H), NCl_3 may also form (eq. (3)), which undergoes decomposition in a subsequent reaction step (eq. (4)). (Kumar et al., 1987)

$$R{-}NH_2 + HOCl = R{-}NHCl + H_2O \quad (1)$$

$$R{-}NHCl + HOCl = R{-}NCl_2 + H_2O \quad (2)$$

$$NHCl_2 + HOCl = NCl_3 + H_2O \quad (3)$$

$$2NCl_3 + 3H_2O = N_2 + 3HOCl + 3Cl^- + 3H^+ \quad (4)$$

The decomposition of these species is of primary concern because of the possible formation of antagonistic products which may find their ways into biological systems(Scully and White, 1991; White, 1992).

The very same kind of chemistry takes place under *in vivo* conditions. In humans, hypochlorous acid is formed in the myeloperoxidase enzyme catalyzed oxidation of Cl^- as part of the defense mechanisms against invading pathogens(Foote et al., 1983; Hazen et al., 1996; How et al., 2017; Laingam et al., 2012; vanDalen et al., 1997). Hypochlorous acid[1] is a strong, non-discriminating oxidant which has a controversial role in biological systems. Essentially it may induce necrosis or apoptosis in the pathogens but also in healthy cells via the same reaction paths(Heinzelmann et al., 1999; Kim et al., 2009; Klebanoff, 2005; Pullar et al., 2000; Zhang et al., 2003). As part of these processes the oxidation of amino acids, as well as peptides and proteins need to be considered. The secondary effects due to the formation of biologically active

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[1] The protolytic equilibrium between HOCl and OCl^- is established at a diffusion controlled rate. Consequently, the concentration ratio of the two forms is determined by the actual pH. In this paper, we use one name or formula – hypochlorous acid, HOCl – for both the acidic and basic forms. Distinction between HOCl and OCl^- is made only when it is required for the clarity of the presentation.

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products via the decomposition of *N*-chloro amino acids may also be relevant(Simon et al., 2019; Szabó et al., 2015). It was shown that HOCl reacts considerably faster with the thiol group than with the amino group, thus, first the available SH substituted compounds are oxidized in a biological system(Armesto et al., 2000; Nagy and Ashby, 2007; Peskin and Winterbourn, 2001; Storkey et al., 2014; Winterbourn, 1985). In the case of cysteine and methionine the products are disulfides and sulfoxides, respectively. In general, oxidation of proteins by HOCl may lead to cross-linking or aggregation(Pullar et al., 2000; Schraufstätter et al., 1990).

Since amino acids and related compounds are present in relatively large excess over HOCl in living systems, only the formation of mono *N*-chloro amino compounds is feasible once the SH groups are oxidized(Panasenko et al., 2013). These species may cause genotoxicity or cytotoxicity in humans(Laingam et al., 2012; Stacey et al., 2012). Earlier studies have explored certain details of the formation of *N*-chloro amino acids. The most thorough studies were reported by Armesto et al. 1993, 1994 The reactions of some of the most common amino acids with HOCl were shown to be very fast exhibiting distinct pH profiles. The pH dependence of the reaction rate was interpreted in terms of the protolytic equilibria of the reactants.

The relative reactivity of amino acids toward HOCl has been the subject of studies under limited conditions and using indirect methods which necessarily introduced uncertainties into the results (How et al., 2016; Na and Olson, 2007; Pattison and Davies, 2001). In the indirect kinetic studies, a mixture of excess amino acids was reacted with hypochlorous acid and the relative rates were estimated on the basis of product distribution long after mixing the reactants. The degradation of the *N*-chloro amino acids and cross-chlorination could easily led to false results in these cases. Most of the previous investigations have been performed at around pH 7.4 by implying that the formation of *N*-chloro amino acids is only relevant at physiological pH. However, the stimulation of the myeloperoxidase enzyme (MPO) catalyzed oxidation of chloride ion to HOCl within the phagosome may also occur under slightly acidic conditions(Di et al., 2006; Hackam et al., 1999; Ip et al., 2010). In addition, water treatment technologies are implemented in a broader pH range. The consumption of chlorinated water may introduce residual chlorine and chloramines into the saliva and the highly acidic gastric fluid(Scully and White, 1991). Consequently, expanding these comparisons to an extended pH range by using a direct kinetic method is of primary importance.

Now, we report detailed kinetic studies on the formation of *N*-chloro species with 17 proteinogenic amino acids by monitoring the chlorination reaction directly in the pH 3–13 range. The reactions of compounds with SH substituents were excluded from this study because our preliminary observations were fully consistent with previous literature results. The results presented here allow the direct comparison of the reactivities of amino acids toward chlorination in a broad pH range. It will also be shown that the chlorination of histidine occurs via two pH dependent competitive reaction paths. The parameters of activation were also determined for these reactions.

2. Experimental

2.1. Chemicals and solutions

All *L*-amino acids used in this study were of analytical reagent grade and used as received without further purification (*Sigma Aldrich*). In order to obtain the rate constants under the same experimental conditions over the entire pH range studied, the ionic strength was adjusted to $I = 1.0$ M by using appropriate amounts of $NaClO_4$ prepared as described earlier(Fábián and Gordon, 1991). The pH was controlled by using 0.01 M phosphate and 0.015 M borax buffers under neutral (pH 5–8.5) and slightly alkaline (pH 9–10.5) conditions, respectively. Higher pH was set by using standardized NaOH solution. Doubly deionized and ultra-filtered (ELGA Purelab Classic system) water was used to prepare the stock solutions and the samples. The temperature was set to 25.0 °C in the experiments except in the temperature-dependence measurements. Chloride ion free sodium hypochlorite solution was prepared as described earlier(Adam et al., 1992; Peintler et al., 1990). The stock solutions of NaOCl were stored at 4 °C in the dark and were standardized before use as follows. After acidification, KI was added to an aliquot of the solution and the iodine formed was titrated by standardized $Na_2S_2O_3$ solution yielding the concentration of hypochlorite ion(Vogel, 1989). Another aliquot of the stock solution was titrated with standardized $HClO_4$ solution. In this titration, two equivalence points were observed allowing the calculation of the concentration of OCl^- and the excess NaOH. An excellent agreement was found in the concentrations of hypochlorous acid obtained from the two analytical methods.

2.2. Methods

pH measurements were performed using a Metrohm 785 DMP Titrino automatic titrator equipped with a 6.0262.100 pH electrode. The electrode was calibrated every day using KH-phthalate (0.05 M) and borax (0.01 M) standard solutions. The readout of the pH meter was converted to $pH = -\log[H^+]$ as described by Irving et al., (1967) Iodometric and pH-metric titrations were made with a Metrohm 888 Titrando system equipped with Metrohm 6.0451.100 combination platinum and Metrohm 6.0262.100 combination glass electrodes, respectively. The acid dissociation constants of the amino acids were determined on the basis of pH-metric titrations by evaluating the data with the dedicated program, SUPERQUAD(Gans et al., 1985).

Kinetic experiments were carried out with an Applied Photophysics SX-20 stopped-flow instrument using a photomultiplier tube (PMT) attached as the detector. The kinetic traces were collected using 10.0 mm optical path length. Kinetic traces were obtained as the average of 3–5 runs. The dead time of the stopped-flow instrument – $t_d = 1.51$ ms – was determined by monitoring the reduction of 2,6-dichlorophenol-indophenol (DCPIP) under pseudo-first order conditions with ascorbic acid in excess(Tonomura et al., 1978).

The first-order kinetic traces were fitted with the controlling software of the stopped-flow instrument. All other data fitting was made with the program package OriginPro (2018) using nonlinear least-squares routines. (2018).

^{1}H NMR measurements were made on a Bruker DRX 400 (9.4 T) NMR spectrometer equipped with a Bruker VT-1000 thermocontroller and BB inverse *z* gradient probe (5 mm). Each solution was prepared in H_2O and DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) in D_2O was added to the sample in a capillary as an external standard for ^{1}H (0 ppm). The spectra were recorded by using the standard Bruker watergate pulse sequence for the suppression of water proton signal. In each ^{1}H NMR experiment, 32 scans were collected with 16K data points using a sweep width of 5995 Hz, a pulse angle of 90°, an acquisition time of 1.366 s and relaxation delay of 2 s. The spectra were analyzed with the Bruker WinNMR software package.

Mass spectra were recorded in the positive mode with a Bruker micrOTOF-Q type Qq-TOF-MS instrument (Bruker Daltonik, Bremen, Germany) instrument. The tuning parameters were optimized to examine the desired mass range (50–500 m/z). Na-formate solution was injected after each sample for internal calibration of each individual analysis (relative mass errors were <2 ppm).

Collision induced fragmentation (CID) was applied in MS/MS investigation of the reaction products. Different collision energies were applied between 5 and 30 eV, and the optimal fragmentation energy was ~13 eV. Mass spectra were recorded with otof Control version 4.1 (build: 3.5, Bruker) and processed by Compass Data Analysis version 4.4 (build: 200.55.2969).

3. Results and discussion

Mixing hypochlorous acid with amino acids in excess yields quickly the corresponding *N*-chloro amino acid which exhibit characteristic absorption maximum at around 255 nm. Representative spectra and the absorption maxima of these species are given in the Supplementary Material (Fig. S1 and Table S1).

The amino acids containing aromatic side chains show specific features and their reactions are discussed in subsequent parts of this paper. The formation of *N*-chloro compound from the rest of the amino acids follows the same kinetic pattern. Under alkaline conditions and in the excess of the amino acid, simple first order kinetic traces were observed in each system. As demonstrated in Fig. 1, the corresponding pseudo-first order rate constants (k^{1st}_{obs}) are linearly dependent on the amino acid concentration with zero intercept. Under the same conditions k^{1st}_{obs} does not depend on the concentration of HOCl. These observations confirm that the reaction is first order for both reactants, i.e. the reaction is overall second order (eq. (5)).

$$\frac{dc_{HOCl}}{dt} = -k^{2nd}_{obs} c_{HOCl} c_{AA} \quad (5)$$

where c_{HOCl} and c_{AA} are the concentrations of hypochlorous acid and the amino acid, respectively.

The formation of the *N*-chloro amino acids becomes increasingly faster by decreasing the pH and eventually the pseudo-first order conditions can not be applied. Under slightly alkaline conditions, the reactants were used in comparable concentrations in the kinetic experiments and the experimental results were evaluated by fitting the kinetic traces with the corresponding second order expression (eq. S(1)). The second order pH dependent rate constants (k^{2nd}_{obs}) obtained from the two types of experiments are consistent with each other and show a bell-type pH dependence (Fig. 2).

To provide a coherent interpretation for these observations it needs to be considered that in principle *N*-chloro amino acids may

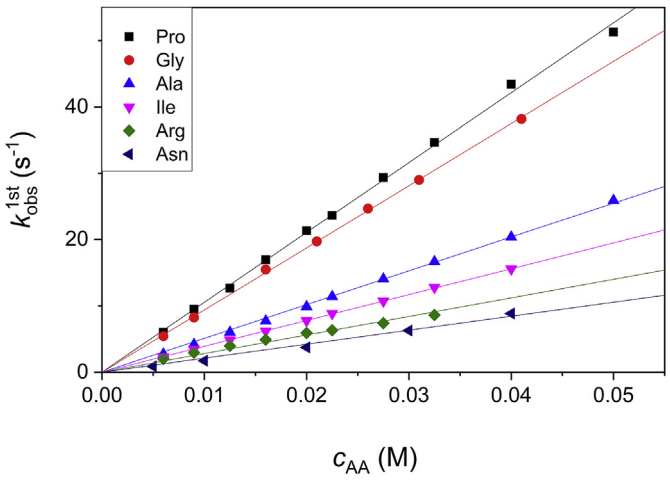

Fig. 1. The dependence of k^{1st}_{obs} on the concentration of amino acids under alkaline conditions. $c^0_{HOCl} = 1.00 \times 10^{-3}$ M, $[OH^-] = 5.00 \times 10^{-2}$ M, $I = 1.00$ M $NaClO_4$, $T = 25.0$ °C.

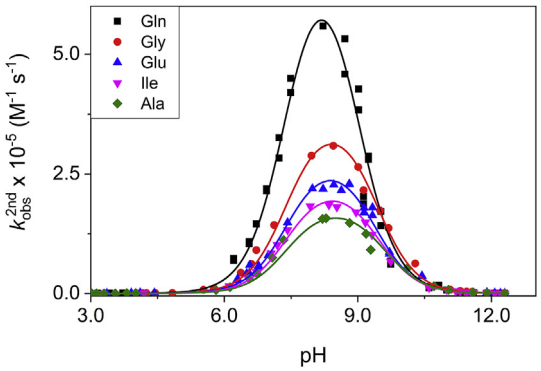

Fig. 2. The pH dependence of k^{2nd}_{obs}. Experimental data (markers) and fitted curves on the basis of eq. (10) (solid lines). $I = 1.00$ M $NaClO_4$, $T = 25.0$ °C.

form via four competing reaction paths as shown in eq.s. (6)–(9).

$$HOCl + RNH_3^+ = RNHCl + H_2O + H^+ \quad (6)$$

$$OCl^- + RNH_3^+ = RNHCl + H_2O \quad (7)$$

$$HOCl + RNH_2 = RNHCl + H_2O \quad (8)$$

$$OCl^- + RNH_2 = RNHCl + OH^- \quad (9)$$

These reactions take into account the acid-base equilibria of HOCl and the NH_2 or NH (in proline) group of the amino acid. (The carboxylic groups of the amino acids are fully deprotonated in the studied pH region – cf. Table S2 – and the corresponding acid-base equilibrium is not considered in the model. The negative charge of this group is not shown either.) The reaction paths between the protonated (eq. (6)) as well as the deprotonated (eq. (9)) forms of the reactants are negligible. Due to proton ambiguity, the other two paths are indistinguishable experimentally. However, the oxidation of the ammonium ion by OCl^- (eq. (7)) is unlikely for two reasons: hypochlorite ion is not a particularly strong oxidant and the third proton protects the amino group from the oxidative attack. Thus, in agreement with literature results it is reasonable to assume that the reaction of HOCl with the deprotonated amino acid (eq. (8)) is dominant and k^{2nd}_{obs} can be expressed as shown in eq. (10)(Qiang and Adams, 2004).

$$k^{2nd}_{obs} = k \frac{K_{AA}[H^+]}{(K_{AA} + [H^+])(K_{HOCl} + [H^+])} \quad (10)$$

where k^{2nd}_{obs}, K_{HOCl} and K_{AA} are the pH dependent pseudo-second order rate constant and the acid dissociation constants of hypochlorous acid and the protonated amino group of the amino acid, respectively.

According to this expression, k^{2nd}_{obs} exhibits a bell-shape pH dependence with the maximum at the average of pK_{HOCl} and pK_{AA}. The acid dissociation constants of the amino acids are listed in the Supplementary Material (Table S2). The pK_a of HOCl was taken from our earlier study ($pK_a = 7.40$)(Adam et al., 1992). Eq. (10) fits the experimental data reasonably well (Fig. 2) and the second order rate constant of eq. (8) was calculated by using a non-linear least squares algorithm with fixed values of the acid dissociation constants of the amino acid and HOCl (Table 1).

In accordance with earlier literature, the results imply that the chlorination of the amide nitrogen is slow and does not interfere with the reaction between HOCl and the aliphatic amino group (glutamine, asparagine)(Deborde and von Gunten, 2008; Hureiki

Table 1
The kinetic parameters for the chlorination of the α-amino group of the amino acids. $I = 1.00$ M $NaClO_4$, $T = 25.0$ °C.

Amino acid	$k \times 10^{-7}$ (M^{-1} s^{-1})	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	$\Delta H^{\ddagger}$ (kJ mol^{-1})
alanine	2.91 ± 0.06	−37.1 ± 4.5	18.4 ± 1.4
arginine	2.15 ± 0.05	−40.5 ± 1.5	18.2 ± 0.5
asparagine	1.74 ± 0.04	−73.0 ± 2.0	9.64 ± 0.63
aspartic acid	2.89 ± 0.05	−40.4 ± 2.2	18.1 ± 0.7
glutamic acid	2.78 ± 0.05	−39.7 ± 0.8	18.3 ± 0.2
glutamine	2.82 ± 0.06	−33.6 ± 6.5	22.4 ± 2.0
glycine	3.94 ± 0.09	−36.8 ± 1.1	18.1 ± 0.3
isoleucine	2.65 ± 0.03	−59.0 ± 0.8	12.3 ± 0.2
leucine	2.90 ± 0.04	−73.1 ± 3.6	7.73 ± 1.1
lysine	4.32 ± 0.08	−36.3 ± 3.3	16.4 ± 1.0
proline	7.02 ± 0.01	−72.2 ± 7.7	24.5 ± 2.4
serine	2.60 ± 0.04	−43.2 ± 1.4	18.0 ± 0.4
threonine	1.55 ± 0.03	−65.5 ± 1.6	12.2 ± 0.5
valine	3.35 ± 0.07	−60.0 ± 1.1	12.1 ± 0.3
tyrosine	1.98 ± 0.19	−59.1 ± 1.9	12.7 ± 0.6
histidine	2.74 ± 0.10	−49.3 ± 1.2	16.4 ± 0.4
phenylalanine	2.53 ± 0.06	−40.6 ± 1.2	18.4 ± 0.4

et al., 1994). In principle, the second acid base equilibrium of arginine and lysine may alter the formation of chloramines leading to two different products at high pH, and the following scheme may be applicable (charges of the amino acid are not indicated for the sake of simplicity).

$$H_2A = HA + H^+ \tag{11}$$

$$K_{A1} = \frac{[HA][H^+]}{[H_2A]}$$

$$HA = A + H^+ \tag{12}$$

$$K_{A2} = \frac{[A][H^+]}{[HA]}$$

$$v = k_1[HA][HOCl] + k_2[A][HOCl] \tag{13}$$

Thus, the following expression can be derived for k_{obs}^{2nd}.

$$k_{obs}^{2nd} = \frac{k_1 K_{A1}[H^+]^2 + k_2 K_{A1} K_{A2}[H^+]}{\left(K_{A1}K_{A2} + K_{A1}[H^+][H^+]^2\right)(K_{HOCl} + [H^+])} \tag{14}$$

where k_1 and k_2 are the rate constants for the reactions of HOCl with the HA and A forms of the amino acid. We assume that the α-amino group is deprotonated and the side chain functional group is protonated in the HA form while A is the fully deprotonated form.

A thorough analysis of the experimental data on the basis of eq. (14) revealed that the second deprotonation step does not affect the reactivity of the amino acid leading to the conclusion that most likely only the chlorination of the α-amino group occurs in these systems. In the case of arginine, this conclusion is corroborated on the basis of the following arguments. The guanidine group is very basic, its deprotonation occurs only under highly alkaline conditions, i.e. the chlorination of this group may be relevant only at high pH. However, the structural features of the guanidine group strongly resemble to those of the amide group which is far less reactive toward HOCl than an aliphatic amino group (Deborde and von Gunten, 2008). Thus, it is unlikely that the guanidine group competes with the amino group in the chlorination process.

In the case of lysine, the ε-amino group is more basic than the α-amino group. According to the corresponding pK_a values, the concentrations of the HA and A forms become comparable above pH 10. It is reasonable to assume that the reactivities of the two amino groups toward HOCl do not differ significantly. Thus, first the experimental data up to pH 9.5 were fitted to eq. (10) by assuming that the second term is negligible in eq. (13). The value obtained by this fitting procedure is listed in Table 1. In order to estimate the contribution of the k_2 path to the overall process, the data in the entire pH range were fitted to equation (14) by including k_1 with the fixed value from the previous fitting procedure. As expected, the estimate for k_2 is very uncertain but its value is of the same order of magnitude as that of k_1. According to these results, it cannot be excluded that a mixture of α-amino and ε-amino group chlorinated products form in the highly alkaline pH range, but it is obvious that only the former species is present at around physiological pH.

The amino acids with aromatic moiety raises the issue of a competing chlorination reaction step involving the aromatic ring. Earlier, the reactions of such amino acids were mainly discussed in the context of modelling their interactions with HOCl or chloramines as part of a peptide chain(Domigan et al., 1995; Fu et al., 2000; Pattison and Davies, 2001, 2005). Thus, the amino group of these species were blocked by converting the α-amino group into a relatively inert amide or model compounds were used by replacing the amino group with an appropriate substituent which is not involved in the chlorination process.

In the case of histidine, it was tested how the chlorinated imidazole ring is involved in trans-chlorination processes(Pattison and Davies, 2005). According to detailed studies, the oxidation of tryptophan and tyrosine leads to the formation of 2-oxindole as well as 3-chlorotyrosine, 3,5-dichlorotyrosine, respectively. It was concluded that the formation of 3-chlorotyrosine is an excellent marker for HOCl mediated protein oxidation. This species may form in the peptide chain via a trans chlorination process by the *N*-chloro amino group of the terminal amino acid in the same species(Domigan et al., 1995; Fu et al., 2000; Kettle, 1996; Pattison and Davies, 2001). There are controversial reports regarding the chlorination of the aromatic ring in free tyrosine when the amino acid is in excess(Fu et al., 2000; Pattison and Davies, 2001). In fact, such a process may occur if we assume, that the chlorination of the α-amino group is less favorable than that of the aromatic ring. This is an unlikely scenario if we consider the very high reactivity of simple amino acids toward HOCl and the relatively slow chlorination reactions of simple aromatic compounds such as the phenols and its derivatives(Deborde and von Gunten, 2008). In any case, we performed a detailed kinetic study on the corresponding systems. As it turned out, each reaction exhibits specific features.

The simplest case is the chlorination of phenylalanine where the reaction is characterized with a typical second order rate constant characteristic for the formation of *N*-chloramines with α-amino groups. In agreement with previous results (Pattison and Davies, 2001), we have shown that HOCl does not react with the aromatic ring of 3-phenylpropanoic acid which was used as a deaminated model compound of phenylalanine.

In tyrosine, the deprotonation of the aromatic OH group may alter the general kinetic pattern of chlorination. The corresponding kinetic data were fitted to eq. (14). In this case, the OH group is intact while the α-amino group is deprotonated in the HA form. On the basis of the fitting process it could be established that the deprotonation of the OH group does not affect the reactivity of the amino group. Finally, the data were fitted by assuming that $k_1 = k_2$.

In order to exclude that the chlorination of the aromatic ring is involved in the overall reaction, we compared the ^{1}H NMR spectra of free and chlorinated tyrosine (Fig. 3). The spectra of the reaction mixtures were recorded as soon as possible, typically within 3–4 min after mixing the reactants. In these studies, ionic strength was not set and the amino acid was used in 20% excess over HOCl.

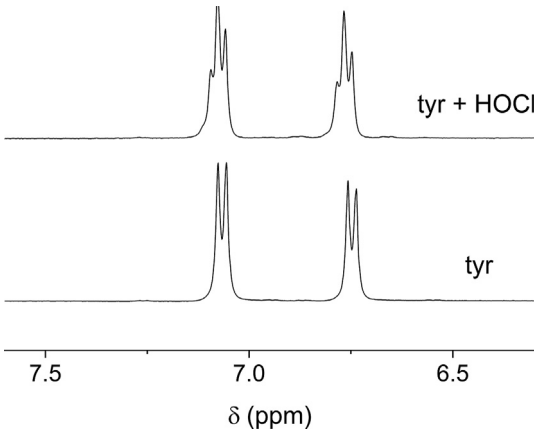

Fig. 3. The aromatic domain of the ^{1}H NMR spectra of tyrosine and the reaction mixture of tyrosine and HOCl a few minutes after mixing the reactants. $c^0_{HOCl} = 5.00 \times 10^{-3}$ M, $c^0_{AA} = 6.00 \times 10^{-3}$ M, $T = 25.0$ °C.

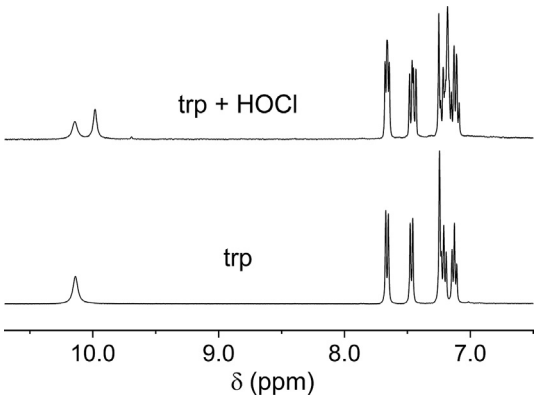

Fig. 4. The aromatic domain of the ^{1}H NMR spectra of tryptophan and the reaction mixture of tryptophan and HOCl a few minutes after mixing the reactants. $c^0_{HOCl} = 5.00 \times 10^{-3}$ M, $c^0_{AA} = 6.00 \times 10^{-3}$ M, $T = 25.0$ °C.

The chlorination reaction went to completion within a few seconds and it was confirmed by time resolved UV–Vis spectrophotometry that the decomposition of the *N*-chloro amino acid is not significant within the time frame of the NMR measurements. An inspection of the aromatic domain of the ^{1}H NMR spectra reveals that the product of chlorination features very similar spectrum to that of the parent compound (Fig. 3). In fact, the peaks of the parent compound and the chlorinated product overlap and significant chemical shift is not seen upon chlorination. Thus, the upper part of Fig. 3 can be interpreted as the sum of the doublets of the two species. It implies that, as expected, the aromatic ring remains intact during the chlorination reaction. However, this does not exclude the possibility that the aromatic ring of tyrosine is chlorinated when its amino group is protected by a peptide bond in a biologically important molecule. Once such a molecule is digested, the final reaction mixture may contain substantial amount of 3-chlorotyrosine. Our results only exclude that the formation of this derivative is feasible in a direct reaction between HOCl and tyrosine.

The chlorination of tryptophan features very complex kinetic pattern as reported earlier(Pattison and Davies, 2001). In this study, first we observed a very fast process within the dead time of the stopped flow instrument. This spectral change is followed by a complex multistep process (Fig. S2). It is quite obvious that the formation and subsequent decay of the chloramine are not separated in time as in the other systems. Quantitative kinetic evaluation of this reaction requires further thorough studies. However, it is noteworthy to mention that the structure of the aromatic part of the ^{1}H NMR spectra does not change significantly as a consequence of the chlorination process. Although there is a strong overlap between the two sets of signals, it can clearly be established that tryptophan and the chlorinated product are characterized by the same spectral features under both neutral and alkaline conditions (Fig. 4). This observation is very similar to that obtained with tyrosine, i.e. the spectrum of the reaction mixture features two sets of very similar peaks in the aromatic domain. Earlier, the formation of 2-oxindole was reported in the oxidation of peptides containing tryptophan. However, the pyrrole ring loses its aromatic character in such a compound, and a significant change in the ^{1}H NMR spectra is expected. In conclusion, it is unlikely that a direct reaction occurs between HOCl and the indole ring of free tryptophan.

The reaction of histidine with HOCl proceeds via two pH dependent parallel reaction paths. Fitting the data to eq. (14) yields $k_1 = (3.7 \pm 0.4) \times 10^5\ M^{-1}s^{-1}$ and $k_2 = (2.74 \pm 0.10) \times 10^7\ M^{-1}s^{-1}$. The value obtained here for k_1 is in reasonable agreement with the rate constant reported for the chlorination of the imidazole ring of *N*-acetylhistidine ($8.0 \times 10^4\ M^{-1}s^{-1}$)(Pattison and Davies, 2001). The contributions of the two paths to the overall reaction as a function of pH are shown in Fig. 5.

In accordance with the results obtained for the other systems (Table 1), the value of k_2 is consistent with the formation of the *N*-chloro-α-amine. As expected, this reaction is dominant in the alkaline pH range. The k_1 path is assigned to the *N*-chlorination of the imidazole side chain. As demonstrated by Fig. 5, the two different chlorinated products should form in comparable amounts at around pH 7.1. An attempt was made to confirm the co-existence of these molecules by recording time resolved UV–VIS spectra in the pH 5.6–8.7 region. Only small changes were observed in the spectra obtained after the full consumption of HOCl (at 1 s) although the absorbance decreased somewhat at the characteristic 255 nm band of the aliphatic *N*-chloro species. This finding does not contradict the kinetic observations but it does not serve direct evidence on the product distribution either.

In order to obtain corroborating proof for the formation of the chlorinated products, MS experiments in positive ion mode were performed with reaction mixtures of pH 6.0, 7.1 and 8.0. In all cases, characteristic peaks were observed at m/z = 190.0379 and 212.0194 which correspond to the H^+ and Na^+ adducts of chlorinated histidine. Since the two products process the same molecular weight

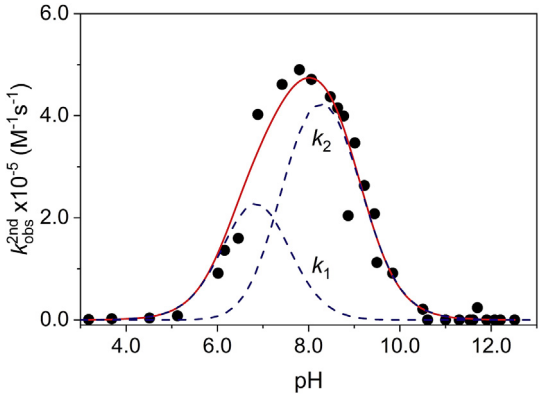

Fig. 5. The pH dependence of k^{2nd}_{obs} in the histidine – HOCl reaction. Experimental data (dots), fitted curve on the basis of eq. (14) (solid line) and the contributions of the two paths to the overall process (dashed lines). $c^0_{HOCl} = 5.00 \times 10^{-4}$ M, $c^0_{AA} = 7.50 \times 10^{-4}$ M, $I = 1.00$ M $NaClO_4$, $T = 25.0$ °C.

and charge, they cannot be distinguished by such experiments. Thus, we also made MS/MS experiments at these peaks hoping that the fragmentation patterns confirm that these peaks belong to two different compounds as a function of pH. According to these experiments, the first fragmentation always leads to the removal of Cl, and the remaining fragment of the amino acid produces the same fragmentation peaks in all cases. Thus, these studies confirmed the formation of the *N*-chloro compounds but otherwise should be termed as inconclusive.

The aromatic domain of pH dependent ^{1}H NMR spectra of the reaction mixtures just after mixing solutions of HOCl and histidine are shown in Fig. 6. The signals show definite shifts as the function of pH presumably due to the acid − base equilibria of the amino acid and its *N*-chloro derivatives. Unfortunately, there is a strong overlap between the corresponding peaks of the δ and ε protons at around pH ~7.0, where the existence of three independent compounds are expected. At pH 7.15 and 7.54, the histidine peaks of the ε proton appear at 7.81 and 7.75 ppm, respectively. The peaks assigned to the ε proton of the chlorinated compounds at 7.98 ppm and 7.86 ppm feature a small shoulder peak. It is also noteworthy that the chemical shifts of the δ and ε protons are more significant at lower pH. This trend may be consistent with the formation of an *N*-chlorinated aromatic ring at lower pH because such a chlorination is expected to exert larger effect on the chemical shifts of the aromatic C−H protons than the formation of the *N*-chloro-α-amino group. Thus, the observations seem to be in line with the existence of two different *N*-chloro products in this system. However, it needs to be emphasized that all of these measurements were made several minutes after mixing the reactants and the primary chlorinated product are involved in subsequent reactions. It was demonstrated earlier that the *N*-chloro-aromatic ring of histidine may chlorinate α-amino groups(Pattison and Davies, 2005). Thus, chlorine transfer either within the imidazole chlorinated species or between the side chain chlorinated histidine and the excess free histidine may significantly alter the primary product ratio.

The temperature dependence of k was studied in 0.05 M NaOH, in the 11−40 °C range. Representative temperature dependencies of k_{obs} are shown in Fig. S3. Under these conditions, $K_{HOCl} \gg [H^+]$ and $K_{AA} \gg [H^+]$ and eq. (10) transforms into eq. (15).

$$k_{obs}^{2nd} = k \frac{1}{[OH^-]} \frac{K_W}{K_{HOCl}} \quad (15)$$

where K_W is the water ionic product.

The temperature dependencies of k, K_W and K_{AA} were taken into account by using the Eyring-Polányi and van't Hoff equations, respectively. Thus, eq. (15) can be rewritten as follows

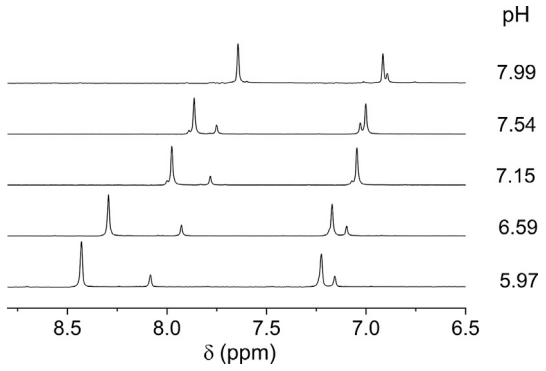

Fig. 6. The aromatic domain of the ^{1}H NMR spectra of the reaction mixture of histidine and HOCl a few minutes after mixing the reactants as a function of pH. $c^0_{HOCl} = 5.00 \times 10^{-3}$ M, $c^0_{AA} = 6.00 \times 10^{-3}$ M, $T = 25.0$ °C.

$$k_{obs}^{2nd} = \frac{k_B T}{h} e^{\frac{\Delta S^{\neq}}{R}} e^{-\frac{\Delta H^{\neq}}{RT}} \frac{1}{[OH^-]} \frac{e^{-\frac{\Delta H_W^{\neq}}{RT}} e^{\frac{\Delta S_W^{\neq}}{R}}}{e^{-\frac{\Delta H_{HOCl}^{\neq}}{RT}} e^{\frac{\Delta S_{HOCl}^{\neq}}{R}}} \quad (16)$$

The experimental data were fitted with eq. (16) and the corresponding parameters of activation ($\Delta H^{\neq}$ and $\Delta S^{\neq}$) are listed in Table 1. The enthalpy and entropy for K_W were obtained by determining its value at different temperatures on the basis of strong acid − strong base titrations and fitting the data with the van't Hoff equation: $\Delta H_W = 48.7 \pm 2.2$ kJ mol^{-1} K^{-1}, $\Delta S_W = -97.8 \pm 6.8$ J mol^{-1} K^{-1}. The thermodynamic parameters for the dissociation of HOCl were obtained from the literature: $\Delta H_{HOCl} = 12.4$ kJ mol^{-1} K^{-1}; $\Delta S_{HOCl} = -99.7$ J mol^{-1} K^{-1}(Adam et al., 1992). The thermodynamic parameters for the acid dissociation reaction and the self-dissociation of water were kept fixed during the calculations.

The second order rate constants for the chlorination of the α-amino group are very similar and on the order of 10^7 M^{-1}s^{-1} for the studied amino acids (Table 1). Accordingly, there is only a very weak correlation between the reactivity and basicity of the α-amino group. (Fig. S4). A similar trend was reported for the chlorination of various amines earlier(Antelo et al., 1995). The parameters of activation do not feature a straightforward trend reflecting structural or other features of the amino acids. Nevertheless, the entropy of activation tends to be more negative and the enthalpy of activation tends to be smaller when the side chain has a bulky moiety close to the α-carbon atom. In this respect, it is interesting to note that the longer side chain of glutamine keeps the amide group farther from the reaction center compared to asparagine. As a consequence, the activation parameters for the reactions of these two closely related amino acids differ significantly. It is reasonable to assume that the bulky side chains close to the α-carbon atom decrease the strength of bonding between the reactants and make the structure more diffuse in the transition state.

Several attempts have been made to establish the order of the reactivities of amino acids toward HOCl in previous literature(Na and Olson, 2007; Pattison and Davies, 2001; Winterbourn, 1985). Quite often these studies were limited to the physiological pH range (7.2−7.4) and the conclusions were reached on the basis of unjustified assumptions. Na and Olson reevaluated earlier literature results and reported k_{obs}^{2nd} values for the chlorination of a few amino acids at pH 7.0(Na and Olson, 2007). However, these results need to be termed irrelevant because the acid - base equilibrium of HOCl was ignored and an erroneous expression was used in the calculations. In the same study, competitive chlorination experiments were made by reacting equimolar mixtures of amino acids with HOCl and analyzing the spent reaction mixtures for residual amino acids 3 h after initiating the reaction. It was concluded that glycine and proline are the less reactive amino acids in the chlorination process. With respect to glycine, our findings are quite different. This discrepancy could be the consequence of the different experimental approaches. In our study, the rate constants were obtained from direct kinetic experiments allowing direct comparison of the reaction rates. In the cited study, the analysis of the reaction mixtures was performed long after the completion of the chlorination process and trans-chlorination (Peskina et al., 2005) or other side reactions could lead to biased conclusions. Such uncertainties are eliminated when the comparisons are based on real kinetic data.

Pattison and Davis made an attempt to model the chlorination of the imidazole ring of histidine by the reaction of HOCl with 4-imidazol acetic acid as model compound and reached the conclusion that the aliphatic amine is less than 30% more reactive than the imidazole moiety at pH 8.0(Pattison and Davies, 2001). Our data confirms an about 6 times difference between the reactivities of these groups at pH 8.0 and that the reaction becomes more

favorable with the imidazole ring only below pH 7.0 (Fig. 5).

The data shown in Table 1 can easily be used to model the reactivity order of amino acids toward HOCl in a wide pH range. To demonstrate this possibility, we assume that HOCl reacts with a solution of 7 amino acids present in equimolar ratios and in excess over the oxidant. The individual chlorination rates can be calculated as a function of pH by substituting the appropriate parameters into eq. (10). The relative contribution of each amino acid to the consumption of HOCl is shown in Fig. 7. Under slightly acidic – neutral conditions $[H^+] \gg K_{AA}$ and the relative rates do not change by varying the pH. This situation changes dramatically when the acid – base equilibria of the α-amino groups are shifted toward deprotonation (above pH 8.5). Under alkaline conditions the relative rates are strongly affected by the actual K_{AA} values of the amino acids. In this respect, it is interesting to note that the largest k was obtained for proline. Nevertheless, it reacts considerably slower with HOCl than the other amino acids at pH 7.0. due to its relatively large pK_{AA}. The relative rate of proline increases significantly by increasing the pH, because the concentration of its deprotonated form gradually becomes comparable with those of the other amino acids.

In accordance with the above considerations, the results for the chlorination of serine represents the opposite trend because the relative rate with this compound significantly decreases by increasing the pH. This amino acid has the smallest pK_a, and as such its deprotonated form is present in the largest concentration in the neutral pH range. Consequently, its reaction with HOCl is relatively fast. Whenever the alkaline pH range is reached, the concentrations of the deprotonated form of all amino acids becomes comparable and their share in the chlorination process is determined by the order of k (Table 1).

4. Conclusions

For the first time, these results offer a possibility for direct comparison of chlorination rates of amino acids in a relatively wide pH-range and are suitable for modelling the primary concentration ratios of *N*-chloro amino acids in water treatment technologies and biological systems. It is well known that *N*-chloro compounds are also involved in trans chlorination processes (Peskina et al., 2005), and the next step along the line of these studies is to provide detailed description how these species interact with various amino acids. These reactions may significantly alter the concentration ratios of the *N*-chloro compounds. Since the abilities of *N*-chloro compounds to penetrate into the cell may differ significantly, the results are expected to provide deep insight into the molecular background of the biological impact of *N*-chloro amino acids.

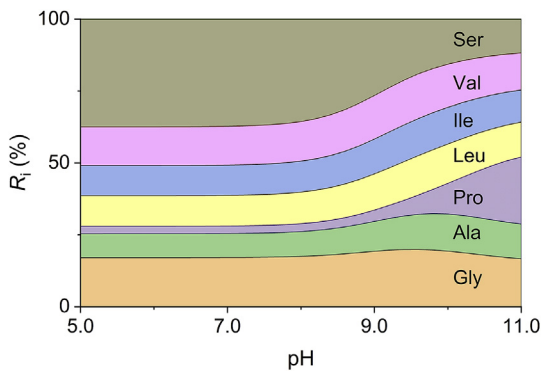

Fig. 7. The calculated relative rates (R_i) of several amino acids by assuming that the amino acids are present in equimolar ratios and in excess over HOCl.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.114994.

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