Intracellular oxidation of dipeptides. Very fast halogenation of the amino-terminal residue

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Peptides undergo a very fast halogenation reaction with aqueous halogens. The process takes place *via* aliphatic electrophilic substitution with bimolecular rate constants of *ca.* 10^7 M^{-1} s^{-1}. The products formed are *N*-halo-peptides, *i.e.*, the halogenation process takes place on the nitrogen atom at the amino-terminal moiety of the amino acid residue. At ratios [halogenating agent]/[dipeptide] ≤ 1, no analytical or kinetic evidence has been found of halogenation on the peptide bond or on the oxygen atom of the carboxy-terminal residue. The intracellular oxidation of peptides to *N*-halo-peptides proceeds by an *in vivo* mechanism to reduce the oxidative stress caused by intracellular oxidants.

Introduction

Halogenation in general, and chlorination in particular, is one of the biochemical mechanisms used by mammalians as protection from pathogens.[1] It is well established that the enzymatic system myeloperoxidase/H_2O_2/X^- ($X^- = Cl^-$, Br^-, I^-)[2] is able to produce *in vivo* substantial amounts of HOCl,[3] which is a powerful cytotoxic oxidant against a variety of microorganisms.[1,4–8] In addition, HOCl reacts with nitrogen compounds, yielding *N*-chloro-derivatives,[9] which are able to oxidize *in vivo* a variety of compounds, such as nucleic acids,[5,10] nucleotides,[11,12] peptides,[13] *etc*.

The presence of organic nitrogenated compounds in natural waters, as peptides,[14,15] has led chemists to investigate their aqueous chemistry. The most widely used water-disinfection method is chlorination,[16] during which *N*-chloro-compounds are formed,[9] in a process entirely similar to the one taking place *in vivo* (see above). The benefits and risks of halogen-based water disinfection are well-known.[16,17]

With aqueous halogens, nitrogenated compounds undergo a very fast halogenation reaction to yield *N*-halo-derivatives, which can then undergo decomposition to yield ammonia, halide ions and the corresponding carbonyl compounds.[9]

In view of the biochemical and environmental relevance of halogenation processes, it seems important to clarify the mechanism of HOCl-mediated oxidation of peptides, as a first step to understanding the oxidative degradation of proteins.

The oxidation of amines and amino acids has been extensively studied.[9,13,18–22] However, that of peptides has received little attention.[23] In a previous communication we presented preliminary results for the oxidation of two peptides: Gly-Gly and Gly-Gly-OEt.† [13] Here, we report a systematic study of the mechanism of halogenation of glycinamide, as a model compound, and of 12 dipeptides (see Scheme 1).

Results and discussion

Under near-neutral conditions, the chlorination of dipeptides takes place in some tenths of a millisecond, as proved by the simultaneous disappearance of the peak for the ClO^- in the UV absorption spectra,[26] and the growth of the characteristic UV band of *N*-chloroamines.[9] At constant pH, the process follows a second order kinetic law: first order in both the chlorinating agent and the dipeptide. The observed rate constant shows a strong dependence on the acidity of the medium, as shown in Fig. 1.

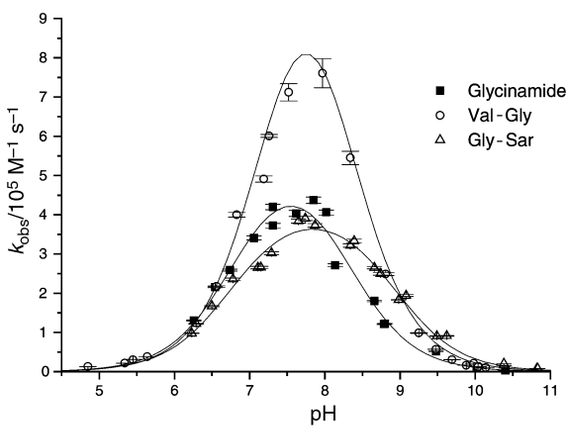

Fig. 1 Influence of the pH on the rate of chlorination of *N*-chloro-dipeptides. Conditions: [chlorinating agent] = [dipeptide] = 1.2 mM, *I* (NaCl) = 0.50 M, *T* = 298.15 K.

This dependence is adequately described by the empirical equation, eqn. (1), where *a*, *b* and *c* are adjustable parameters and *r* is the reaction rate.

$$r = k_{obs}[\text{chlorinating agent}][\text{dipeptide}]$$
$$= a\frac{[H^+]}{(b + [H^+])(c + [H^+])}[\text{chlorinating agent}][\text{dipeptide}] \quad (1)$$

A proper description of the reaction mechanism requires that all the species that may participate in the peptide hydrolysis in aqueous media are considered. These are shown in Scheme 2 (only acid–base equilibria are indicated).

Similarly, hypochlorous acid and the hypochlorite anion

† The IUPAC three-letter system has been used to denote α-amino acids. Dipeptides are named following the names of the corresponding parent amino acids (Aa) as Aa_1-Aa_2.[24,25]

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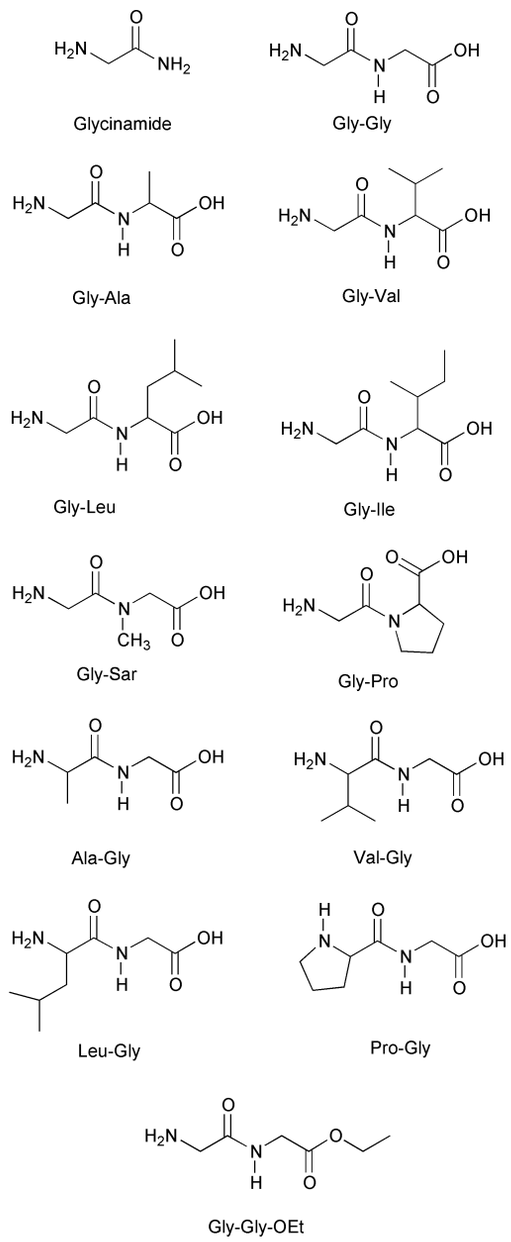
coexist under the reaction conditions as the equilibrium $HClO \rightleftharpoons ClO^- + H^+$.

Taking into account all the possible species and reaction sites, more than thirty different processes arise as mechanistic possibilities for the chlorination of dipeptides.



Scheme 1

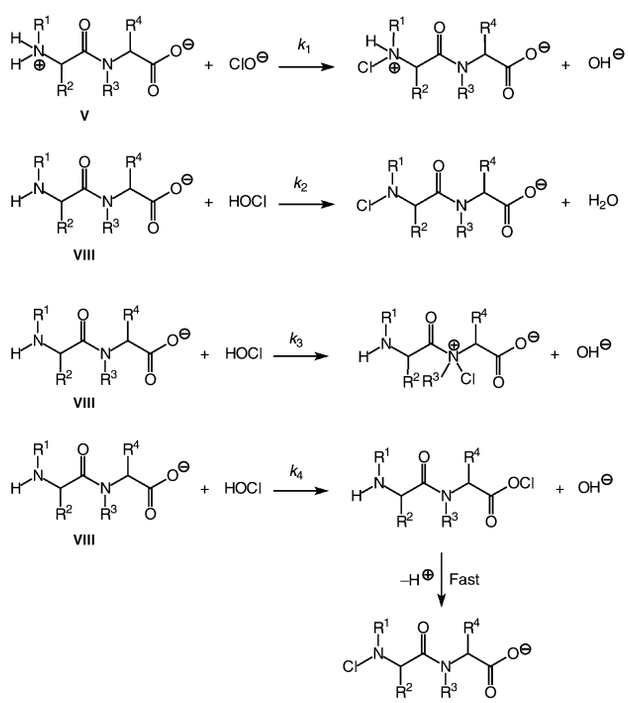
If the conditions of pH used in this work (4.5–13.0) and the pK_a values obtained for these compounds (pK_{a1} *ca.* 1.5–2.0, pK_{a2} *ca.* 8.5–9.0) are considered,[27,28] it seems reasonable to expect that species **I**, **II**, **III**, **IV**, **VI** and **VII** will be found in very low concentration.

Moreover, if the observed rate constants are considered, the corresponding bimolecular rate constant for chlorination involving those species should be higher than that of the diffusion limit.[29]

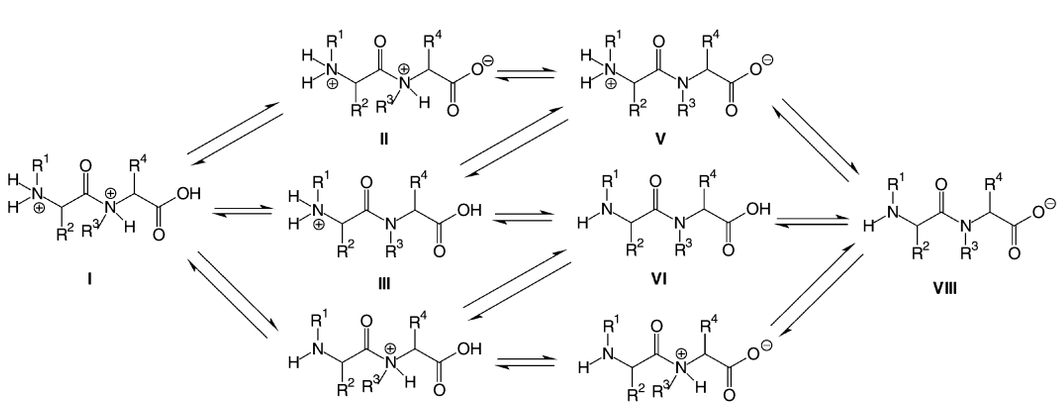
Bearing this in mind, only species **V**, **VIII**, HOCl and ClO^- remain as possible reacting species. As **V** and **VIII** both have three possible reaction centres, several elementary processes can be put forward. If the k_{obs} *vs.* pH observed profiles are considered, only four of these processes are coherent with the obtained results, one taking place between ClO^- and species **V**, and three taking place between HOCl and species **VIII**, at different reaction sites, as shown in Scheme 3.

The general expression (2) can be deduced for the observed rate constant, where k_{obs} is the second order rate constant.

$$k_{obs} = k_i K_J \frac{[H^+]}{(K_{Am} + [H^+])(K_{HOCl} + [H^+])} \qquad (2)$$



Scheme 3 Possible rate determining steps for the chlorination of dipeptides.



Scheme 2 Possible monoprotonation equilibria for dipeptides.

Table 1 Bimolecular rate constants for the chlorination of dipeptides and pK_{Am} values for the studied dipeptides, $T = 298.15$ K, $I = 0.5$ M (KCl)

Dipeptide	$10^{-6}\ k_2/M^{-1}\ s^{-1}$	pK_{a_2} (Experimental)	pK_{Am} (Optimised)	pK_{Am} (Literature)
Glycinamide	4.3 ± 0.3	—	7.90 ± 0.07	7.95,[b] 7.96[c]
Gly-Gly-OEt	7.5 ± 0.2	8.075 ± 0.008	—	7.75,[b] 7.710,[d] 7.76[e]
Gly-Gly	9.1 ± 0.3	8.25 ± 0.01	—	8.10,[f] 8.25,[g] 8.27,[d] 8.33,[b] 8.18,[h] 8.17,[i] 8.12,[j] 8.20,[k] 8.15,[e] 8.25[l]
Gly-Sar	12.6 ± 0.6	—	8.58 ± 0.07	8.55,[m] 8.59,[e] 8.61[n]
Gly-Ala	8.8 ± 0.9	8.310 ± 0.005	—	8.29,[o] 8.23,[p] 8.19,[i] 8.21[l]
Gly-Val	9.5 ± 0.7	—	8.3 ± 0.1	8.33,[q] 8.25,[r] 8.22[e]
Gly-Ile	6.4 ± 0.5	—	8.1 ± 0.1	8.15,[s] 8.00[l]
Gly-Leu	6.4 ± 0.4	—	8.13 ± 0.08	8.14[c]
Gly-Pro	15 ± 5	—	—	8.41,[t] 8.69,[b] 8.65,[k] 8.50,[e] 8.53[u]
Ala-Gly[a]	8.5 ± 0.8	8.272 ± 0.003	—	8.27,[v] 8.18,[p] 8.21[l]
Val-Gly	6.0 ± 0.4	—	7.94 ± 0.06	7.89,[w], 8.02[e]
Leu-Ala	8.0 ± 0.7	—	7.96 ± 0.09	8.10[x]
Pro-Gly	18 ± 3	—	8.7 ± 0.1	8.97,[s] 8.97[w]

[a] k_{obs}/s^{-1} at different temperatures: 20.79 (286.0 K), 34.15 (292.5 K), 41.46 (297.0 K), 56.44 (303.0 K), 74 (308.0 K), 97.46 (314.0 K). [HOCl] = 0.002 M, [Ala-Gly] = 0.1 M, [NaOH] = 0.1 M, I ($NaClO_4$) = 0.5 M. [b] Ref. 30. [c] Ref. 31. [d] Ref. 32. [e] Ref. 33. [f] Ref. 34. [g] Ref. 35. [h] Ref. 36. [i] Ref. 37. [j] Ref. 38. [k] Ref. 39. [l] Ref. 40. [m] Ref. 41. [n] Ref. 42. [o] Ref. 43. [p] Ref. 44. [q] Ref. 45. [r] Ref. 46. [s] Ref. 47. [t] Ref. 48. [u] Ref. 49. [v] Ref. 50. [w] Ref. 51. [x] Ref. 52.

If the elementary reaction between species **V** and ClO^- were considered, k_i would be k_1 (Scheme 3) and K_J would correspond to the ionisation constant for HOCl (K_{HOCl}). On the other hand, accepting the process would involve species **VIII** and HOCl, k_i could be k_2, k_3 or k_4 and K_J would be K_{Am}, *i.e.*, the ionisation constant for the terminal amino group of species **VIII**.

Although the processes depicted in Scheme 3 are kinetically indistinguishable, it is hard to think of a bond-breaking/bond-making mechanistic sequence for a direct transfer of chlorine from ClO^- to the positively charged amino group. Therefore, we propose that the elementary process in the halogenation of dipeptides by aqueous halogen under the experimental conditions must be the bimolecular reaction between the anionic form of the dipeptide (species **VIII**) and hypochlorous acid.

Thus, the rate equation becomes eqn. (3).

$$r = k_i K_{Am}\left\{\frac{[H^+]}{(K_{Am} + [H^+])(K_{HOCl} + [H^+])}\right\}[HOCl][\mathbf{VIII}] \quad (3)$$

It follows that the maximum rate constant observed in Fig. 1 must occur at the arithmetic mean of the pK_a values of both reactants: $pH_{max} = (pK_{Am} + pK_{HOCl})/2$. This prediction is in good agreement with the observed behaviour.

Using eqn. (3) it is possible to estimate the bimolecular rate constant k_i, as well as the values of the ionisation constants K_{Am} and K_{HOCl}. These are shown in Table 1. The experimentally obtained pK_{Am} values agree with those optimised from the equation. There is also good agreement with the values available in the literature for this kind of compound (Table 1).

The observed rate constant obtained for glycinamide is of the same order of magnitude as those for dipeptides. Assuming a parallel between basicity and nucleophilicity scales, and an absence of any kind of α-effect for dipeptides, if the chlorination were to take place on the nitrogen of the amide, then the expected rate constant would be much smaller, since the pK_a of an amide is much lower than that of the amino group [pK_{a2}-(glycinamide) = *ca.* −1.78].[53] On the other hand, dipeptides with substituents R^3 on the peptidic nitrogen (Gly-Sar and Gly-Pro in this study) show rate constants even higher than those obtained for dipeptides with R^3 = H.

It has been previously established that chlorination on the peptidic unsubstituted nitrogen is negligible provided the ratio [chlorinating agent]/[dipeptide] < 1.[23] The authors of this study estimated that the chlorination of *N*-acetyl-Gly is at least five orders of magnitude slower than the chlorination of the parent Gly. A detailed analysis of the reaction products by GC/MS showed no evidence of chlorination on the peptidic nitrogen of *N*-acetyl-L-Ala.[54] A similar result was obtained when HOCl was generated by means of the enzymatic system myeloperoxidase/H_2O_2/Cl^-.[55] All this evidence allows us to conclude that halogenation on the peptidic nitrogen is negligible.

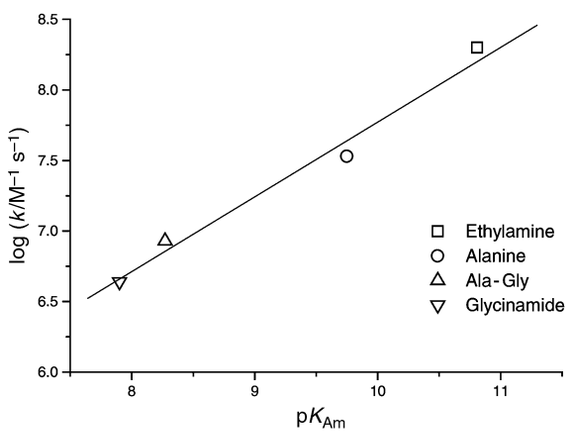
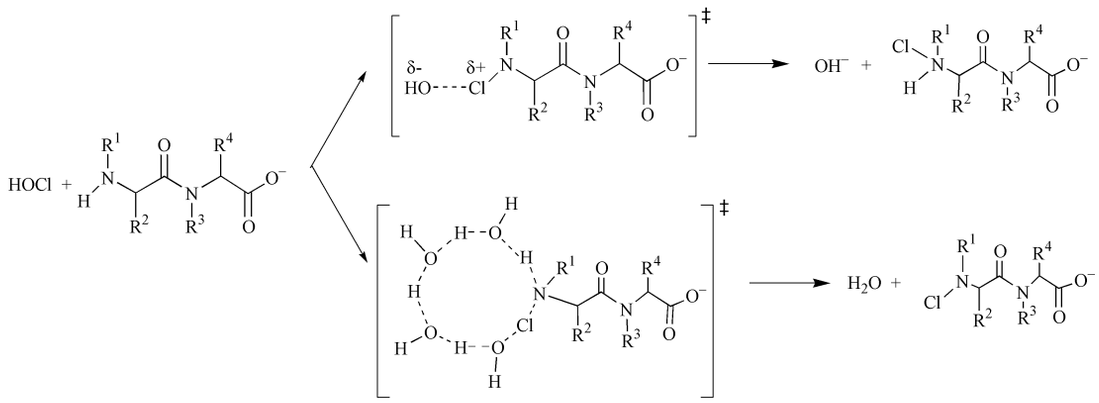


Fig. 2 Dependence of the bimolecular rate constant of chlorination (k_2) with the pK_{Am} of the amino nitrogen ($r = 0.99$).

Another mechanistic alternative (see Scheme 3) would be halogenation taking place first on the oxygen atom of the carboxylate, with a subsequent fast transfer of the halogen to the nitrogen atom of the amino group. However, such an alternative pathway can be discounted, since the observed rate constants are of the same order of magnitude for dipeptides and their corresponding esters (Table 1). In addition, the behaviour observed in the chlorination of primary and secondary aliphatic amines is entirely analogous to that observed for dipeptides.[18]

Consequently, the chlorination of dipeptides must take place *via* transfer of the *chlorine* from HOCl to the *nitrogen* of the unprotonated terminal amino residue of the dipeptide (k_2 in Scheme 3), *i.e.* $k_i = k_2$ in eqn. (3) and the process can be classified as an aliphatic electrophilic substitution.

Such a conclusion is supported by the fact that log (k) is linearly dependent ($\rho = 0.54 \pm 0.05$) on the pK_a of the amino nitrogen (pK_{Am}), and therefore on its nucleophilicity (*vide supra*). As shown in Fig. 2, the more basic the amino group, the higher the rate of chlorination. Fig. 2 is formally similar to a Brønsted plot.[56] Accepting that the process is an aliphatic electrophilic substitution, where a positively charged chlorine atom is being transferred, the classical interpretation of a Brønsted plot for proton transfer could be extended to the present case. Under this assumption the chlorine atom is approximately half transferred at the transition state (TS) and there is a decrease in electron density on the nitrogen and an increase on the oxygen.



Scheme 4

The obtained bimolecular rate constants k_2 (Table 1) show high values, but are still well below the diffusion control limit.[29] However, in the case of primary and secondary aliphatic amines and even amino acids, it has been postulated that the process may be diffusion controlled. This statement was based on the values found for the enthalpy of activation, less than 15 kJ mol^{-1}. $\Delta H^{\ddagger} \approx 0$ kJ mol^{-1} has been estimated for the chlorination of Ala-Gly and other dipeptides.[13] The decrease in the bimolecular rate constant as the basicity/nucleophilicity of the compound decreases rules out this hypothesis.

Highly negative values have been found for the entropy of activation in the chlorination of Ala-Gly (-141 ± 7 J mol^{-1} K^{-1}), as well as other dipeptides[13] and other nitrogenated compounds such as ammonia (-97 ± 5 J mol^{-1} K^{-1}) and primary and secondary aliphatic amines.[18] Such activation entropy values imply that the TS is more ordered than the reactants. Considering the nature of the reaction, this fact should be mostly associated with solvation effects and/or participation of the solvent in the TS.

Looking in detail at the proposed rate determining step, at least the two possibilities depicted in Scheme 4 should be considered. Both pathways could explain the observed $\Delta S^{\ddagger}$ values. Negative $\Delta S^{\ddagger}$ values are expected to be due either to formation of a cyclic structure or generation of a charged species.

The low $\Delta H^{\ddagger}$ values support the formation of a cyclic structure at the TS, where the favourable interactions within the cycle lower the energy requirements of the reaction.

The observed activation parameters agree with the process in the Scheme 4 where, in addition to the transfer of the chlorine from HOCl to the nitrogen of the unprotonated terminal amino residue of the dipeptide, there is a direct participation of water molecules at the TS. The formation of a cyclic structure in the TS allows a rather synchronous transfer of Cl and H atoms, avoiding the formation of charged species and reducing charge development on the reaction centres at the TS.

Theoretical studies on ammonia chlorination by HOCl point to the participation of at least three water molecules at the TS.[57]

As shown in Table 1 the rate constants for chlorination of Gly-Sar and Gly-Pro are higher than expected. This difference cannot be attributed to changes in their basicity/nucleophilicity. A plausible explanation for this observation is the greater availability of the terminal amino group for reaction, *i.e.* peptides unsubstituted in the peptide bond may show a hydrogen-bond interaction between the terminal amino group and the hydrogen of the peptide bond, which, in the case of Gly-Sar or Gly-Pro, results in the reaction becoming faster.

Conclusions

The oxidation of peptides by aqueous halogens takes place through a second-order process in which the halogen is transferred from the oxygen of HOX to the terminal amino residue of the peptide with direct participation of water molecules at the TS. Despite the high rate constants found for the elementary process, this is controlled by its activation energy, and not by the diffusion of the reagents. The rate of the process is controlled by the basicity/nucleophilicity of the terminal amino group of the dipeptide. The available data agree with an aliphatic electrophilic substitution mechanism.

Experimental

Reagents

All peptides were purchased from Sigma, and used as received, without further purification. All other reagents used were Merck *pro analysi* products.

Aqueous chlorine solutions, prepared as described elsewhere,[58] were used as the chlorinating agent, their concentration being spectrophotometrically determined.[59]

The pH was adjusted using different buffer solutions ($H_2PO_4^-/HPO_4^{2-}$, HCO_3^-/CO_3^{2-}) and NaOH. The ionic strength was kept to 0.50 M using NaCl or $NaClO_4$. Twice-distilled water was used in all cases to make up all solutions.

Kinetic studies

The reactions were monitored spectrophotometrically by measuring either the increase of UV absorbance at $\lambda = 250$ nm, where the *N*-chloro-dipeptides show a maximum absorption,[13] or the decrease of UV absorbance at 292 nm, the absorption maximum of ClO^-.

In the range $6 < pH < 12$, the rate of the chlorination process became so fast that the use of Hi-Tech stopped-flow spectrophotometers became necessary. In a typical stopped-flow kinetic run the reaction was followed at 250 nm by mixing equal amounts of both reactants ($[HOCl + ClO^-] = [Dipeptide] = 1.2 \times 10^{-3}$ mol dm^{-3}) properly adjusted to the working pH. At each pH the rate constant is an average of 5 to 10 kinetics runs.

Outside of that pH range it was possible to work under pseudo-first-order conditions, with the concentration of dipeptide at least 50-fold that of the chlorinating agent, and to use a conventional UV spectrophotometer. This time each rate constant has been measured twice.

pH values lower than *ca.* 4.5 were avoided in order to eliminate possible interference of direct chlorination by Cl_2 (aq). Kinetic runs were not carried out at $pH > 13$ to avoid simultaneous chlorination of the *N*-chloro-dipeptides' decomposition products.[60]

Most kinetic runs were followed at 25.0 °C. The reagents and cell holders were water-flow thermostated to within ±0.1 °C. Kinetics between 15.0 and 40.0 °C were carried out to study the chlorination of ammonia ($\Delta H^{\ddagger} = 4 \pm 1$ kJ mol^{-1} and $\Delta S^{\ddagger} = -97 \pm 5$ J mol^{-1} K^{-1}) and Ala-Gly.

First and second order rate equations were adequately fitted to the kinetic data using the Marquardt non-linear optimisation algorithm.[61] The same algorithm was used in fitting rate eqn. (3)

to (k_{obs}, pH) data. For each dipeptide between 10 and 19 pairs of values (k_{obs}, pH) were used to estimate the bimolecular rate constants (k_2) and the optimised pK_a values reported in the Table 1. In this way, satisfactory standard deviations from the experimental data were achieved, as shown in Fig. 1.

pH and pK_a determinations

pH measurements were carried out using a conventional, properly calibrated, combined-glass electrode. Routine potentiometric techniques were used to determine the pK_a values of HOCl (7.16 ± 0.03 at $I = 0.5$ M and 298.15 K) and the dipeptides under the same conditions of ionic strength, type of electrolyte and temperature used in this work (Table 1). A combined-glass electrode was used to directly read the pH during the titration of the dipeptides in aqueous hydrochloric acid against standard aqueous sodium hydroxide.[62,63] The program MINIQUAD was used to estimate the corresponding pK_a values.[64]

Reaction products

The product of chlorination of Pro-Gly was identified by MS as (*N*-Cl)-Pro-Gly. For this purpose, a Fisons Instruments' VG cuattro mass spectrometer was used (70 eV, EIMS): *m*/*z* 207/209 (M^+ 13/4%), 70.1 (100) [C_4H_8N], 147 (8) [$M - CH_2CO_2H]^+$, 162 (8) [$M - CO_2H]^+$, 190 (5) [$M - OH]^+$, 105 (33) [$C_4H_7NCl]^+$.

N-Chloro-dipeptides are not stable for long, and undergo base-promoted decomposition. The decomposition products of *N*-chloro-dipeptides were found to be the corresponding 2-[*N*-alkyl-*N*-(2-imino-2-alkylethanoyl)amino]-2,2-dialkylethanoic acid, ammonia or primary amines and chloride anion.[18,55,58,65]

Acknowledgements

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