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First Steps in the Oxidation of Sulfur-Containing Amino Acids by Hypohalogenation: Very Fast Generation of Intermediate Sulfenyl Halides and Halosulfonium Cations

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Abstract—Sulfur-containing amino acids show an extraordinary binding towards $HOCl/ClO^-$. During the process, the Cl is transferred from the *O* to the *S* of the amino acid. Met reacts with HOCl one order of magnitude faster than the non-*S* containing amino acids ($k_{(Met+HOCl)}=8.7\cdot10^8$ mol^{-1} dm^3 s^{-1}). Instead, Cys reacts as its thiolate (RS^-), two orders-of-magnitude faster ($k_{(RS^-+HOCl)}=1.2\cdot10^9$ mol^{-1} dm^3 s^{-1}). Cys reacts also with ClO^- ($k_{(RS^-+ClO^-)}=1.9\cdot10^5$ mol^{-1} dm^3 s^{-1}). Such processes take place much more readily than the corresponding *N*-halogenation of the non-sulfur containing amino acids. To our knowledge, these are the first kinetic measurements of the rate of formation of sulfenyl halides and halosulfonium cations in aqueous solution. Sulfenyl chlorides and chlorosulfonium ions derived from amino acids are elusive, and sulfide-type amino acids (Met) eventually yield sulfoxides (MetO), while thiol-type amino acids (Cys) lead to disulfides (Cys^Cys) and sulfonic acids (Cya). The fate of sulfur-containing amino acids upon oxidation with $HOCl/ClO^-$ seems to be related to their mutagen-inactivation ability.

Introduction

Halogens in their different aqueous forms, and particularly chlorine derivatives, are disinfectants used worldwide, with well-known benefits and risks that have been the subject of controversy and debate for a long time.[1] The direct correlation between the halogen-based water disinfection and carcinogenicity/mutagenicity of different toxic compounds commonly present in water is well documented.[2]

The last years have seen a blossom of literature concerned with the in vivo halogenation via the myeloperoxidase/H_2O_2/Cl^- system, which generates HOCl (in general, HOX, if X^- is present),[3] giving rise to processes and products entirely similar to those taking place during water treatment. Such processes are relevant in relation to many important biological processes, like aging.[4]

In connection with these processes, different authors have reported on the sulfur-based inactivation of some of the mentioned mutagens found in treated tap water,[3f,j,5] as well as on the activity of sulfur-containing compounds against the toxicity derived from other substances.[6]

A relatively abundant chemical literature is available on the use of halogen electrophiles to oxidize various sulfur compounds, for example: sulfur-containing amino acids and peptides,[7] thiols,[8] sulfides,[9] sulfoxides,[10] sulfones,[9c] etc. Surprisingly, no detailed mechanistic studies of these reactions have been carried out; particularly remarkable is the lack of kinetic evidence for the mechanistic involvement of sulfenyl halides and halosulfonium cations, usually claimed as the reaction intermediates for these oxidations.[11]

Considering the biochemical relevance of these processes, and in the framework of a wider project aiming to clarify the reaction mechanisms of model sulfur compounds toward halogen electrophiles and the reasons for their implication in the reduction of the mutagenicity, we have studied the mechanism of oxidation of two essential sulfur-containing amino acids, cysteine (Cys) and methionine (Met),[12] by aqueous chlorine.

Results and Discussion

The chlorination of Cys takes place in some milliseconds, as proven by the disappearance of the ClO^- absorption band. In the case of Met, the disappearance of ClO^- was also observed, the process being slower than for Cys. In turn, the chlorination under similar conditions of analogous amino acids and peptides containing no *S* is much slower, ranging from tenths of a second to seconds.[13]

Keywords: sulfenyl halides; halosulfonium cations; in vivo halogenation; amino acids; water chlorination.

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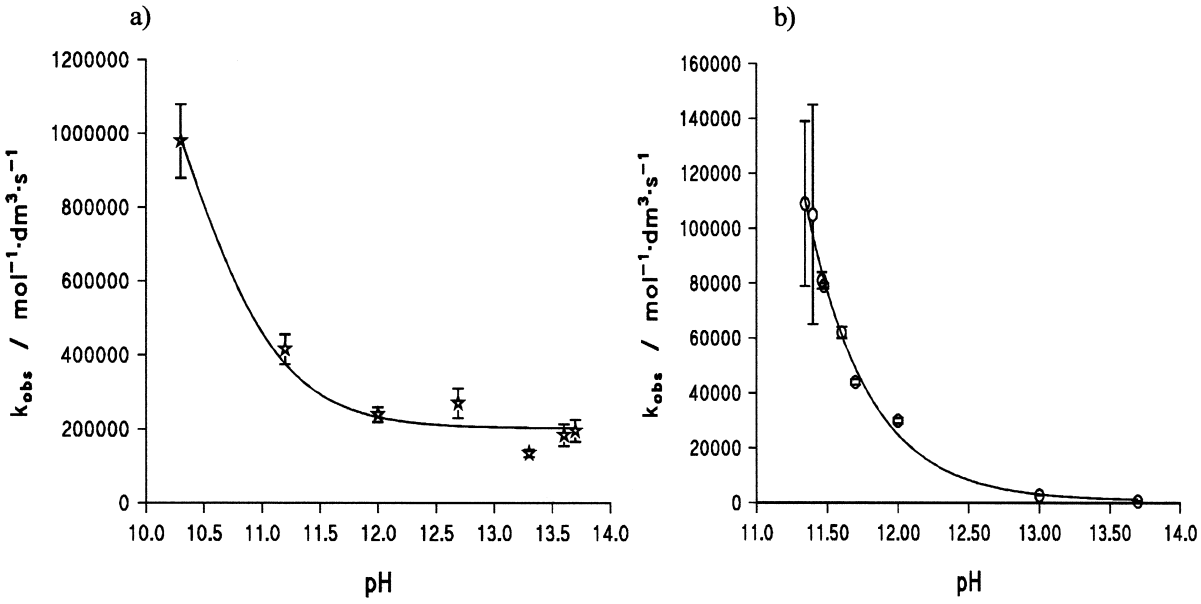


Figure 1. Dependence of k_{obs} for reaction of: (a) Cys, (b) Met, and HOCl/ClO^- with the acidity of the medium. I=0.50 mol dm^{-3}, T=298.0 K.

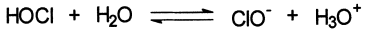
The kinetic data were accurately fit by a second-order kinetic law, first-order with respect to each reagent:

rate=k_{obs}·[*S*-compound]·[chlorinating agent]

The observed rate constant depends strongly on pH, as shown in Fig. 1(a) and (b).

In a mildly basic or near-neutral medium the reactions were too fast to be monitored, even on the stopped-flow time scale. For this reason, the kinetics had to be carried out at higher pH values in all cases.

In order to interpret these kinetic results, the different species present in aqueous solution must be taken into account. The chlorinating agent has two possible species related by the equilibrium depicted in Scheme 1 [pK_a(HOCl)=7.26±0.04] :[13d]



Scheme 1. Possible chlorinating species.

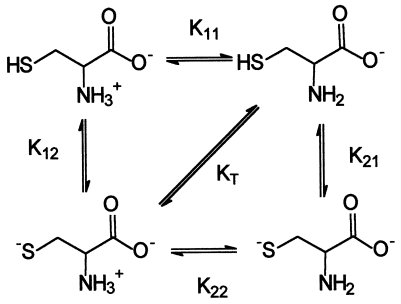
The prominence of HOCl as an active agent at the pH values at which the kinetic studies were carried out will, obviously, depend on its oxidizing power. Since this is known to be much higher than that of ClO^-, it must be taken into account for mechanistic purposes.[13a]

Cys could be present in the form of eight different species, interrelated by three macroscopic, twelve microscopic and six tautomerization equilibria.[14] The case of Met is simpler: four species could be present, with two macroscopic, four microscopic and one tautomerization equilibria. Considering the possible species for both reagents, 16 processes for Cys and 8 for Met could take place. However, under the conditions of basicity used in this study, and considering the pK_a values[15] for the different ionization sites of Cys [pK_a(–CO_2H)=1.88±0.02, pK_a(–NH_3^+)=8.15±0.06, pK_a(–SH)=10.29±0.08] and Met [pK_a(–CO_2H)=2.20±0.04, pK_a(–NH_3^+)=9.05±0.02], the conclusion can be drawn that only the species shown in Schemes 2 and 3 should be considered, i.e. four species in the case of Cys and two in the case of Met.

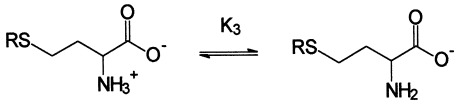
The number of possible elementary processes reduces to eight in the case of Cys and four for Met. Scheme 4 exemplifies such processes for Cys (notice that the chlorination process could, in principle, take place either in the *S* or in the *N*, or in both).

However, the mechanistic possibility of chlorination in the amino group can be discarded for these compounds on the basis of the following pieces of evidence:

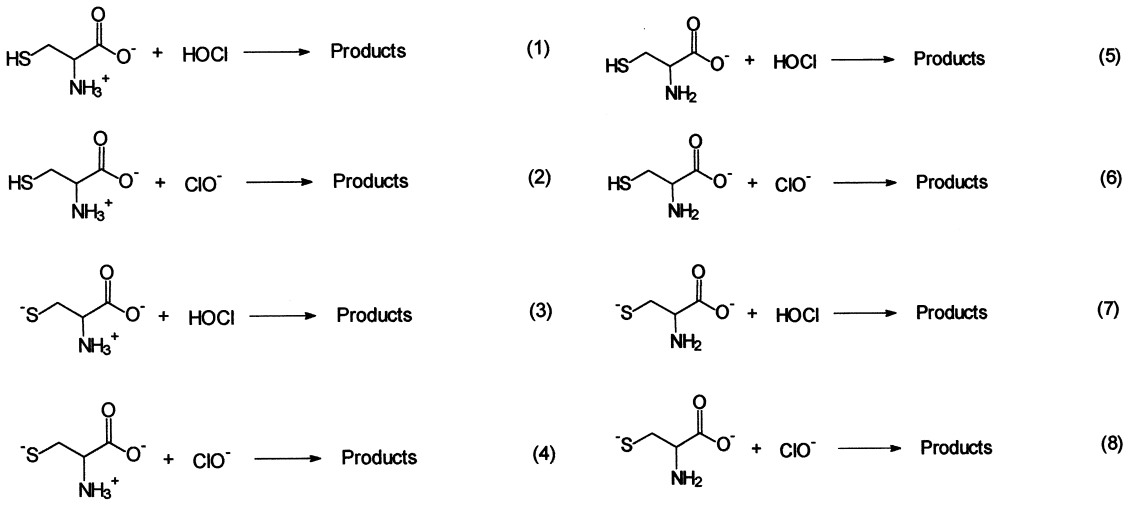
1. The well-known UV bands corresponding to the (*N*-Cl)-compound at ca. 255 nm are not observed.[13,16,17]



Scheme 2. Simplified ionization scheme for Cys.



Scheme 3. Simplified ionization scheme for Met.

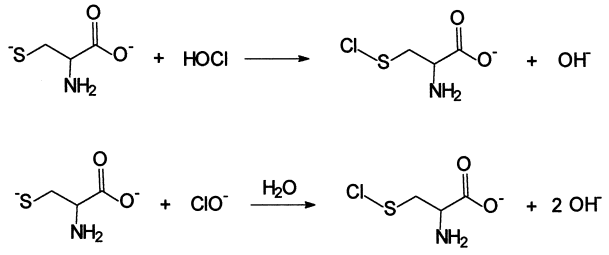


Scheme 4. Possible processes for the chlorination of Cys.

2. Under similar conditions of acidity, the oxidation of Cys and Met by aqueous chlorine is at least one order-of-magnitude faster than that for the non-sulfur containing amino acids with similar pK_{a2}.
3. Using the rate constants known for nitrogenated compounds with similar p$K_{a2,}$ much lower values for reaction rate are predicted, so the k_{obs} values cannot be explained on the basis of a mechanism via chlorination on the amino group.[13]
4. (*N*-Cl)-amines are known to undergo different processes, yielding aldehydes, ketones, α-keto acids and nitriles as final reaction products, depending on the acidity of the medium.[17] None of these compounds were found as the products of oxidation of Met or Cys. In turn, MetO (a sulfoxide) was found as the product of oxidation of Met (a sulfide), while cystine (Cys^Cys, a disulfide) and cysteic acid (Cya, a sulfonic acid), were the products of oxidation of Cys (a thiol).

Mechanism of chlorination of Cys

Processes (1)–(5) can be quickly discarded for Cys, since they do not lead to the observed dependence of k_{obs} on the acidity of the medium. Processes (6) and (7) are kinetically indistinguishable, and is therefore necessary to use chemical reasoning to discern between them. In a similar case, namely the chlorination of amines, the mechanism has been shown to take place through transfer of Cl from the oxygen atom of HOCl to the free amino group.[13] It is well established that the oxidizing power of HOCl is much higher than that of ClO^-,[13] while an RS^- species is some 10^4 times more reactive as a nucleophile than the protonated analogue RSH.[18] These facts allow process (6) in Scheme 4 to be discarded. Of course, it is possible to claim the possibility of a proton transfer taking place within the solvent cage, a thermodynamically favorable process, since pK_a(HOCl)<pK_a(–SH), but this would be entirely equivalent to process (7) in Scheme 4.



Scheme 5. Proposed mechanism for the chlorination of Cys.

On the other hand, it is clear from the observed dependence of k_{obs} with the pH that at high pH values, k_{obs} is different from zero reaching a constant value of ca. $2\cdot10^5$ mol^{-1} dm^3 s^{-1} (Fig. 1). This would represent a pH-independent pathway, which can only be explained by accepting the existence of a reaction between ClO^- and the $-S^-$ group of Cys, i.e. process (8) in Scheme 4. The occurrence of this process must be due to the high nucleophilicity of RS^- species.

Hence, the mechanism should take place through processes (7) and (8), i.e. via Cl transfer from the oxygen of HOCl and ClO^- to the negatively charged sulfur of Cys to yield the corresponding sulfenyl chloride, (*S*-Cl)-Cys, as depicted in Scheme 5.

The rate equation that can be derived for the process of chlorination of Cys is:

$$\text{rate}_{Cys}=\left[\left(k_{(RS^-+HOCl)}\cdot\frac{[H^+]}{K_C+[H^+]}+k_{(RS^-+ClO^-)}\cdot\frac{K_C}{K_C+[H^+]}\right)\cdot\frac{K_S}{K_S+[H^+]}\right]\cdot[HOCl]_0\cdot[Cys]_0$$

where RS^- represents the thiolate form of Cys, $k_{(RS^-+HOCl)}$ and $k_{(RS^-+ClO^-)}$ are the second-order rate constants for such elementary processes, K_C is the ionization constant for HOCl, K_S the ionization constant for the thiol group of Cys and $[HOCl]_0$ and $[Cys]_0$ are the total concentrations of the reagents. The kinetic data are adequately fit by this equation, as shown in Fig. 1(a).

This mechanism is in agreement with the observation of Cys^Cys and Cya as products of the oxidation of Cys by HOCl and also with results from the literature (see Experimental). The initially formed (*S*-Cl)-Cys could further react with Cys or with itself, or undergo hydrolysis, eventually leading to Cys^Cys and Cya, although such processes are not the object of this study. Thiols are known to oxidize to disulfides and subsequently to sulfonic acids.[19] The observation of increasing concentration of Cl^- after the oxidation, leading to a quantitative recovery of Cl^- (based on $[HOCl]_0$) ca. 20 min after oxidation is in agreement with the observed reaction products, which is an evidence for the decomposition of (*S*-Cl)-Cys. The mechanism leading to Cys^Cys formation could be similar to the decomposition of (*S*-NO)-thiols.[20] Cys^Cys can be then further oxidized to Cya,[19] which, on the contrary, is in agreement with the lower yield observed for Cya relative to Cys^Cys. The detailed mechanism of decomposition of these sulfenyl chlorides in this kind of reaction is currently under study.

Mechanism of chlorination of Met

In the case of Met, only processes that are analogous to (1), (2), (5) and (6) in Scheme 4, but replacing the –SH group by a $-SCH_3$, are possible. Processes (1), (2) and (6) are readily discarded since they do not lead to the observed dependence of k_{obs} with the acidity of the medium. Hence, the chlorination of Met takes place through Cl transfer from the oxygen of the HOCl to the sulfur of Met to yield the corresponding chlorosulfonium cation, as depicted in Scheme 6.

The rate equation derived for the process of chlorination of Met is:

$$\text{rate}_{\text{Met}} = k_{(\text{Met}+\text{HOCl})} \cdot \frac{[H^+]}{K_C + [H^+]} \cdot [\text{HOCl}]_0 \cdot [\text{Met}]_0$$

where k is the second-order rate constant for the elementary process and K_C, $[HOCl]_0$ and $[Met]_0$ are defined as in the case of Cys. The kinetic data are adequately fit by this equation, as shown in Fig. 1(b).

This mechanism is also in agreement with the observation of MetO as the oxidation product (see Experimental). Sulfides are known to oxidize to sulfoxides under various conditions.[9,16,21] The fact that the concentration of Cl^- continuously increases after oxidation until a quantitative recovery of Cl^- (based on $[HOCl]_0$) is reached ca. 20 min after oxidation is in agreement with the observed reaction product. Hydrolysis of the intermediate chlorosulfonium cation should eventually yield MetO. The detailed mechanism of decomposition of these chlorosulfonium cations in this kind of process is currently under study.

Final considerations

Using the experimental values for K_C and K_S, the second-order rate constants can be obtained for the different processes. Thus, for Cys:

$$k_{(RS^- + HOCl)} = 1.2 \cdot 10^9 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$$

$$k_{(RS^- + ClO^-)} = 1.9 \cdot 10^5 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$$

and for Met:

$$k_{(\text{Met}+\text{HOCl})} = (8.7 \pm 0.2) \cdot 10^8 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$$

The rate constant $k_{(RS^- + HOCl)}$ obtained for the reaction between the thiolate form of Cys and HOCl is presumably not within the diffusion-control limit, indicating that such process is mostly chemically-controlled, although with a low energy barrier for recombination of both reagents.

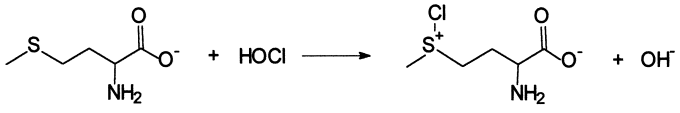
To our knowledge, these are the first kinetic measurements of the rate of formation of chlorosulfonium cations or sulfenyl chlorides in aqueous solution.

In both mechanisms OH^- is produced during the chlorination process. In principle, this is not thermodynamically favored, but in a way similar to the case of non-sulfur containing amino acids, several water molecules are expected to participate in the transition state,[13a] so that additional stabilization is achieved by solvating OH^-, lowering the bonding angle strain and avoiding a bent proton transfer.

When these rate constants are compared with those for non-sulfur containing amino acids,[13e] it turns out that in the case of Met the process is ca. one order-of-magnitude faster, while for Cys (in its RS^- form) it is two orders-of-magnitude faster. This difference in reactivity is due to the fact that sulfides are ca. 10^5 times less reactive as nucleophiles than thiolates.[18]

The observed reaction of Cys (RS^- form) with ClO^- is not detected in the case of the reaction with nitrogenated compounds.[13] Such difference must again be attributed to the enormously higher nucleophilic reactivity of thiolates compared to amines (ca. four orders-of-magnitude higher).[18] The rate constant for this process, $k_{(RS^- + ClO^-)}$, is roughly two orders-of-magnitude lower than those obtained for the most nucleophilic amines,[13] but still rather higher than, for example, those of aromatic amines or amides.[13b]

The much higher reactivity observed for HOCl toward Cys and Met relative to non-sulfur containing amino acids has important biochemical implications; when sulfur-containing amino acids are present the halogen electrophiles will react preferably with them, and the major products formed will be those derived from such a reaction. The sulfenyl chlorides or the halosulfonium ions formed by reaction of thiols and



Scheme 6. Proposed mechanism for the chlorination of Met.

sulfides with halogenating agents are short-lived intermediates and react readily with water yielding, respectively, disulfides, sulfonic acids and sulfoxides.[7,8] In turn, (*N*-halo)-amines, compounds with a relatively long lifetime,[17] are known to lead to toxic[22] and/or mutagenic compounds.[2] Hence, the observed sulfur-based mutagen inactivation[3,5] must be attributed to differences in toxicity between the products generated upon *S*- and *N*-chlorination.

Conclusion

The reaction between sulfur-containing amino acids and aqueous chlorine takes place through a second-order process in which the Cl is transferred from the oxygen of HOCl to the sulfur atom of the amino acid. Sulfide-type amino acids, like Met, react with HOCl ca. one-order of magnitude faster than the non-sulfur containing amino acids ($k_{(Met+HOCl)}=(8.7\pm0.2)\cdot10^8$ mol^{-1} dm^3 s^{-1}), yielding sulfoxides. Thiol type amino acids, like Cys, react ca. one–two orders-of-magnitude faster ($k_{(RS^-+HOCl)}=1.2\cdot10^9$ mol^{-1} dm^3 s^{-1}). We found that thiol-type amino acids, like Cys, can also react at a relatively high rate with ClO^- ($k_{(RS^-+ClO^-)}=1.9\cdot10^5$ mol^{-1} dm^3 s^{-1}). The products of oxidation of thiols are disulfides and sulfonic acids. These are the first kinetic measurements of the rate of the first elementary steps for the formation of sulfenyl chlorides or chlorosulfonium cations. On the basis of the proposed mechanisms, the available observations of mutagen inactivation by sulfur-containing amino acids must be attributed to greater nucleophilicity and to the different toxicity of the products generated upon *S*-chlorination and *N*-chlorination.

Experimental

Reagents

Aqueous chlorine solutions were prepared by adjusting appropriate NaOCl solutions to the desired pH. Acid pH values were avoided in order not to have interference from $Cl_{2\,(aq)}$. The way in which the concentration of aqueous chlorine was titrated, as well as the detailed experimental procedure, have been described elsewhere.[16] All other chemicals were commercially available (Fluka, Merck) and used without further purification. The pH of the medium was adjusted with standard NaOH solutions. The ionic strength was kept to 0.50 mol dm^{-3} with $NaClO_4$. In order to slow down the process, all the kinetics were followed in basic or alkaline medium.

Kinetic studies

The reactions were monitored by measuring the decrease in the absorption of the ClO^- ion at 292 nm in a Hi-Tech Scientific SF-61 MX stopped-flow spectrophotometer was used. The reagents and the cell holder were water-flow thermostated to within ±0.1 K.

The experimental data were accurately fit by a second-order kinetic rate equation. The values reported here for the second-order rate constants are an average of those obtained for 5–10 kinetic runs (standard deviations from the experimental data are shown in Fig. 1).

Product analysis

1 mM Met and Cys were treated with 1 mM HOCl at different pH values between 7 and 12, under conditions similar to those used for the kinetic studies, except for the ionic strength, that was not controlled for the sake of simplicity. The reactions were started and left to proceed for ca. 15–20 min. Then, without any further work-up procedure, the aqueous samples were used as such and the reaction products analyzed by TLC, HPLC and Cl^- ion selective detection.

For the TLC analysis, 10 cm silica gel plates were used, and *n*-BuOH/AcOH/H_2O (80:20:20 cm^3) and *n*-PrOH/H_2O (70:30 cm^3) mixtures used as eluents for development of the plates. Using the first eluent, Methionine sulfoxide (MetO) was found as the product of oxidation of Met with HOCl. In the case of Cys, Cys^Cys and Cya were found as the products of oxidation using both eluents.

The HPLC analyses were performed with a system equipped with a flow unit, automatic injection, column oven with temperature-control and photodiode array UV–Vis and scanning fluorescence detectors. The flow rate used was 1 cm^3 min^{-1} and 5 μL of the sample were injected in all cases. The linearity of response of the detector to all analyzed products was checked, and the so-obtained calibrations used for calculation of reaction yields.

For the HPLC analysis of the products of oxidation of Met with HOCl, a reversed-phase 250 mm length, 3.9 mm internal diameter Alltech column, packed with Partisil OD53, 5 mm, was used with a 7.5 mm length, 4.6 mm internal diameter Alltech precolumn, filled with Partisil OD53, 5 mm. The mobile phase was 5:95 MeOH/H_2O at 298.0 K. The detection was carried out at 210 nm. Under such conditions, 74, 76, 76 and 75% yield of MetO ($t_R\approx2.8$ min) were obtained at the pH values of 6, 7, 8, 12, after oxidation of Met ($t_R\approx3.5$ min) with HOCl.

For the HPLC analysis of the products of oxidation of Cys with HOCl, two different procedures were used.

1. A normal-phase 300 mm length, 3.9 mm internal diameter Waters column packed with Porasil 125 Å, 10 mm was used. The mobile phase was 70:30 *n*-PrOH/H_2O, at 298.0 K. The detection was carried out at 210 nm. Under such conditions, a 22% yield of Cys^Cys ($t_R\approx7.6$ min) was obtained after oxidation of Cys ($t_R\approx4.4$ min) with HOCl, with evidence for the presence of Cya ($t_R\approx3.0$ min).
2. A derivatization procedure was followed using *o*-phthalaldehyde/2-mercaptoethanol (OPA/MCE), to generate fluorescent derivatives, following an established procedure that permits quick and straightforward fluorimetric assay of amino acids down to the nanomole range.[23] A reversed-phase 250 mm length, 3.9 mm internal diameter Alltech column packed with Partisil OD53, 5 mm was used with a 7.5 mm length, 4.6 mm internal diameter Alltech precolumn filled with Partisil

OD53, 5 mm. The mobile phase was a 72:20:8 mixture of H_2O/0.25 mol dm^{-3} sodium propionate (pH≈6.5)/ CH_3CN at 298.0 K. The fluorescence detection was carried out with $\lambda_{excitation}$=360 nm and $\lambda_{emission}$=455 nm. The minimal fluorescence yield of cysteine/cystine OPA/MCE derivatives was solved by pre-treatment with iodoacetic acid.[24] The yields of the fluorescent OPA/MCE amino acid derivatives were quantified by comparison with the peak produced by homoserine (t_R≈30.0 min), that was used as internal standard. Under these conditions, and for the different pH values used, a mean of 31% yield of Cya (t_R≈5.0 min) and 11% of Cys^Cys (t_R≈12.0 min) were obtained after oxidation of Cys (t_R≈12.0 min) with HOCl. However, these yields must be taken as lower limits, since the stability of the OPA/MCE derivatives of Cys, Cya and Cys^Cys is not high, according to the available studies.[23b] Moreover, it is worth noting that using this method the peaks corresponding to Cys and Cys^Cys would elute at the same time, since the latter is reduced to the former.

The concentration of Cl^- generated after oxidation of Met and Cys by aqueous chlorine was measured with a Cl^- selective electrode. Both for the oxidation of Cys and Met with HOCl, a quantitative recovery (100%) of Cl^- was obtained ca. 20 min after starting the reaction, i.e. following decomposition of the initial oxidation product.

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