Formation of S-Alkylcysteines as Artifacts in Acid Protein Hydrolysis, in the Absence and in the Presence of 2-Mercaptoethanol

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Alkylations of cysteine by thiols and thioethers have been studied under the conditions of acid protein hydrolysis using radioactively labeled compounds. Such alkylation, with second-order rate constants of the order of magnitude of 0.1 - 1 M⁻¹ h⁻¹ at 120 °C, lead to the formation of S-alkylated cysteines, e.g. S-methylcysteine and lanthionine, not present in the original protein, but do not seriously impair the analysis of the common amino acids. When a protein is hydrolyzed in the presence of 2-mercaptoethanol, without prior alkylation of its cysteine residues, cysteine reacts with this thiol giving S-(2-hydroxyethyl)cysteine and S,S'- (1,2-ethylene)-bis-cysteine.

To subject a protein in vacuo to 6 M HCl for 15 - 48 h at 110 - 120 °C is widely recognized as the most satisfactory method of hydrolyzing it into its amino acid constituents, despite the fact that not all amino acids are completely recovered after such treatment.

Among the amino acids that suffer losses during acid hydrolysis are the sulfur-containing amino acids: methionine, cysteine and cystine. These losses have been primarily attributed to the readiness with which cysteine and methionine are oxidized to cystic acid and methionine-sulfoxide, respectively.¹

An optimal recovery of methionine, whether present originally as methionine or as its sulfoxide, is obtained when the reducing agent 2-mercaptoethanol (1:2000 v/v) is employed, as described by Keutmann and Potts.² Excellent recoveries of all amino acids, except for cystine, in a standard mixture were reported, cysteine being determined as S-carboxymethylcysteine.

The lability of the sulfur-carbon bond when exposed to concentrated mineral acids is a second factor causing impaired recoveries of the sulfur-containing amino acids. In fact, one method for assaying the methionine content of proteins, often used before the advent of chromatographical methods, relies on the quantitative formation of methyl iodide when methionine is refluxed in concentrated hydriodic acid.³

In this laboratory a method has been developed for determining the content of S-(2-hydroxyethyl)cysteine in haemoglobin.⁴ This alkylation product exists in very small amounts in comparison to those of the common amino acids in the haemoglobin of mice that have been exposed to hydroxyethylating agents, e.g. ethylene oxide. At the initial stage of the work, 1 % by volume of mercaptoethanol was added to the haemoglobin before hydrolysis. However, as it became evident that haemoglobin from non-exposed mice also furnished appreciable amounts of S-(2-hydroxyethyl)cysteine, attention was directed towards the possible role of 2-mercaptoethanol in the formation of this artifact. In a model experiment it was shown that, under the hydrolytic conditions employed, cysteine reacts with 2-mercaptoethanol to give S-(2-hydroxyethyl)cysteine and S,S'- (1,2-ethylene)-bis-cysteine, which is formed from S-(2-hydroxyethyl)-cysteine in a subsequent reaction with cysteine.
The finding that cysteine is alkylated by 2-mercaptoethanol in 6 M HCl prompted us to investigate whether reactions of cysteine with thiols and thioethers constitute a third pathway by which the recoveries of the sulfur-containing amino acids are reduced during acid protein hydrolysis. It is the object of this communication to discuss the importance of such reactions on a semi-quantitative basis.

MATERIALS AND METHODS

**Chemicals.** L-[Methyl-14C]-methionine (50.6 mCi/mmol) and L-[3H]-cystine (40.6 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. The L-[3H]-cystine was obtained from L-[3H]-cystine by reduction with NaBH4 (Merck) and subsequent purification on a Dowex 50 column.

L-Cystine, L-methionine, S-methyl-L-cysteine and 2-mercaptoethanol (type I) were obtained from Sigma Chemical Co., St. Louis, Mo. L-Cysteine.HCl was from BDH Biochemicals, Poole, England, DL-lanthionine from ICN Pharmaceuticals Inc., Plainview, N. Y., and hydrochloric acid (Suprapur) and ethylene glycol (p.a.) from Merck, Darmstadt, Germany.

S-(2,3-Dihydroxyethyl)-L-cysteine was prepared according to Zilkha and Rappoport.6 S-(2-Chloroethyl)-L-cysteine and S,S'-[(1,2-ethylenedi-bis-cysteine (homodienkolic acid) were prepared by a modification of the method described by Connors and Ross.8

**Reaction conditions.** 6 M HCl was prepared from distilled water and 5.45 M HCl. The reactions were performed in pyrex glass tubes. Oxygen was removed by alternately evacuating the tubes to 5 x 10-4 mmHg and letting in argon gas. After sealing, the tubes were placed in an oven at 120 °C (± 5 °C) for selected periods of time. The reactions were interrupted by evaporating the samples to dryness in a rotatory evaporator at 60 °C. If S-(2-hydroxyethyl)-L-cysteine was to be analyzed, the residue was treated with water overnight at 25 °C in order to convert S-(2-chloroethyl)-L-cysteine to S-(2-hydroxyethyl)-L-cysteine.

**Ion-exchange chromatography.** The amino acids were chromatographed on a Dowex 50W-X4 column (90 x 1 cm) which was eluted with 660 ml of 1 M HCl followed by 660 ml of 2 M HCl. The retention volume on this column for the analyzed amino acids were as follows: S-(2-Hydroxyethyl)-L-cysteine 220 ml of 1 M HCl, L-cysteine 240 ml of 1 M HCl, S-methyl-L-cysteine 290 ml of 1 M HCl, L-methionine 460 ml of 1 M HCl, DL-lanthionine 600 ml of 1 M HCl, L-cystine 90 ml of 2 M HCl, S,S'-[(1,2-ethylenedi-bis-cysteine 235 ml of 2 M HCl. For the analysis of radio-labeled lanthionine, it was necessary to separate the appropriate fractions on two additional columns: (a) an Aminex A-5 (BioRad) column (40 x 0.9 cm) eluted first with 170 ml of 0.1 M sodium citrate pH 3.2 and then with the same buffer at pH 4.25. This column was connected to a Cheminert CMP-1 pump (LDC); (b) a Dowex 50W-X4 (23 x 1.5 cm) developed with 0.1 M sodium citrate pH 4.25.

**Radioactivity determinations.** Samples were assayed for radioactivity were mixed with Instagel (Packard) and counted in an Intertechnique SL 30 scintillation spectrometer. The counting efficiency was determined using the automatic external standardization.

**Gas chromatography—mass fragmentography.** The method used for identification of S-(2-hydroxyethyl)-L-cysteine is described in a previous publication.4

**Thin-layer chromatography.** Amino acids in the eluted fractions were detected and identified by thin-layer chromatography on silica gel plates. The chromatograms were developed with 2-propanol-water (7:3) and sprayed with a ninhydrin reagent.

RESULTS

**Experimental layout.** Three series of experiments were conducted. In the first series, methionine, [14C]-labeled in the methyl carbon and unlabeled cysteine were mixed and subjected to the conditions of acid protein hydrolysis in the presence and in the absence of 2-mercaptoethanol. The amounts of radio-labeled S-methylcysteine and methionine were determined after column chromatography. The experimental data are presented in Table 1.*

In the second series of experiments, [3H]-cysteine was incubated at 120 °C alone and in the presence of protein, ethylene glycol or 2-mercaptoethanol. One sealed tube with cysteine in 6 M HCl was stored in the refrigerator and analyzed as a blank. Cysteine and the reaction products cystine, lanthionine, S-(2-hydroxyethyl)cysteine and S,S'-[(1,2-ethylenedi-bis-cysteine were determined by means of radioactivity measurements after column chromatography. S-(2-hydroxyethyl)-cysteine was identified by means of GC-MS in the experiment with added protein.

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* The following abbreviations in accordance with the recommendations of IUPAC-TUBS (J. Biol. Chem. 247 (1972) 977) are used in the tables:

Cys, cysteine; Met, methionine; Cys Cys, cystine; Lan, lanthionine; Cys(Me), S-methylcysteine; Cys-\(\mathrm{EtOH}\), S-(2-hydroxyethyl)cysteine; Cys Cys, S,S'-[(1,2-ethylenedi-bis-cysteine. 

Table 1. Incubation of [methyl\textsuperscript{3H}]methionine with cysteine at 120 °C for 24 h in 6 M HCl in the absence and in the presence of 2-mercaptoethanol.

<table>
<thead>
<tr>
<th>Added to the reaction mixture</th>
<th>Found after incubation (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Cys] mM [Met] mM [HSCH\textsubscript{2}CH\textsubscript{2}OH] mM [Cys(Me)] mM [Met] mM</td>
<td>4.2 \times 10^{-2} \sim 0.24</td>
</tr>
</tbody>
</table>

\(a\) No products other than \(S\)-methylcysteine and methionine were determined in this experiment. Most of the radioactivity was eluted at the front after cation exchange separation, possibly as methanol.

Experimental data are presented in Table 2.

In the final series of experiments, a trace amount of \(^{35}S\)-labeled cysteine was added to a solution of unlabeled \(S\)-(2-hydroxyethyl)cysteine in 6 M HCl and the reaction mixture was analyzed for radiolabeled \(S\)-(2-hydroxyethyl)-cysteine and \(S\),\(S\)\'-(1,2-ethylene)-bis-cysteine after selected periods of time. The experimental data are presented in Table 3.

Reaction kinetics. Data derived from Tables 1–3 have been used for rough estimates of the second-order rate constants, \(k\), for the formation of some of the products found after incubation at 120 °C. These constants are summarized in Table 4.

The reaction constants \(k\), for the formation of the products C, which have been calculated using the equation

\[
[C]/[A][B] = kt
\]

Table 2. Incubation of \(^{35}S\)-cysteine at 120 °C for 15 h in 6 M HCl in the presence of protein, ethylene glycol or 2-mercaptoethanol and alone.

<table>
<thead>
<tr>
<th>Added to the reaction mixture</th>
<th>Found after incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Cys] mM Added compound</td>
<td>[Cys] mM [Cys Cys] mM [Lan] mM [Cys(EtOH)] mM [Cys(Et)] mM</td>
</tr>
<tr>
<td>1.68 5 mg of protein/ml</td>
<td>0.25 0.65 4.0 \times 10^{-4} (a)</td>
</tr>
<tr>
<td>1.01 HOCH\textsubscript{2}CH\textsubscript{2}OH 160 mM</td>
<td>0.37 0.25 3.9 \times 10^{-4} (a)</td>
</tr>
<tr>
<td>1.01 HSCH\textsubscript{2}CH\textsubscript{2}OH 130 mM</td>
<td>0.44 0.065 11 \times 10^{-4} 8 \times 10^{-4} 3.9 \times 10^{-2}</td>
</tr>
<tr>
<td>1.94 –</td>
<td>0.24 1.6 1.1 \times 10^{-4} (a)</td>
</tr>
<tr>
<td>2.48 (not heated)</td>
<td>2.1 0.1 &lt;2.3 \times 10^{-3} (a)</td>
</tr>
</tbody>
</table>

\(a\) Not determined.

Table 3. Incubation of \(^{35}S\)-cysteine with \(S\)-(2-hydroxyethyl)cysteine in 6 M HCl at 120 °C for selected periods of time. To the reaction mixture was added 25.5 mM [Cys(EtOH)] and 14 300 dpm [Cys].

<table>
<thead>
<tr>
<th>Found after incubation</th>
<th>Reaction activity, [Cys(EtOH)] dpm [Cys] dpm, Reaction time, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 400</td>
<td>80 (a)</td>
</tr>
<tr>
<td>10 600</td>
<td>280</td>
</tr>
<tr>
<td>8 300</td>
<td>1300</td>
</tr>
<tr>
<td>8 300</td>
<td>880</td>
</tr>
<tr>
<td>11 400</td>
<td>10 600</td>
</tr>
<tr>
<td>8 300</td>
<td>8 300</td>
</tr>
</tbody>
</table>

\(a\) Not determined.

Discussion

Two of the demonstrated reactions, 1 and 2 (Scheme 1), are likely to take place whenever a protein is subjected to acid hydrolysis without prior oxidation or alkylation of its cysteine residues.

Table 4. Estimated second-order reaction rate constants for the reactions \( A + B \xrightarrow{k} C + (D) \) in 6 M HCl at 120 °C.

<table>
<thead>
<tr>
<th>Reaction No.</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>( k ) (M(^{-1}) h(^{-1})) Using initial [Cys] Using final [Cys]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cys</td>
<td>Met</td>
<td>Cys(Me)</td>
<td>0.5(^{(1.1)})(^b)</td>
</tr>
<tr>
<td>2</td>
<td>Cys</td>
<td>Cys</td>
<td>Lan</td>
<td>0.03(^c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>((0.07 &gt; k &gt; 0.002)^d)</td>
</tr>
<tr>
<td>3</td>
<td>Cys</td>
<td>HSCH(_2)CH(_2)OH</td>
<td>Cys(EtOH)</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td>4</td>
<td>Cys</td>
<td>Cys(EtOH)</td>
<td>Cys</td>
<td>0.3(^e)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>((0.8 &gt; k &gt; 0.2))</td>
</tr>
<tr>
<td>5</td>
<td>[^{35}\text{S}]\text{Cys}</td>
<td>Cys(EtOH)</td>
<td>[^{35}\text{S}]\text{-Cys(EtOH)}</td>
<td>0.2(^f)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>((0.3 &gt; k &gt; 0.2))</td>
</tr>
</tbody>
</table>

\(^a\) For reaction numbers, cf. Schemes 1, 2 and 4. \(^b\) Calculated under the assumption that the concentration of methionine dropped exponentially to its final value. \(^c\) Average of four determinations. \(^d\) 95 % confidence limits. \(^e\) Average of three determinations. \(^f\) Average of three determinations, the value obtained for 48 h (Table 3) was not used.

To the best of our knowledge, \( S \)-methylcysteine has not previously been demonstrated to be an artifact in acid protein hydrolysis.

Reaction 2, however, has been shown by Dowling and MacLaren \(^7\) to occur when wool is subjected to acid hydrolysis after reduction, but without alkylation, of its cysteine residues. In contrast to our findings, the authors did not obtain any lanthionine when free, non-protein bound, cysteine was subjected to the conditions of acid hydrolysis, and argue that some of the cysteine residues in wool may be optimally arranged for lanthionine formation.

A great variation in the rate constants for reaction 2 is observed (cf. Table 4) especially when the rate constants are based on the initial concentration of cysteine. This variation becomes smaller when the calculation is based on the final concentration of cysteine. This might be explained by a rapid initial decrease of the concentration of cysteine due to oxidation in some of the experiments.

For unknown reasons, \( S \)-methylcysteine was not found when cysteine was incubated with methionine in the presence of 2-mercaptoethanol; Table 1.

The importance of reactions 1 and 2 can be estimated tentatively using the rate constants from Table 4. In order for the recovery of cysteine to be decreased by one per cent through reaction 1, the concentration of methionine in the protein hydrolysate needs to be about 0.01/15 h \times 0.5 \text{M} \text{h}^{-1} = 1 \times 10^{-3} \text{ M}, and for the same decrease to occur due to reaction 2, the concentration of cysteine needs to be about 0.01/15 h \times 0.3 \text{M} \text{h}^{-1} = 2 \times 10^{-3} \text{ M} (using the rate constant obtained from the final concentration of cysteine). Provided the concentration of protein is not higher than 5 mg/ml, which is the concentration suggested by

\[ \text{Scheme 1.} \]

Scheme 2.

Moore and Stein, and provided the protein does not contain more than about 5% each of methionine and cysteine, the recovery of cysteine will not be decreased by more than 2% through reactions 1 and 2.

It can thus be concluded, that if the purpose of hydrolyzing a protein is to determine the amounts of the common amino acids it contains, the demonstrated side reactions will not seriously impair the analyses. However, if the aim is to assay nanogram or micrograms of methylated amino acids in gram amounts of proteins, reaction 1 may be of paramount importance.

Reactions 3 and 4 (Scheme 2) are expected to occur whenever a protein is subjected to acid hydrolysis in the presence of 2-mercaptoethanol without prior alkylation of its cysteine residues. A detailed mapping of the reaction pathways leading to the formation of S-(2-hydroxyethyl)cysteine and S,S'- (1,2-ethylene)-bis-cysteine is beyond the scope of this investigation. However, it is known that 2-mercaptoethanol in 3.2 M HCl is in a state of equilibrium with 2-chloroethanethiol and thiranium ion, compounds that polymerize to polyethylenesulfide and p-dithiane. In analogy with these reactions, it is reasonable to suggest that cysteine reacts first with thiranium ion (or 2-chloroethanethiol) (Scheme 3) yielding S-(2-mercaptoethyl)cysteine which, after protonation, may either regenerate cysteine and thiranium ion or generate hydrogen sulfide and a thiranium ion involving cysteine. This thiranium ion, finally, may alkylate either water, chloride ion, or cysteine yielding S-(2-hydroxyethyl)cysteine, S-(2-chloroethyl) cysteine and S,S'- (1,2-ethylene) -bis-cysteine, respectively. For the equilibrium between S-(2-hydroxyethyl)cysteine and S-(2-chloroethyl)-cysteine see Refs. 4, 6 and 9. That S,S'- (1,2-ethylene)bis-cysteine can actually be formed from S-(2-hydroxyethyl)cysteine and cysteine (reaction 5, Scheme 4) is shown in Table 3.

The rate constant for the formation of S-(2-hydroxyethyl)cysteine (reaction 3) is probably somewhat underestimated for a number of reasons: (a) the possible breakdown of S-(2-mercaptoethyl) cysteine into its reactants; (b) the polymerization of 2-mercaptoethanol; (c) the fact that only the alkylation of 34S-labeled cysteine has been analyzed. Since 2-mercaptoethanol and thiranium ion act as nucleophiles in 3.2 M HCl one would expect them to be alkylated by cysteine. If that is the case, a resulting product with cysteine would be S-(2-hydroxyethyl)cysteine (though unlabeled).

It is implied by Keutmann and Potts that the reduction product of cystine obtained after acid hydrolysis in the presence of 2-mercaptoethanol is cysteine, which seems reasonable considering the ability of 2-mercaptoethanol to reduce cystine to cysteine in a neutral solution. Hence, reactions 3 and 4 will occur also when cysteine is in the form of cystine at the onset of hydrolysis.

Hydroxyl groups of ethylene glycol do not serve as leaving groups even in 6 M HCl, as shown by the fact that S-(2-hydroxyethyl)cysteine and S,S'- (1,2-ethylene) -bis-cysteine, were not found when cysteine was incubated in the presence of ethylene glycol (Table 2).

Reaction 6 (Scheme 4), finally, demonstrates that alkyl groups of S-2-hydroxyethylated cysteine "migrate" from one molecule of cysteine to another during the course of acid hydrolysis, provided that non-alkylated cysteine molecules are available.
Scheme 3. Possible pathway for the formation of S-(2-hydroxyethyl)cysteine and ethylene-bis-cysteine in acid protein hydrolysis in the presence of 2-mercaptoethanol.

From our finding that cysteine acts as a nucleophile even under the conditions of acid protein hydrolysis, the question arises as to what extent other amino acids also do this. As to methionine, this seems to be the case, since Gundlach et al. obtained homoserine-lactone and S-carboxymethylhomocysteine, the degradation products of carboxymethylated methionine, when methionine was subjected to 6 M HCl in the presence of iodoacetic acid. Moreover, Floyd et al. obtained methylmethionine sulfonium chloride after treatment

Scheme 4.

of methionine sulfoxide in 6 M HCl. The authors suggested that the methylmethionine sulfonium chloride was formed by a nucleophilic attack of methionine on methyl chloride, both of which they argued were liberated from methionine sulfoxide, and not directly by an attack of one methionine molecule on the 6-methyl carbon of another.

Another question which arises from the finding that cysteine acts as a nucleophile in 6 M HCl, is whether that could give a partial answer to why the amino acids serine and threonine suffer minor losses during acid hydrolysis. Alkylations of cysteine by serine and threonine would lead to the formation of lanthionine and \( \beta \)-methyl-lanthionine, respectively. The experiments of Lavine et al.\(^4\) are, however, somewhat discouraging in this respect. Unlike simple alcohols, serine and threonine failed to give sulfonium salts with methionine in 50 \% \( \text{H}_2\text{SO}_4 \).

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REFERENCES


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