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# Regeneration of Methionyl Residues from Their Sulfonium Salts in Peptides and Proteins†

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ABSTRACT: The reaction of methionylsulfonium derivatives of the model peptide Gly-Met-Gly with several nucleophiles was found to result in the regeneration of an intact methionyl residue. Sulfonium salts derived from this peptide were especially susceptible to attack by sulfur nucleophiles. The rate of regeneration of the methionyl side chain depends on the nature of both the sulfonium salt and the attacking nucleophile. Reaction with mercaptoethanol was found to reactivate chymotrypsin alkylated at Met-192 with phenacyl bromide. Regeneration by thiols may be used to distinguish the alkylation of methionyl residues from that of other amino acid residues in biologically active proteins.

Modification of proteins is often accomplished using various alkylating agents. Such reagents have been found to attack methionyl residues and yield alkylsulfonium derivatives (Stark, 1970). Methionylsulfonium salts are generally stable to performic acid oxidation (Neumann *et al.*, 1962) and cyanogen bromide cleavage (Spande *et al.*, 1970) but decompose on acid hydrolysis to give a variety of products (Gundlach *et al.*, 1959; Goren *et al.*, 1968). Sulfonium salts prepared from acylated methionine were found by Toennies and Kolb (1945a,b) to yield several materials when incubated with different nucleophiles at high temperature for long periods of time.

During a recent investigation it was found that β-galactosidase can be inactivated by the alkylation of a methionyl residue near its active site (Naider *et al.*, 1972; Yariv *et al.*, 1971). On incubation with mercaptoethanol the protein regained more than 90% of its enzymatic activity, suggesting the recovery of the methionyl residue. In view of the findings of Toennies and Kolb (1945b) the decomposition of the methionylsulfonium salt was not expected to result in one product. It is therefore of particular importance to determine whether "thiolysis" is a general method for the regeneration of the methionyl side chain from methionylsulfonium salts. In this report we present evidence that reaction with sulfur nucleophiles leads to the quantitative regeneration of methionyl residues from sulfonium salts in peptides and proteins.

## Experimental Section

*Materials.* Glycyl-L-methionylglycine was purchased from Mann Laboratories (lot R1956). It was homogeneous in paper electrophoresis at pH 3.5. *Anal.* Calcd for $C_9H_{17}N_3O_4S$: N, 15.98; S, 12.12. Found: N, 15.91; S, 13.22. Amino acid analysis after performic oxidation indicated 1.07 methionyl residues/2 glycyl residues.

Chymotrypsin was purchased from Worthington (three-times crystallized, lot CDI SLK). All other materials employed in this investigation were reagent grade chemicals.

## Methods

*Electrophoresis.* Electrophoresis was carried out with a Model LT-36 electrophoresis tank, EC-123 coolant, and an HV-5000 power supply (Savant Instruments). Pyridine–acetate buffer (pH 3.5) was prepared from glacial acetic acid–pyridine–water (10:1:89, v/v). Samples were applied to Whatman No. 3MM paper and run at a gradient of 50 V/cm for 45–60 min. After electrophoresis, the paper was dried, dipped in a solution of ninhydrin (0.5% w/v) in 95% aqueous acetone, and developed by heating at 80–90°.

*Amino Acid Analysis after Performic Acid Oxidation.* Samples of 10–25 μl were mixed with 0.5 ml of performic acid (Moore, 1963) and kept at 0° for 4–6 hr. The samples were then dried under reduced pressure using a rotary evaporator at 40°. The last traces of performic acid were removed by adding water (0.5 ml) and again evaporating to dryness. Removal of excess performic acid by the addition of HBr (Moore, 1963) was found to decompose some of the sulfonium salts investigated to yield methionine sulfone. The oxidized samples were hydrolyzed with 6 N HCl for 22 hr in evacuated sealed tubes at 110°. Analyses were carried out using a Beckman 121 automatic amino acid analyzer.

*Assay of Chymotrypsin.* Chymotryptic activity was measured in a pH-stat (Radiometer, Copenhagen) with acetyl-L-tyrosine ethyl ester as substrate. Assays were performed in solutions $2 \times 10^{-3}$ M in substrate, 0.2 M in KCl and 0.1 M in $CaCl_2$, at pH 8.0 and 24°.

*Preparation of Sulfonium Salts.* A solution of Gly-Met-Gly (0.05 M) in 0.05 M sodium acetate buffer (pH 4) was made 0.1 M in alkylating agent. The reaction was allowed to proceed at

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30° and the liberation of bromide ion was followed by titration of aliquots according to the procedure described by Cheng (1959). Alkylation was 95–100% complete in 15 hr. The reaction with phenacyl bromide was carried out in a mixture of equal volumes of ethanol and 0.05 M acetate buffer (pH 4). The reaction mixture was initially turbid and clarified as the product formed. The sulfonium salts studied were found to be stable for 3–4 days at pH 4 and only minor degradation products were observed on electrophoresis. No attempt was made to isolate or purify the sulfonium salts and they were used directly in the regeneration experiments.

*Reaction of Nucleophiles with Sulfonium Salts.* One volume of the sulfonium salt solution was diluted with two volumes of a 0.2 M sodium carbonate buffer (pH 9.6). The solution was made 0.1 M in the selected nucleophile, its pH was measured, and the reaction was allowed to proceed at 30°. The course of the reaction was followed by subjecting aliquots of the reaction mixture to paper electrophoresis at pH 3.5 and/or by amino acid analysis after performic acid oxidation.

*Inactivation and Reactivation of Chymotrypsin.* Chymotrypsin was reacted with phenacyl bromide at pH 5.1 and 20° as described by Schramm and Lawson (1963). The time required to obtain 50% inactivation was 12 min. After 5 hr the residual activity was 13%. Schramm and Lawson (1963) report a half-time for inactivation of 15 min and a residual activity of 1–2% as determined using a different assay procedure.

Inactivated chymotrypsin was reactivated by mixing aliquots of the inactivation reaction mixtures with equal volumes of 0.2 M $Na_2HPO_4$ containing various concentrations of mercaptoethanol. The final pH of all reactivation media was 7.5 (enzyme concentration, 0.5 mg/ml).

## Results

The sulfonium salts formed by the reaction of Gly-Met-Gly with various alkylating reagents are converted to Gly-Met-Gly on treatment with sulfur nucleophiles (Figures 1 and 2, Table I). Figure 1 (numbers 2 and 3) presents the electrophoretic behavior of the sulfonium salt formed from Gly-Met-Gly and bromoacetamide before and after incubation with 0.12 M mercaptoethanol at pH 8.9 for 24 hr at 30°. This reaction results in the complete disappearance of the sulfonium compound and the appearance of a compound whose electrophoretic mobility is identical with that of Gly-Met-Gly. In the absence of mercaptoethanol incubation of the sulfonium salt at pH values as high as 10 does not result in the appreciable recovery of Gly-Met-Gly (Figure 1, no. 4). The regeneration of Gly-Met-Gly from the sulfonium salt formed using *N*-bromoacetyl-β-D-galactosylamine, an affinity reagent for β-galactosidase, is observed to be nearly complete after 3 hr of reaction with mercaptoethanol (Figure 1, no. 7 and 8). Under similar conditions the sulfonium salt formed with phenacyl bromide, an affinity label for chymotrypsin, was found to revert rapidly to the starting peptide. Inspection of electrophoretograms after 2-hr reaction with mercaptoethanol showed complete disappearance of the sulfonium salt, and amino acid analysis indicates that the reaction is more than 50% complete in 10 min. As illustrated in Figure 1 (no. 5 and 6) the sulfonium salt derived from bromoacetic acid is much more resistant to thiolysis than that derived from bromoacetamide. It can be seen that only a small part of the Gly-Met-Gly has been recovered after incubation of the former sulfonium compound with mercaptoethanol for 1 day.

The effect of different nucleophiles on the sulfonium salt derived from Gly-Met-Gly and bromoacetamide is illustrated in Figure 2. After incubation for 3 hr at 30° the reaction with all of the sulfur nucleophiles (mercaptoethanol, mercaptoacetic acid, and thiophenol) is nearly complete whereas after 20 hr most of the sulfonium salt remains after reaction with

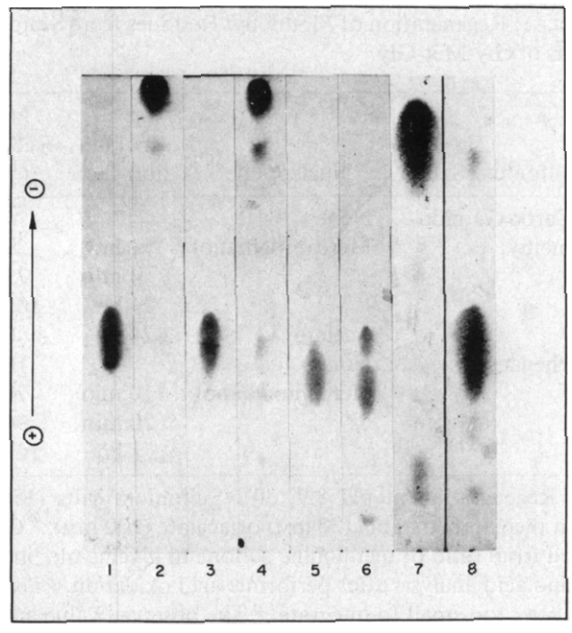



FIGURE 1: Regeneration of Gly-Met-Gly from various sulfonium salts using mercaptoethanol at pH 8.9. Paper electrophoresis, pH 3.5. (1) Gly-Met-Gly, (2–4) Gly-Met(*S*-carboxyamidomethyl)-Gly: (2) untreated, (3) after 24 hr incubation with mercaptoethanol, (4) after 24 hr at pH 10 without mercaptoethanol; (5 and 6) Gly-Met(*S*-carboxymethyl)-Gly; (5) untreated, (6) after 24 hr incubation with mercaptoethanol; (7 and 8) sulfonium salt from Gly-Met-Gly and β-D-galactosylamine: (7) untreated, and (8) after 3-hr incubation with mercaptoethanol.

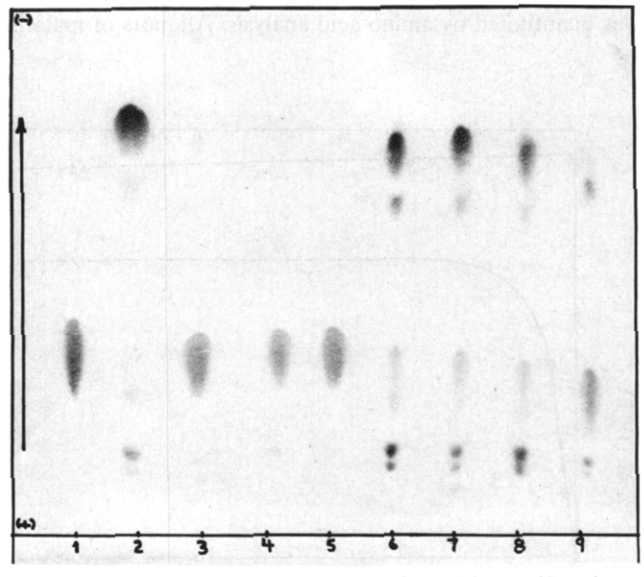



FIGURE 2: Regeneration of Gly-Met-Gly from Gly-Met(*S*-carboxyamidomethyl)-Gly using different nucleophiles. Paper electrophoresis at pH 3.5. (1) Gly-Met-Gly; (2–9) Gly-Met(*S*-carboxyamidomethyl)-Gly: (2) untreated, after incubation for 20 hr with: (3) mercaptoethanol (pH 8.9), (4) mercaptoacetic acid (pH 7.4), (5) thiophenol (pH 9.5), (6) sodium acetate (pH 9.4), (7) phenol (pH 9.9), (8) hydroxylamine (pH 9.2), and (9) hydrazine (pH 10.1). The concentration of all nucleophiles was 0.1 M and the measured pH is given in parentheses.

TABLE I: Regeneration of Methionyl Residues from Sulfonium Salts of Gly-Met-Gly.

| Sulfonium Salt | Nucleophile[a] | Incubation Time | Met Recovd[b] (%) |
|---|---|---|---|
| *S*-Carboxyamidomethyl | None | | 4.1 |
| | Mercaptoethanol | 1 hr | 29.7 |
| | | 4 hr | 73.1 |
| | | 24 hr | 102.0 |
| | Acetate | 24 hr | 0[c] |
| *S*-Phenacyl | None | | 16.9[d] |
| | Mercaptoethanol | 10 min | 78.1 |
| | | 20 min | 94.2 |
| | | 1 hr | 101.5 |

[a] Regeneration at pH 8.9, 30°. Sulfonium salts (15 mM) with mercaptoethanol (120 mM) or acetate (100 mM). [b] Calculated from ratio of methionine sulfone to glycine obtained by amino acid analysis after performic acid oxidation. [c] Peak on analyzer too small to integrate. [d] The observed value appears too high in view of the results of bromide titration (see Experimental Section) and from inspection of electrophoretograms. It is possible that this reactive sulfonium salt partly is decomposed by performic acid, to give some methionine sulfone. Slight decomposition of sulfonium salts by performic acid was also observed by Gundlach *et al.* (1959).

acetate ion, hydroxylamine and phenol. Only in the case of hydrazine is a significant amount of Gly-Met-Gly observed after 20 hr. Several spots which do not correspond to either Gly-Met-Gly or the sulfonium salt appear on the electrophoretogram. These spots are especially intense after reaction with the nonsulfur nucleophiles.

In several experiments the recovery of the starting peptide was quantitated by amino acid analysis. Aliquots of material

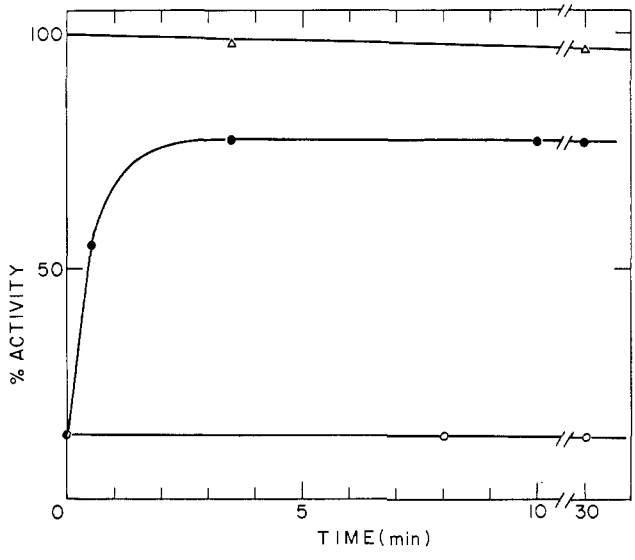
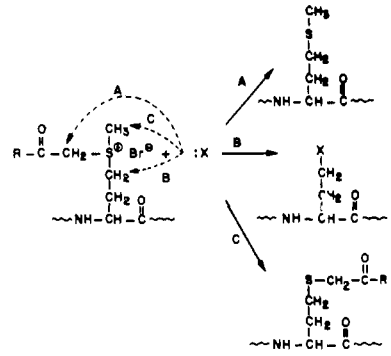



FIGURE 3: Regain of the activity of chymotrypsin inactivated with phenacyl bromide. (●) Inactivated enzyme in 0.006 M mercaptoethanol at pH 7.5; (Δ) native enzyme in 0.006 M mercaptoethanol at pH 7.5; (○) inactivated enzyme at pH 7.5.

SCHEME I



were removed from the sulfonium salt mixtures before and after regeneration, oxidized with performic acid, hydrolyzed in 6 N hydrochloric acid, and analyzed. Under these conditions methionyl residues are quantitatively converted to methionine sulfone. In contrast although methionylsulfonium salts yield several decomposition products these do not include methionine sulfone (Goren *et al.*, 1968). The recoveries of methionyl residues on incubation (of several sulfonium salts) with mercaptoethanol and with acetate ion, computed from the ratio of methionine sulfone to glycine, are summarized in Table I. The results indicate that the rate of decomposition of the *S*-phenacylsulfonium salt is much higher than that of the *S*-carboxyamidomethyl derivative. The observation that acetate ion is not an effective nucleophile in this reaction is also substantiated.

Reactivation with mercaptoethanol was investigated using chymotrypsin inactivated with phenacyl bromide. This reagent was shown to alkylate specifically Met-192 (Schramm and Lawson, 1963). Incubation of the inactivated chymotrypsin with 0.006 M mercaptoethanol at pH 7.5 resulted in the recovery of 72% of chymotryptic activity in approximately 2 min (Figure 3). No further activation was achieved by prolonging the incubation time with mercaptoethanol. With higher concentrations of mercaptoethanol (0.01–0.05 M) similar regains in chymotryptic activity were observed in less than 30 sec and activity did not increase further on prolonged incubation. Native chymotrypsin when incubated with mercaptoethanol under similar conditions showed no change in activity.

## Discussion

The reaction of sulfonium derivatives of methionine with nucleophiles can proceed by three distinct pathways (Scheme I), all of which have been observed in model systems (Toennies and Kolb, 1945b). Methionylsulfonium residues in peptides and proteins can also undergo intramolecular cyclization to homoserine lactone as observed in cyanogen bromide cleavage reactions (Spande *et al.*, 1970).

In the present study thiolysis of sulfonium salts derived from Gly-Met-Gly by mercaptoethanol, thiophenol, or β-mercaptoacetic acid results predominantly in the regeneration of the starting peptide. Inspection of paper electrophoretograms gives a definite indication that nucleophilic breakdown of the sulfonium salt by sulfur nucleophiles proceeds exclusively by pathway A. If the attacking nucleophile were incorporated into the peptide (pathway B, Scheme I) the products obtained using different nucleophiles would be expected to move very differently in an electric field. This is especially

true in the case of β-mercaptoacetic acid whose incorporation would add an extra negative charge into the peptide. Furthermore, the nucleophilic decomposition of the various sulfonium derivatives of Gly-Met-Gly examined in this study results in the same final product suggesting that reaction pathway C is not followed. The recovery of the methionyl side chain is further supported by the results of the amino acid analyses (Table I).

The decomposition of the sulfonium salts studied here apparently involve the nucleophilic attack on the carbon α to the carbonyl function in the following structure: $>S^{+}C_{\alpha}H_2$-C(O)R. Such a reaction is expected to occur by an SN2 mechanism (Pocker and Parker, 1966) and its rate would then depend on the nature of both R and the attacking nucleophile. Among the sulfonium salts that we investigated the ease of the nucleophilic attack changes with R in the following manner; $C_6H_5 > NH_2 > OH$. This is similar to the rate of nucleophilic attack in SN2 reactions involving alkyl halides which is known to depend on the electrophilicity of the α-carbon atom (Hine, 1956). In all cases examined sulfur nucleophiles are found to decompose the sulfonium derivatives at accelerated rates and with greater specificity when compared to oxygen and nitrogen nucleophiles. The superiority of the "soft" sulfur nucleophiles when compared to the "hard" nitrogen and oxygen nucleophiles is expected on the basis of trends observed in other nucleophilic substitution reactions (Pearson, 1969).

The primary importance of the regeneration of methionyl residues from their sulfonium salts relates to the utilization of this reaction in protein chemistry. In order to ascertain whether the reaction of methionylsulfonium salts with thiols can be carried out on proteins containing disulfide bonds without affecting their biological activity, we investigated the reactivation of chymotrypsin inactivated by the alkylation of one methionyl residue. β-Galactosidase, in which this reaction was first observed, is atypical in this respect since it does not have disulfide bridges (Craven *et al.*, 1965). We found that the incubation of alkylated chymotrypsin with mercaptoethanol results in the rapid recovery of 72% of its original activity (Figure 3). Native chymotrypsin does not show any loss of activity under the conditions used for reactivation. Our inability to reactivate the enzyme completely may, therefore, have been due to the alkylation of other residues during the lengthy inactivation reaction. It should be pointed out that reactivation is not conclusive proof that an intact methionyl side chain is regenerated. Regain of enzymatic activity might conceivably occur if reaction pathway B (Scheme I) were followed, thus leading to formation of *S*-hydroxyethylhomocysteine. In view of the results with the *S*-phenacyl derivative of Gly-Met-Gly, however, we feel that this possibility is unlikely.

The reactivation of alkylated chymotrypsin is rapid at very low concentrations of mercaptide ion ($10^{-3}$ M mercaptoethanol, pH 7.5) (Figure 3). The regeneration of the methionyl side chain is much slower ($t_{1/2}$ 5–10 min, $10^{-1}$ M mercaptoetharol, pH 8.9) in the corresponding model *S*-phenacylsulfonium derivative (Table I). The enhanced reactivation rate in the enzyme may, perhaps, be ascribed to some strain in the sulfonium salt resulting from the interaction of the phenacyl moiety with the substrate binding site. Similarly the reaction with mercaptoethanol of β-galactosidase, alkylated with an active-site-directed reagent, was found to be 12 times more rapid than that of enzyme alkylated with iodoacetamide (Naider *et al.*, 1972). In contrast the decomposition of the analogous sulfonium derivatives from the model peptide Gly-Met-Gly proceeds at approximately the same rates. It thus appears that in proteins there are several factors in addition to the nature of the nucleophile and the electrophilicity of the carbon atom attacked, which affect the facility of the reactivation process.

This investigation presents evidence that methionylsulfonium derivatives in peptides and proteins may be decomposed by thiols to regenerate an intact methionyl skeleton. The regeneration of biological activity upon thiolysis of sulfonium salts may offer a convenient method for the study of the importance of methionine in the active sites of proteins. Thiolysis occurs rapidly at 25°, in the pH range of 7–9, with thiol concentrations of $5 \times 10^{-3}$ to $10^{-1}$ M, conditions where most proteins are stable. The products of alkylation of other functional groups of proteins (imidazoyl, sulfhydryl, phenol, amino, and carboxyl) are not expected to be susceptible to thiolysis under these conditions. This identification of methionyl modification may be particularly useful since methionylsulfonium salts decompose during acid hydrolysis (Goren *et al.*, 1968) so that their determination by amino acid analysis is complicated and tedious. The occurrence of this reaction must also be considered when planning structural studies on alkylated proteins since reduction with thiols, often employed as a preliminary step in sequence determinations, may decompose sulfonium groups if present.

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