helpful and illuminating discussions, and Mr. Marcel Knossow for his computational assistance.

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Selective Covalent Binding of Methionyl-Containing Peptides and Proteins to Water Insoluble Polymeric Reagent and Their Regeneration†

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ABSTRACT: A polymeric reagent of the type Ⓟ ~ $NHCOCH_2Cl$ (where Ⓟ is Bio-Gel P-100) was prepared. This polymer covalently bound peptides and proteins specifically at methionine residues, under acidic conditions in the presence of a small amount of sodium iodide. Treatment of the polymer-peptide conjugate with 2-mercaptoethanol resulted in essentially complete removal of the peptide with regeneration of intact methionyl residues. In an alternative way, the polymer was suspended for 2 h in boiling water. This treatment resulted in the conversion of the bound methionyl residues to homoserine residues and cleavage and liberation of the bound peptides. The polymeric reagent was successfully applied to the separation of methionyl peptides from peptide mixtures and for specific covalent binding of enzymes and biologically active proteins via their exposed methionyl residues, with the retention of their biological activity.

Gundlach et al. (1959) and Stark and Stein (1964) showed that alkylating agents react with methionyl residues in peptides and proteins over a wide pH range and may be considered as specific agents toward methionine at a low pH where other reactive groups are protonated. The products formed, 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). *S*-Carboxamidomethyl (CM[1])-methionylsulfonium peptides undergo quantitative cleavage with formation of homoserine lactone on heating in a sealed tube for 2 h (Tang and Hartley, 1967). Naider and Bohak (1972) have shown that reaction of methionine sulfonium derivatives with several nucleophiles, particularly with sulfur nucleophiles, results in the regeneration of intact methionyl residues.

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[1] Abbreviations used: 8CM-lysozyme, lysozyme derivative in which all four disulfide bonds were reduced and carboxymethylated; CM, *S*-carboxamidomethyl; Z, benzyloxycarbonyl; BAEE, α-*N*-benzoyl-L-arginine ethyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester; Chloramine-T, *N*-chloro-*p*-toluenesulfonamide; GSH, glutathione; Ⓟ~$NHCOCH_2Cl$, Bio-Gel P-100 derivative in which 2-chloroacetic acid was covalently attached through ethylenediamine; 1-MetO-α-lactalbumin, α-lactalbumin derivative in which the single methionyl residue was oxidized to methionine sulfoxide; 2-MetO-Kunitz-trypsin inhibitor, Kunitz trypsin inhibitor derivative in which both methionyl residues were oxidized to methionine sulfoxide; 1-MetO-ribonuclease, ribonuclease derivative in which Met-29 was oxidized to methionine sulfoxide; Ⓟ~Met-Val, Met-Val, covalently linked through its methionyl residue to Ⓟ~$NHCOCH_2Cl$ (the same kind of abbreviation is used for free methionine or other methionyl peptides, e.g., Ⓟ~Met); Hse, homoserine; RT, room temperature; DEAE, diethylaminoethyl.

Studies involving selective alkylation of methionine residues in proteins at acidic pH have been widely documented, as in the contributions on ribonuclease (Gundlach et al., 1959; Neumann et al., 1962; Goren and Barnard, 1970), trypsinogen and trypsin (Holeysovsky and Lazdunski, 1968), horse heart cytochrome *c* (Tsai and Williams, 1965), and isocitrate dehydrogenase (Colman 1968, 1969).

Water-insoluble polymeric reagents which react covalently with selected amino acid residues in peptides and proteins and are released afterward with a suitable reagent have been described for cysteine (Brocklehurst et al., 1974; Lin and Foster, 1975; Sluyterman and Wijdenes, 1974) and tryptophan (Rubinstein et al., 1976).

In view of the specificity of alkylating agents toward methionyl residues at acidic pH and the possibility of regenerating intact methionyl residues from methionine sulfonium derivatives by sulfur nucleophiles, a polymeric reagent for the reversible covalent binding of methionyl residues was prepared. Its properties and applications to peptides and proteins are reported.

Experimental Section

Materials. Bovine α-lactalbumin was isolated from raw skimmed milk by modification of the methods of Aschaffenburg and Drewry (1957) as described by Castellino and Hill (1970). Bovine pancreatic ribonuclease, hen egg-white lysozyme, bovine trypsin, L-(1-tosylamido-2-phenyl) ether chloromethyl trypsin, and Kunitz soybean trypsin inhibitor were purchased from Worthington Biochemical Corp. Pyruvate kinase type 1 from rabbit skeletal muscles (Sigma Chemical Co.) contained nucleotide diphosphokinase and lactate dehydrogenase necessary for the spectrophotometric assay of UDP. 8CM-lysozyme was prepared and digested by trypsin as described earlier (Shechter et al., 1972). Bowman-Birk trypsin inhibitor was prepared from untreated soybeans by the method of Frattali (1969); its purification with DEAE-cellulose was repeated twice. Partially purified galactosyl transferase (A protein) was isolated by a combination of the methods used by Brodbeck and Ebner (1966) and Brew et al. (1968). Cyanogen bromide, Chloramine-T, 2-mercaptoethanol, 2-chloroacetic acid, 2-iodoacetic acid, *N*-hydroxysuccinimide, ethylenediamine, and dicyclohexylcarbodimide were Fluka products. Dithioerythritol was from Calbiochem. BAEE, ATEE, Z-Trp-Ala, and Z-His-Phe were purchased from Miles Yeda (Rehovot, Israel). The peptides Met-Val, Met-Leu-Gly, Met-Asp, and Gly-Met were from Schwarz/Mann laboratories. The peptide Tyr-Thr-Lys-Pro-Arg was a gift from Mr. Y. Stabinsky of our department. Bio-Gel type P-100 was from Bio-Rad Laboratories. The standard calibration mixture of amino acids type I (which does not contain cysteine) was purchased from Spinco-Beckman. Prior to use, the mixture was lyophilized and dissolved in the appropriate buffer, and an equimolar amount of tryptophan was added to it. 2-MetO-Kunitz-trypsin inhibitor, 1 MetO-α-lactalbumin and 1-MetO-ribonuclease were prepared by oxidizing the proteins with 20 equiv of Chloramine-T in 0.1 M Tris-HCl (pH 8.5) essentially as described by Shechter et al. (1975). Under these conditions, Met-29 in ribonuclease is selectively oxidized (Y. Shechter and Y. Burstein, to be published).

Chloroacetamidoethylpolyacrylamide ($\text{Ⓟ}\sim NHCOCH_2Cl$) was prepared as follows: A solution of dicyclohexylcarbodiimide (5.5 g, 25 mmol) in ethyl acetate (50 mL) was combined with an ice cold solution of chloroacetic acid (2.36 g, 25 mmol) and *N*-hydroxysuccinimide (2.95 g, 25 mmol) in ethyl acetate (200 mL). The mixture was left for 1 h at 0 °C and 1 h at room temperature. Dicyclohexylurea was filtered off and the filtrate evaporated to dryness. The residue was taken in dioxane (50 mL) and filtered into a suspension of aminoethylated polyacrylamide beads (Inman and Dintzis, 1969) (6 g, Bio-Gel P-100) in water (250 mL). The suspension was stirred for 20 min at room temperature; the polymer was then filtered off, washed with water, and acetylated according to Inman and Dintzis (1969). The polymeric reagent was finally washed by methanol–water mixtures with a gradual increase of the methanol concentration up to 100%. Analysis of the dry polymer gave 2.72% Cl (0.77 mmol/g).

Spectrophotometric measurements were performed with a Beckman Acta V spectrophotometer. Quartz cells of 1-cm light path were used. The following extinction values ($E_{280}^{1\%}$) for the native proteins were used: trypsin, 15, 4; chymotrypsin, 20, 4; lysozyme, 26, 9; α-lactalbumin, 19, 0; ribonuclease, 6, 5; Kunitz trypsin inhibitor, 10, 13; Birk-Bowman trypsin inhibitor, 4, 6.

Amino acid analyses were performed on the Beckman-Spinco Model 120C automatic amino acid analyzer after hydrolysis in 6 N HCl for 22 h at 110 °C. To determine the total amount of peptide or protein covalently bound to the polymeric support, 3–5 mg of polymer was hydrolyzed in 6 N HCl for 48 h at 110 °C and the amino acid content was determined. Protein concentrations were calculated using published values for amino acid composition. To determine homoserine content, the acid hydrolyzate was incubated prior to analysis in 0.1 M NaOH for 1 h, acidified, and applied to the long column of the analyzer. Elution was performed at 45 °C. This procedure resulted in good separation of homoserine from threonine and glutamic acid. The integration constant used was 70% of the value for aspartic acid (Eshdat et al., 1974).

Methionine sulfoxide content in proteins was determined as described earlier (Shechter et al., 1975).

Trypsin and α-chymotrypsin activities were assayed with BAEE and ATEE, respectively, according to the spectrophotometric method of Schwert and Takenaka (1955).

Binding capacities of trypsin and α-chymotrypsin by immobilized Kunitz and Birk-Bowman soybean trypsin inhibitors were determined by suspending 1–5 mg of the corresponding polymer–inhibitor conjugates in 1.0 mL of solution of trypsin or α-chymotrypsin (0.3 mg/mL) at pH 8.0 for 10 min. The polymer was then filtered off and an aliquot from the supernatant was withdrawn for enzymic activity and compared with the enzymic activity before suspending the polymer. As a control, the same experiment was performed with a polymeric reagent which does not contain a covalently bound inhibitor.

Ribonuclease activity was assayed with yeast RNA in 0.1 M acetate buffer at pH 5.0 according to the method of Anfinsen (1954). Immobilized ribonuclease was suspended in the assay mixture and removed by filtration prior to the precipitation stage of the undigested yeast RNA.

α-Lactalbumin activity was assayed as described earlier (Shechter et al., 1973). By this method UDP formation in the lactose synthesis reaction was followed spectrophotometrically at 340 nm according to Davidson (1959). Immobilized α-lactalbumin was suspended in the assay mixture for a given period of time and removed by filtration prior to absorbancy measurement.

General Procedure for Coupling Methionine, Methionyl Peptides, or Proteins to $\text{Ⓟ}\sim NHCOCH_2Cl$. To a solution of methionine (0.5-2 μmol/mL), methionyl peptides (0.4–1.5 μmol/mL), or a protein (0.2–0.5 μmol/mL) in 0.1 M acetic acid which contained 0.1% sodium iodide (pH 3.3), the poly-

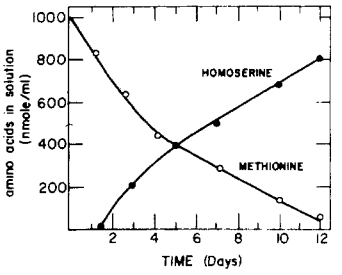


FIGURE 1: Course of the reaction of methionine with Ⓟ~NHCOCH$_2$Cl in 0.1 M acetic acid–0.1% NaI (pH 3.3). Reaction was carried out at methionine concentration of 1 μmol/mL with 40 mg of Ⓟ~NHCOCH$_2$Cl at 37 °C. Aliquots were filtered off and assayed for their methionine (○) and homoserine content (●).

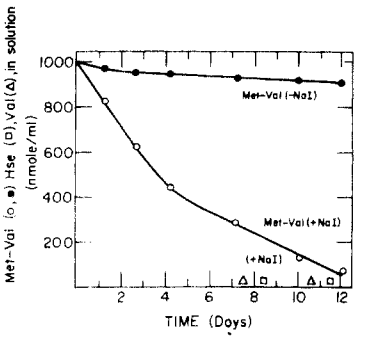


FIGURE 2: Course of the reaction of Met-Val with Ⓟ~NHCOCH$_2$Cl in 0.1 M acetic acid–0.1% NaI (pH 3.3). Reaction was carried out at Met-Val concentration of 1 μmol/mL, with 40 mg of Ⓟ~NHCOCH$_2$Cl at 37 °C. Aliquots were filtered off and assayed with and without acid hydrolysis for the content of Met-Val (○) homoserine (□) and valine (△). Reaction was also carried out in the absence of NaI (●).

meric reagent was added (40 mg/mL). The reaction was carried in the dark at 37 °C with constant stirring. During the course of the reaction, aliquots were removed and filtered off, and the supernatant was analyzed for its content. The reaction was terminated by centrifuging down the polymeric reagent. The polymer was then washed repeatedly with 0.1 M acetic acid, H_2O, 0.5 M NaCl, H_2O, 0.1 M Tris-HCl buffer (pH 8.0), H_2O, and dried under reduced pressure.

Removal of Covalently Bound Methionyl Peptides by 2-Mercaptoethanol. The peptide bound polymeric reagent (5 mg) was suspended in 1.0 mL of 0.1 M ammonium bicarbonate which was brought to pH 9.0 by NH_4OH. To this suspension, 2-mercaptoethanol was added to a final concentration of 0.2 M. The reaction was performed in a closed glass tube for 3 h at 37 °C or 15 h at room temperature in the dark with constant stirring. The polymeric reagent was then centrifuged down and the supernatant lyophilized.

Removal of Covalently Bound Peptides and Proteins by Cleavage at Methionyl Residues. Polymer–peptide conjugate (5 mg) was suspended in 1.0 mL of distilled water and incubated in a sealed glass tube for 2 h at 105 °C. The polymer was then removed by filtration and the filtrate lyophilized. To determine the number of methionyl residues involved in binding of proteins to the polymeric support, the polymer–protein conjugate (5 mg) was suspended in 1.0 mL of performic acid reagent (95 volumes of 99% formic acid and 5 volumes of 30% hydrogen peroxide) for 2 h at room temperature. The reagent was then removed by evaporation and the polymer was suspended in 1.0 mL of distilled water and incubated in a sealed glass tube for 20 h at 105 °C. The polymer was then removed, and the filtrate was acid hydrolyzed and analyzed for its homoserine content.

Results

Reaction of Ⓟ~NHCOCH$_2$Cl with Free Amino Acids and Glutathione (GSH). Treatment of standard calibration mixture of amino acids (1 μmol/mL) in 0.1 M acetic acid containing 0.1% NaI with 40 mg of Ⓟ~NHCOCH$_2$Cl for 12 days at 37 °C resulted in the complete absence of methionine from the supernatant and appearance of 0.8 residue of homoserine. The values for all other amino acids were within experimental error (standard deviation less than 3%) identical with those of the solution prior to the addition of Ⓟ~NHCOCH$_2$Cl and are omitted for the sake of brevity.

Treatment of GSH (1 μmol/mL) with Ⓟ~NHCOCH$_2$Cl under the conditions applied to the amino acid mixture resulted in the covalent binding of approximately 20% of the peptide within 12 days.

Reaction of Ⓟ~NHCOCH$_2$Cl with a Mixture of Peptides. To a mixture of peptides containing 0.5 μmol of each Met-Leu-Gly, Z-Trp-Ala, Met-Asp, Z-His-Phe, and Tyr-Thr-Lys-Pro-Arg in 1.0 mL of the above medium, 40 mg of Ⓟ~NHCOCH$_2$Cl was added and the reaction was carried out under the conditions described above for 12 days. The polymeric reagent was then centrifuged down and the supernatant examined for its amino acid content after acid hydrolysis. The polymer was extensively washed as described under the Experimental Section; an aliquot was acid hydrolyzed, and the free amino acids liberated were analyzed. Leucine, glycine, and aspartic acid (the amino acids constituents of the methionyl peptides) were absent in the supernatant. These amino acids were present in the hydrolyzed polymeric fraction in equimolar amounts. In addition, the three decomposition products of acid-hydrolyzed carboxymethylmethionine, namely methionine, homoserine, and carboxymethylhomocysteine (Gurd, 1972), were also present in small quantities. No other amino acids were present in this fraction.

Course of the Reaction of Ⓟ~NHCOCH$_2$Cl with Free Methionine and Methionyl Peptide. During the course of the reaction of Ⓟ~NHCOCH$_2$Cl with L-methionine (Figure 1) and Met-Val (Figure 2) in 0.1 M acetic acid–0.1% NaI (pH 3.3) at 37 °C, aliquots were removed at intervals and centrifuged down and the supernatant was examined for its amino acids content. With Met-Val the supernatant was examined for its amino acid content with and without acid hydrolysis. As can be seen from Figure 1, methionine was dissipated from the supernatant but simultaneously the bound methionine was released to the medium as homoserine. By adding the amount of methionine left to the amount of homoserine released to the medium, it was calculated that approximately 20% of the methionine remained bound to Ⓟ~NHCOCH$_2$Cl during most stages of the reaction. In contrast to free methionine, the dipeptide Met-Val was completely bound to the polymeric reagent within 12 days (Figure 2). No release of homoserine or valine was detected. When the same reaction was performed in the absence of sodium iodide, only 10% of the peptide was bound to Ⓟ~NHCOCH$_2$Cl within 12 days (Figure 2, upper curve).

Stability and Conditions for Removal of Methionine and Methionyl Peptides Covalently Bound to Ⓟ~NHCOCH$_2$Cl. Methionine or methionyl peptides covalently bound to Ⓟ~NHCOCH$_2$Cl were suspended in different media and subjected to various conditions in order to determine stability and optimal conditions for removal of the bound material from the polymeric support. Results are summarized in Table I. Under the reaction conditions used for binding methionyl residues to Ⓟ~NHCOCH$_2$Cl (0.1 M acetic acid–0.1% NaI

TABLE I: Stability and Conditions for Removal of Methionine and Methionyl Peptides, Covalently Bound to Ⓟ~$NHCOCH_2Cl$.

No.	Derivative Designation[a]	Medium	pH	Temp (°C)	Time	Product Liberated (yield, %)[b]
1	Ⓟ~Met	0.1 M acetic acid–0.1% NaI	3.3	37	4 days	Hse (100)
2	Ⓟ~Met	H_2O	7.0	100	2 h	Hse (80)
3	Ⓟ~Met	0.1 M NH_4HCO_3–0.2 M 2-mercaptoethanol	9.0	37	4 h	Met (50), Hse (18)
4	Ⓟ~Met-Val	0.1 M KCl–HCl	2.0	37	12 days	None
5	Ⓟ~Met-Val	0.1 M acetic acid–0.1% NaI	3.3	37	12 days	None
6	Ⓟ~Met-Val	0.1 M Na_3PO_4	6.5	37	12 days	None
7	Ⓟ~Met-Val	0.1 M Tris-HCl	8.5	37	12 days	Hse (20), Val (20)
8	Ⓟ~Met-Val	H_2O	7.0	100	2 h	Hse (80), Val (80)
9	Ⓟ~Met-Val	0.1 M NH_4HCO_3–0.2 M 2-mercaptoethanol	9.0	RT	15 h	Met-Val (100)
10	Ⓟ~Met-Asp	0.1M acetic acid–0.1% NaI	3.3	37	12 days	None
11	Ⓟ~Met-Asp	H_2O	7.0	100	2 h	Hse (70), Asp (70)
12	Ⓟ~Met-Asp	0.1 M NH_4HCO_3–0.2 M 2-mercaptoethanol	9.0	RT	15 h	Met-Asp (100)
13	Ⓟ Gly-Met	0.1 M acetic acid–0.1% NaI	3.3	37	4 days	Gly-Hse (100)

[a] Polymeric samples (5 mg) were suspended in 1.0 ml of the described medium and stirred in the dark in a closed glass tube. [b] Percent of total bound amino acids or peptides. Total amount of bound peptide was determined by acid hydrolysis of a polymer sample as described in the Experimental Section. Bound methionine was estimated by the difference between the initial amount of methionine and the combined amount of unreacted methionine and homoserine released to the medium, during preparation of the conjugate.

at 37 °C), bound methionine was liberated to the medium as homoserine (Table I, No. 1). Bound Gly-Met was liberated to the medium as Gly-Hse (Table I, No. 13). In contrast, polymer-bound methionyl peptides in which the carboxylic group of the methionyl residue participates in a peptide bond (for example, in Met-Val or Met-Asp) are conclusively stable under the reaction conditions (Table I, No. 5 and 10). The same stability was observed at a pH range of 2.0–6.5 (Table I, No. 4–6). At pH 8.5, 12 days of incubation at 37 °C released 20% of the polymer-bound Met-Val in the form of homoserine and free valine (Table I, No. 7).

The course of homoserine release from polymer–methionine conjugate (Ⓟ~Met) was examined at two different pH values. It was found that almost twice the amount of homoserine is released at pH 4.15 as compared with the amount released at pH 1.16 (Figure 3).

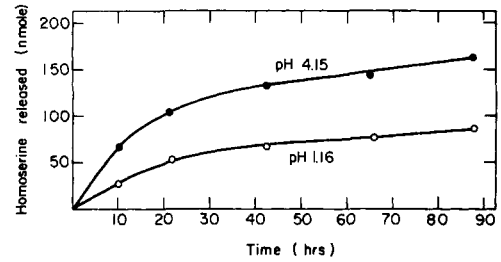


FIGURE 3: Liberation of homoserine from Ⓟ~Met at two different pH values. Ⓟ~Met (8 mg) was suspended in 1 mL of 0.1 M HCl–KCl (pH 1.16) or 0.1 M sodium acetate (pH 4.15) and incubated at 37 °C. Aliquots were filtered off and assayed for their homoserine content.

Removal of the bound material is achieved by two ways. Incubation of the polymer for two h at 100 °C resulted in the conversion of the bound methionine to homoserine, cleavage and liberation of homoserine and free amino acids, in 70–80% yield (Table I, No. 2, 8, 11). In an alternative way the polymer was treated with 2-mercaptoethanol in 0.1 M NH_4HCO_3 (pH 9.0). Under these conditions, 50% of the polymer-bound methionine is regenerated as intact methionine and 18% of the bound methionine is released as homoserine (Table I, No. 3). In contrast, the methionyl peptides covalently bound to the polymeric support are released quantitatively as the intact peptides (Table I, No. 9 and 12). No cleavage of the peptides occurred as evidenced by the absence of homoserine or free amino acids.

Isolation of the Methionyl Peptides from the Tryptic Peptides Mixture of 8CM-Lysozyme. One of the practical applications of Ⓟ~$NHCOCH_2Cl$ is demonstrated by the one-step isolation of the methionyl peptides from the tryptic peptides mixture of 8CM-lysozyme. The solution of peptides (0.5 μmol/mL) was allowed to react with 40 mg of Ⓟ~$NHCOCH_2Cl$ under the conditions described for 12 days. The polymer was then repeatedly washed as described in the Experimental Section, and dried under reduced pressure.

The bound peptides were removed either by treatment with 2-mercaptoethanol or by cleavage at methionyl residues and the amino acid content of the peptides removed was determined in each case. These results are summarized in Table II. As can be seen from the table, the amino acid composition of the material released by mercaptoethanol is in considerable agreement with the composition expected for 1:1 ratio of both methionyl containing tryptic peptides, namely, residues 6–13 and 98–112 in 8CM-lysozyme. The reaction of Ⓟ~$NHCOCH_2Cl$ was quantitative as judged by calculating the total amount of polymer-bound methionyl peptides, based on lysine and arginine content. Removal of the bound peptides by 2-mercaptoethanol treatment and by 2 h of boiling proceeded in 80 and 40% yield, respectively.

Reaction of Ⓟ~$NHCOCH_2Cl$ with Proteins. Enzymes and biologically active proteins were covalently bound to Ⓟ~$NHCOCH_2Cl$ under the conditions specified in the Experimental Section for 6 days. The extent of binding, number of methionine residues involved and the biological activity of the immobilized proteins examined, is given in Table III. With the exception of egg lysozyme, all native proteins examined reacted with Ⓟ~$NHCOCH_2Cl$. Under the experimental conditions applied, the amount of the covalently bound protein was in the range of 6–8.5 nmol/mg. Almost no reaction was observed when 1-MetO-α-lactalbumin (a derivative in which the single methionyl residue was oxidized to methionine sulfoxide) was allowed to react with Ⓟ~$NHCOCH_2Cl$. The same results were observed with 2-MetO-Kunitz trypsin inhibitor, a derivative in which both its methionyl residues were oxidized to methionine sulfoxide. An active derivative of ribonuclease in which methionine-29 was selectively oxidized to methionine

TABLE II: Amino Acid Content and Composition of Polymer-Bound Methionyl Tryptic Peptides Derived from 8CM-Lysozyme.

Amino Acid	Polymer (nmol/mg) Initially Bound to Polymer[a]	Removed by Boiling[b]	Removed by 2-Mercaptoethanol[c]	No. of Residues Calcd[d]	No. of Residues Found[e]
Lys	12.5	5.0	10	1	1.0
His					
Arg	12.4	4.8	9.7	1	1.0
Asp	40	17	32	3	3.2
Thr					
Ser	14	5.5	11.4	1	1.14
Glu	12.4	5.0	10.0	1	1.0
Pro					
Gly	27	11	21.0	2	2.1
Ala	53	20	44.0	5	4.4
Val	20	7.0	15	2	1.5
Met	7		14	2	1.4
Ile	14	5.6	11	1	1.1
Leu	12	5.1	12	1	1.2
Tyr	2	0.8	1.0		0.1
Phe	2		2.0		0.2
S-Carboxy-Me-Cys	9	3.0	6.0	1	0.6
Hse	4	8			
Carboxy-Me-Hcy	ND				

[a] A polymer sample (5 mg) was suspended in 6 M HCl and hydrolyzed for 48 h at 110 °C. [b] A polymer sample (10 mg) was incubated in H_2O (2.0 mL) for 2 h at 100 °C and centrifuged. The supernatant was acid hydrolyzed. [c] A polymer sample (5 mg) was incubated in 0.1 M NH_4HCO_3–0.2 M 2-mercaptoethanol for 15 h at room temperature. The polymer was then removed and the supernatant lyophilized and acid hydrolyzed. [d] For peptides comprising 6–13 and 98–112 in 8CM-lysozyme. [e] Based on the amino acid composition of the peptides removed by 2-mercaptoethanol. Assuming lysine as 1 residue.

TABLE III: Extent of Binding, Biological Activity, and Number of Methionine Residues Involved in Binding of Proteins to Ⓟ~$NHCOCH_2Cl$.

Protein	Amount of Covalently Bound Protein[a] (nmol/mg)	No. of Methionine Residues Involved[b] (mol/mol)	Biological Act. (%)
Ribonuclease	8.5	1.4	12
1-MetO-ribonuclease[c]	8.0	1.2	2
Egg white lysozyme	0.5		ND
α-Lactalbumin	6.0	1.0	40
1-MetO-α-lactalbumin[c]	0.1		ND
Birk Bowman trypsin inhibitor	7.2	0.95	58[d] 65[e]
Kunitz-trypsin inhibitor	8.0	1.0	100[e]
2-MetO Kunitz trypsin inhibitor[c]	0.3		ND

[a] Determined by acid hydrolysis and amino acid analysis. [b] Determined by suspending a performic acid oxidized polymeric sample (5 mg) in H_2O (1.0 mL) for 20 h in 105 °C. The polymer was then removed; the filtrate was hydrolyzed and analyzed for its homoserine content. [c] Reaction conditions are identical with the conditions applied for the corresponding native proteins. [d] Determined as the binding capacity for α-chymotrypsin, assuming a 1:1 molar complex. [e] Determined as the binding capacity for trypsin, assuming a 1:1 molar complex.

sulfoxide reacted with the polymeric reagent to the same extent as the native protein but its enzymic activity was only one-sixth of that of immobilized ribonuclease prepared by reaction of the native protein with Ⓟ~$NHCOCH_2Cl$. With the exception of ribonuclease, the covalently bound proteins preserved 40–100% of their biological activity, and about 1 mol of methionine per mol of protein is involved in covalent binding of the protein examined to the polymeric support.

Discussion

The polymeric reagent Ⓟ~$NHCOCH_2Cl$ was found to be selective toward methionyl residues in non-SH peptides and proteins under acidic conditions in the presence of sodium iodide. In the absence of sodium iodide, only 10% of the peptide Met-Val was covalently bound to Ⓟ~$NHCOCH_2Cl$ within 12 days (Figure 2, upper curve), suggesting that the polymeric form participating in the reaction is Ⓟ~$NHCOCH_2I$, formed by exchange reaction of the chloride anion by iodide anion (eq 1).

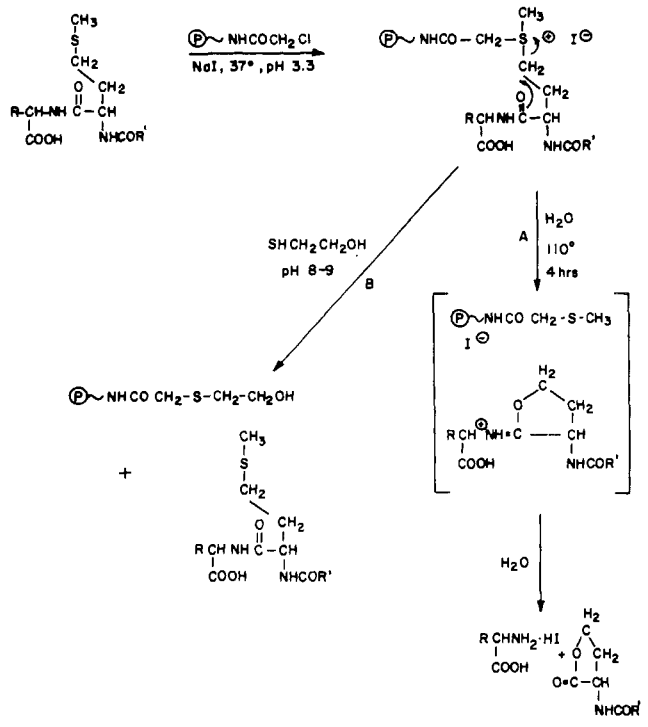
$$Ⓟ\sim NHCOCH_2Cl + I^- \rightarrow Ⓟ\sim NHCOCH_2I + Cl^- \quad (1)$$

It should be mentioned that, under the experimental conditions (pH 3.3, 37 °C, 12 days), no cleavage of peptide bonds adjacent to aspartic acid residues took place, as concluded by the absence of free aspartic acid in solution during the reaction of Ⓟ~$NHCOCH_2Cl$ with Met-Asp, and during incubation of Ⓟ~Met-Asp under the applied experimental conditions (Table I, No. 10). This type of cleavage, described by Shultz, takes place under much more drastic conditions (Schultz, 1967).

With the exception of free methionine and C-terminal methionine peptides, the covalently bound methionyl-containing peptides remained bound to the polymeric reagent under the reaction conditions applied. The removal of covalently bound methionyl peptides was achieved by two alternative ways. Treatment with 2-mercaptoethanol resulted in nearly quantitative removal of the bound material by "thiolysis" (Scheme I, pathway B). Under these conditions, the starting peptide is regenerated. These results coincide with the work of Naider and Bohak (1972), showing that the nucleophilic breakdown of sulfonium salts by sulfur nucleophiles proceeds exclusively via a pathway regenerating intact methionine.

By boiling the water suspension of the polymer for 2–20 h, peptides and proteins were removed by a mechanism involving intramolecular cyclization of methionine sulfonium residues to homoserine lactone and cleavage (Scheme I, pathway A) essentially as described by Lawson et al. (1962) and Tang and Hartley (1967), for the cleavage mechanism of *S*-carboxyamidomethyl(CM)methionine peptides. Methionyl peptides covalently bound to the polymeric reagent, in which the methionyl residues are not located at the C-terminal position, are stable at the pH range of 2–6.5 at 37 °C and the removal via intramolecular cyclization takes place only at elevated temperatures. However, in the case of covalently bound methionine or methionyl peptides in which the methionyl residues do occupy the C-terminal position, the intramolecular cyclization proceeds easily at 37 °C or even at lower temperatures, suggesting that the carboxylate anion is superior to the amide carbonyl in performing the nucleophilic attack on the γ carbon of the substituted methionine. The pH dependency of homoserine release from methionine covalently bound to the polymeric reagent may also suggest that the carboxylate rather than the protonated form is the attacking nucleophile.

Scheme I



Ⓟ~NHCOCH$_2$Cl may have a great potential for the selective isolation of methionyl peptides, particularly for the determination of overlaps between cyanogen bromide fragments during amino acid sequence analysis. The use of Ⓟ~NHCOCH$_2$Cl for the isolation of methionyl peptides from a peptide mixture was demonstrated in this work. It should be pointed out that, among the various nucleophilic groups present in peptides and proteins which are liable to react with haloacetates, the methionine side chain is the only group that can be regenerated from its sulfonium derivative by treatment with 2-mercaptoethanol. This is an additional factor contributing to the isolation of highly purified methionyl peptides. Even if some minor reaction of Ⓟ~NHCOCH$_2$Cl did occur with histidyl, cysteinyl, or lysyl peptides under acidic conditions, these peptides would not be removed from the polymeric carrier hy 2-mercaptoethanol, thus ensuring exclusive isolation of the methionyl peptides.

Protein derivatives in which the methionyl residues were quantitatively oxidized to methionine sulfoxide residues did not react with Ⓟ~NHCOCH$_2$Cl, thus confirming that selective covalent binding of proteins to the polymeric reagent occurs via the methionyl residues. We chose to bind proteins such as α-lactalbumin, Kunitz, and Birk-Bowman trypsin inhibitors, in which specific chemical modification of methionyl residues is known not to affect their biological activity (Castellino and Hill, 1970; Kowalski et al., 1974; Odani and Ikanaka, 1973). Indeed appreciable amounts of activity were retained on immobilization of α-lactalbumin, Kunitz trypsin inhibitor, and Birk-Bowman trypsin inhibitor. With ribonuclease, it has been shown that, at acidic pH, methionine-29 was primarily alkylated by methyl iodide, yielding a fully active derivative (Link and Stark, 1968). On prolonged exposure to halo acids, other methionyl residues were also modified with concomitant loss of enzymic activity (Neumann et al., 1962; Stark and Stein, 1964). We assume that the relatively low activity of immobilized ribonuclease resulted from the participation of other methionyl residues in covalent linking, in addition to methionine-29. This was further supported by the covalent binding of Ⓟ~NHCOCH$_2$Cl to 1-MetO-ribonuclease, a derivative in which methionine-29 was selectively oxidized. Native lysozyme did not react with Ⓟ~NHCOCH$_2$Cl, indicating that both its methionyl residues were buried within the interior of the protein molecule and were therefore unavailable for reaction.

A variety of methods have been devised for covalent linking of proteins to polyacrylamide (Inman, 1974). In all these methods, a large number of side chains are involved in binding, and the specific activity of the bound protein can be adversely affected by establishing too many bonds to each protein molecule. In contrast, if a protein molecule is attached to the matrix by the fewest possible bonds, this will increase the probability that the attached macromolecule will retain its native tertiary structure, and its properties may more clearly resemble those of the native protein in solution (Cuatrecasas, 1969).

In the present study, we have demonstrated a way to covalently attach proteins under controlled conditions where a small number of side chains participate in binding of the protein molecule to the polymeric matrix. The number of methionyl residues involved in binding was determined. Sites of linkage may be located as well, by quantitative end-group analysis of the new amino-terminal amino acids formed on removal of the protein by cleavage at methionyl residues (Y. Shechter, M. Rubinstein, and A. Patchornik, in preparation).

Acknowledgment

We thank Dr. Yair Degani for his interest and very helpful suggestions and discussions.

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Primary Structure of Rat Lysozyme†

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ABSTRACT: For evolutionary reasons, we determined the primary structure of rat lysozyme. The chymotryptic peptides from the reduced and carboxymethylated protein were sequenced and aligned by homology with the sequence of human lysozyme. Overlaps were confirmed by partial structures of tryptic peptides and an automatic sequencer run on the whole protein. By comparing this lysozyme sequence with those of human and baboon and taking into account paleontological estimates of the times of divergence of these species from one another, an approximate estimate of the average rate of lysozyme evolution was made. This rate is not significantly different from the average rate of lactalbumin evolution in mammals—a finding which is at variance with Dickerson's [Dickerson, R. E. (1971), *J. Mol. Evol. 1,* 26] and Dayhoff's [Dayhoff, M. O., Ed. (1972), Atlas of Protein Structure and Sequence, Vol. 5, Silver Spring, Md., The National Biomedical Research Foundation] conclusion that lactalbumin evolution has been faster than lysozyme evolution. Our finding raises the possibility that the gene duplication event responsible for the origin of lactalbumin from lysozyme was more ancient than is generally supposed. Furthermore, from comparison of the rates of lysozyme evolution in rodents and primates, it is suggested that generation time is not a key factor in lysozyme evolution.

To understand the mechanism of evolution, it is essential to study the rates at which evolutionary change has taken place in the sequences of macromolecules. It is important from this point of view to examine cases in which a protein has undergone a radical evolutionary change in biological function. Does the amino acid sequence evolve much faster than normal under these conditions and, if so, by how much? By focusing on this question, one may gain a better understanding of the driving force for sequence change in proteins. Lysozyme, a bacteriolytic enzyme, and lactalbumin, a regulator of lactose synthesis, provide an opportunity to study this problem. Sequence evidence indicates that the lactalbumin gene arose by duplication of the gene for lysozyme (Hill and Brew, 1975). The claim that the lactalbumin gene arose at the outset of mammalian evolution and subsequently experienced accelerated sequence evolution has been widely accepted (Dickerson, 1971; Hood et al., 1975; Florkin, 1975). However, an alternative model, consistent with similar rates of change in the two proteins since their divergence, suggests that the duplication event may be much more ancient.

Support for the recent divergence model and a speed-up in lactalbumin evolution originally came from the observation that the rate of sequence change in mammalian lactalbumins was two or three times that of bird lysozymes (Dayhoff, 1972). However, later immunological work indicated the rate of lysozyme evolution in mammals was more rapid than in birds (Hanke et al., 1973). To check on this point we decided to sequence rat lysozyme. By comparing the rat sequence with that of other mammalian lysozymes and considering the probable times of divergence involved, we estimated that the average rate of lysozyme evolution among mammals is comparable to

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