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Immobilization of Proteins as a Tool for Studying Primary Structure around Their Cysteinyl Residues

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Abstract

The primary structure around the single cysteinyl residue of chicken pepsin was investigated by binding the protein via this residue to an insoluble carrier. Carriers stable towards reagents used for the fragmentation of proteins and sequence analysis were prepared by coupling a spacer arm to poly *N*-hydroxymethyl acrylamide using a thioether bond that is potentially cleavable by mercuric ions (*1*). Phenacyl bromide group, attached to the free end of the spacer, reacted rapidly and specifically with the cysteinyl residue of chicken pepsin. Up to 300 mg of the enzyme were bound to 1 g of carrier.

The polymer-bound protein was cleaved by trypsin or by cyanogen bromide or by a sequence of both. Fragments of 40–120 amino acid residues, depending on the method of cleavage, remained attached to the polymer through the cysteinyl residue. The compositions and partial sequences of these fragments revealed that the cysteinyl residue is located within or in the vicinity of a loop in the molecule formed by a disulfide bond.

Index Entries Immobilization, of proteins; protein, immobilization of; primary structure, of proteins; structure, primary, of proteins; cysteinyl residue, of immobilized proteins.

Introduction

Chicken pepsin is unique among the acid proteases in that it contains a free sulfhydryl group (*2*). Though this group does not participate directly in the catalytic activity, it has been observed that the chemical modification of the SH can en-

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hance markedly the catalytic efficacy of this enzyme (*3*). As part of structural studies on the region of the protein molecule containing the cysteinyl residue, we decided to attach the enzyme via its SH to an insoluble carrier, on the assumption that subsequent cleavages of the bound protein will directly yield the pepties containing the cysteinyl residue of interest.

Attachment to insoluble carriers by disulfide bond formation (*4*) has been used for the isolation and purification of cysteine-containing proteins and peptides (*5*). The ease of cleavage of the disulfide link by reduction is a major advantage in isolation procedures, but it rules out the use of this bond for structural studies, which usually necessitate reduction and carboxymethylation of the protein. To enable structural investigations on an immobilized protein, the insoluble carrier and the linking arm must be inert towards the chemical and enzymic reactions routinely employed in these studies. Furthermore, the matrix to be employed must have a high porosity and swell in aqueous media in order to ensure access of the protein to be bound as well as of the enzymes to be used as reagents. A high binding capacity is advantageous for structural studies since it obviates the operational difficulties associated with chemical reactions with large beds of resin. Polyacrylamide beads were chosen for the present investigation since they possess, many of the desirable characteristics (*6*). A thioether bond, potentially cleavable with mercuric ions (*1*), served to link the backbone to a spacer arm, with the expectation–not realized so far in practice–that the detachment and recovery of peptides will be achieved at will. The reactive end of the spacer arm was a phenacyl bromide group, which reacts readily with SH groups under mild conditions.

In the present communication, we describe the tryptic and cyanogen bromide fragments that remained attached to the carrier on cleavage of chicken pepsin immobilized through its SH group. The data provides information on the overall structure of a domain of the molecule about 160 amino acid residues long that contains the cysteinyl group, as well as one disulfide loop.

Materials and Methods

Materials

Chicken pepsin was purified (*7*) from a commercial preparation obtained from Development and Production of Enzymes Co. (Emek-Hefer, Israel). Monellin was a gift from Dr. N. Weinshanker (Dynapol, Palo Alto, Cal., USA), and its individual chains were separated as described (*8*).

The following commerical materials were used: Polyacrylamide beads (Biogel P-150, Biorad, Cal., USA), soluble polyacrylamide (mw 500 kD, Polysciences Inc., USA), water-soluble carbodiimide (EDC·HCl, Sigma, USA), trypsin (TPCK treated), carboxypeptidases A and B (Worthington, Freehold, NJ, USA). Other chemicals were reagent grade. The following commerical reagents were recrystallized before use: chromotropic acid (*9*) and *N*-hydroxymethyl acrylamide (*10*).

All glassware that came in contact with the resins was siliconized with siliclad (Clay Adams, NJ, USA). Laboratory ware made of polyethylene (Belart, Pequannock, NJ, USA) was used whenever possible.

Synthetic Procedures

N-hydroxymethylation of polyacrylamide (I). Polyacrylamide beads were suspended in water and left to swell overnight. Fifty grams of swollen resin (4 g dry matter, 60 mmol amide groups) were suspended in 100 mL water, and 5.25 mL of 38% formaldehyde (70 mmol) were added. The suspension was brought to pH 9.5 with 0.1 *N* NaOH and stirred 3 h at 50°C, adjusting the pH to 9.5 every 30 min. The resin ws recovered by filtration, dried by successive washing with 25%, 50%, and 100% methanol, and kept in vacuum in a desiccator over P+d22O_5.

The product was found (see below) to contain 2.0 mmol *N*-hydroxymethyl groups/g dry matter. Several repetitions of this preparative procedure yielded products with 2.1 ± 0.3 mmol *N*-hydroxymethyl groups/g.

Soluble polyacrylamide (mw 500 kD) was reacted with formaldehyde under identical conditions, then dialyzed for three days against distilled water. The product contained 4.9 mmol *N*-hydroxymethyl groups/g dry matter.

Binding of cysteamine to poly N-hydroxymethyl acrylamide beads (II). To a suspension of 3.5 g of the dry hydroxmethylated derivatve (7.3 mmol *N*-hydroxymethyl groups) in 60 mL 0.3 *M* HCl was added 1 g of cysteamine hydrochloride (88 mmol) and the pH was adjusted to 0.5 with conc. HCl. The suspension was stirred for 18 h at room temperature. The resin was washed on a sintered glass filter with 0.3 *M* HCl, then with H_2O until no amine was detected [TNBS test (*11*)] in the washing. The resin was suspended and stirred overnight in 0.5 *M* Na_2SO_3 to decompose unreacted *N*-hydroxymethyl groups then washed extensively with 0.3 *M* HCl and water.

Analysis for sulfur revealed 4.01% S (≡ 1.25 mmol/g) and titration of bound chloride gave 1.24 meg/g.

Binding of other SH compounds to poly *N*-hydroxymethyl acrylamide was carried out under similar experimental conditions. The results are given in the text and summarized in Table 1.

Preparation of p-succinamido phenacyl bromide (IV). *p*-Aminoacetophenone (6.75 g, 50 mmol) was dissolved in molten succinic anhydride (7.5 g, 50 mmol), and kept for 30 min at 200°C in an oil bath. The reaction mixture was poured into water and 1 *N* NaOH as added to bring the pH to 10, whereupon a clear solution was obtained. The *p*-succinamidoacetophenone was precipitated by addition of conc. HCl to pH 3 and recrystallized from boiling absolute ethanol. Equivalent weight (acidimetry), 240 (theory, 235).

p-Succinamidoacetophenone (III) was brominated with one equivalent of Br_2 using the procedure described for the preparation of *p*-acetamidophenacyl bromide (*12*). The *p*-succinylamidophenacyl bromide thus obtained was recrystallized from boiling 95% ethanol. Yield, 63%; Br, 26.57% (theory, 25.44%).

TABLE I
Binding Capacities of Polyacrylamide Derivatives

Carrier	Active group, μmol/g	Bound, μmol/g				
		Cysteine	Cysteamine	Reduced glutathione	Monellin (chain II)	Chicken pepsin
Poly *N*-hydroxymethyl acrylamide	2000	616	1240	438	7.8	0.2
Phenacyl bromide derivative	900	23.2		10.3	12.8	5.7

Attachment of phenacyl bromide side chain to polymer II. Polymer II was washed several times with 100 mL of 0.1*M* NaCl in a mixture of H_2O:DMF (3:1 v/v), and suspended in the same solution to give 0.5 g dry matter in 60 mL suspension. A solution of 0.8 g *p*-succinamidophenacyl bromide (IV) in 5 mL DMF was added followed by 840 mg of solid *N*-ethyl-*N'*-(3-dimethylamino) propylcarbodimide hydrochloride (EDC·HCl). The pH was brought to 4.7 and the suspension was stirred for 16 h at room temperature. The resin was washed with about 100 mL of 0.2 *M* NaCl, water, then successively with 25%, 50%, 100% methanol and oven dried at 60° for 24 h. Analysis revealed 7.01% Br (≡ 0.88 mmol/g).

Immobilization of Chicken Pepsin, and Reactions of the Bound Protein

Binding of chicken pepsin to the phenyacyl bromide containing resin. To a solution of 230 mg chicken pepsin in 14 mL 0.1*M* phosphate buffer, pH 7.2, was added 230 mg of dry resin. The suspension was mixed slowly by tumbling.

The progress of the reaction was monitored by stopping the agitation, withdrawing an aliquot (25–50 μL) of the clear liquid and determination of A280 after dilution with 1 mL 0.02*N* HCl.

After 20 min of reaction, the solid was collected by filtration, and washed consecutively with 0.1*M* phosphate buffer, pH 7.2, 0.2*M* NaCl, and 0.03*M* HCl.

Amino acid analysis after acid hydrolysis showed that 250–270 mg of protein were bound to 1 g of carrier, based on the known composition of the protein (2).

The bound enzyme was usually processed immediately after preparation. When samples of bound enzyme had to be stored, they were suspended in 0.03*M* HCl and kept at 4°C.

Reduction and carboxymethylation. Wet filter cake containing 1.5 μmol of bound enzyme was suspended in a solution of 8*M* urea in 0.35*M* Tris·HCl buffer, pH 8.6 in a 15 mL polyethylene vial and 0.5 mL of β-mercaptoethanol was added. The suspension was flushed with N_2, and the vial was capped and tumbled for 4 h at room temperature. The mixture was filtered on a sintered glass filter and the cake

was washed with buffered deaerated 8*M* urea, care being taken to leave some liquid over the cake throughout this operation.

The cake was resuspended in 10 mL of 8*M* urea in 0.35*M* Tris·HCl buffer, pH 8.0, and 0.5 mL of 1*M* sodium iodoacetate was added. After agitation for 30 min, the solid was filtered off, and washed successively with buffered 8*M* urea, 0.2*M* NaCl, and finally with 0.03*M* HCl.

Tryptic digestion, CNBr cleavage, and end group determination of bound proteins and peptides. These reactions were carried out by following precisely the same experimental procedures used for the degradation of soluble proteins (*13, 14*). The reaction mixtures were made up to contain 10 mg of dry matter (corresponding to about 0.06 μmol bound protein or peptide)/mL reaction mixture. For enzymic degradations the substrate-to-enzyme weight ratio was in the range of 1:20 to 1:30.

Carboxypeptidase digests were analyzed after 2, 4, and 16 h of incubation. The terminal amino acids were obtained in 90 ± 10% yield and the order of the terminal 2–3 residues was readily established from the order of their appearances in the digest. the cyanogen bromide fragments yielded, on action with carboxypeptidase A, nearly 0.5 equivalents of each homoserine and homoserine lactone.

Sequential Edman degradations were carried out manually by the procedure described for solid-phase sequencing (*15*).

Analytical Methods

Determination of N-hydroxymethyl groups. *N*-hydroxymethyl groups were determined by reaction with chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid) as described for the determination of free formaldehyde (9). This reaction is carrid out in strong acid where *N*-hydroxymethyl groups are unstable (*16*). Preliminary experiments with *N*-hydroxymethylacrylamide showed (Fig. 1) that the release of formaldehyde and its reaction with the chromotropic acid can be achieved simultaneously. Solutions or suspensions of poly *N*-hydroxymethyl derivatives containing 0.3–2.5 μmol methylol groups in 0.5 mL H_2O were mixed with 0.5 mL 10% chromotropic acid in H_2O, conc. H_2SO_4, 5 mL, was added with vigorous shaking, and the mixture was kept in boiling water for 30 min. The mixture was cooled, diluted with H_2O to 50 mL, and the absorbance at 570 nm was determined. Parent polyacrylamide served as blank.

Amino acid analysis. Polymer bound proteins and peptides were hydrolyzed with 6*N* HCl in sealed evacuated tubes at 110° for 24 h. Solids were removed by filtration on fine sintered glass filters and washed with 6N HCl. The combined filtrate and washings were evaporated to dryness and the residue analyzed by standard amino acid analysis procedures (Beckman Autoanalyzer model 121).

The amino acid composition reported does not include tyrosine, tryptophan, and histidine. Tyrosine was not detected in the hydrolyzates, in accord with another report (*17*) that it is destroyed on acid hydrolysis of tyrosyl peptides bound to

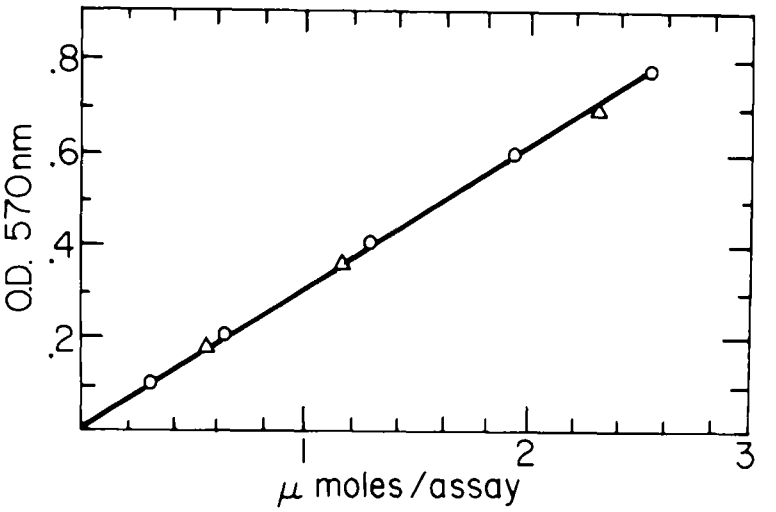

Fig. 1. Determination of *N*-hydroxymethyl groups with chromotropic acid. The procedure for the determination of formaldehyde (*9*) was followed using fomaldehyde (○) or *N*-hydroxymethyl acrylamide (△).

polyacrylamide beads. Histidine could not be determined accurately because it was poorly resolved on the analyzer from the large amounts of ammonia released from the resin on acid hydrolysis.

Amino acid analyses were also used to follow the binding of cysteine and cysteinyl peptides through their SH group to poly *N*-hydroxymethyl acrylamide. In accord with published data obtained with low molecular weight compounds (*18*), it was found that cysteine bound to poly *N*-hydroxymethyl acrylamide is converted quantitatively on acid hydrolysis to thioproline. Thioproline chromatographed in the position of threonine, and was readily determined after calibration with an authentic sample.

Results and Discussion

Preparation and Properties of Supports Based on Poly N-Hydroxmethyl Acrylamide

The synthesis of the support employed in the present investigation is summarized in Fig. 2. The key reactions in this procedure are: (i) the reaction of the amide groups with formaldehyde to form *N*-hydroxymethyl acrylamide and (ii) the attachment of mercaptans to the latter polymer. Both reactions are known to proceed well with low molecular weight compounds (*1*, *19*) and their usefulness for the preparation of functional derivatives of poly-acrylamide ws therefore investigated.

The progress of the reaction between formaldehyde and the amide groups of cross linked polyacrylamide beads is shown in Fig. 3. About 15% of the amide groups reacted in 3 h, and further reaction was very sluggish. For comparison we reacted a linear, non-crosslinked polyacrylamide of mw 500 kD with formalde-

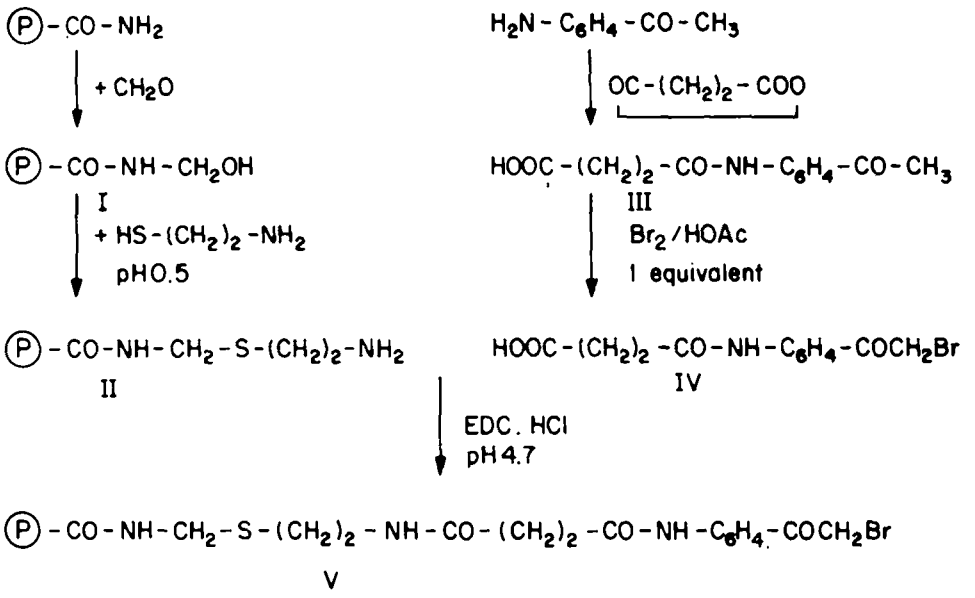

Fig. 2. Preparation of carrier with phenacyl bromide-bearing side arm.

hyde, and found that about 35% of the amide groups were hydroxymethylated in 3 h. The extent of modification of the amide groups in the crosslinked polymer seems therefore to be limited by the matrix. Even so, the poly *N*-hydroxymethyl acrylamide contained 2 meq/g of functional groups, a high level when compared with other carriers used for immobilization of proteins (*20*). The reaction of poly *N*-hydroxymethyl acrylamide beads with sulphydryl compounds at pH 0.5 (*1*) was found to be very slow (Fig. 4) and a day or more were required until half of the hydroxymethyl groups reacted with cysteine or cysteamine. Furthermore, the extent of binding decreased sharply when the cysteinyl residue was part of a long pep-

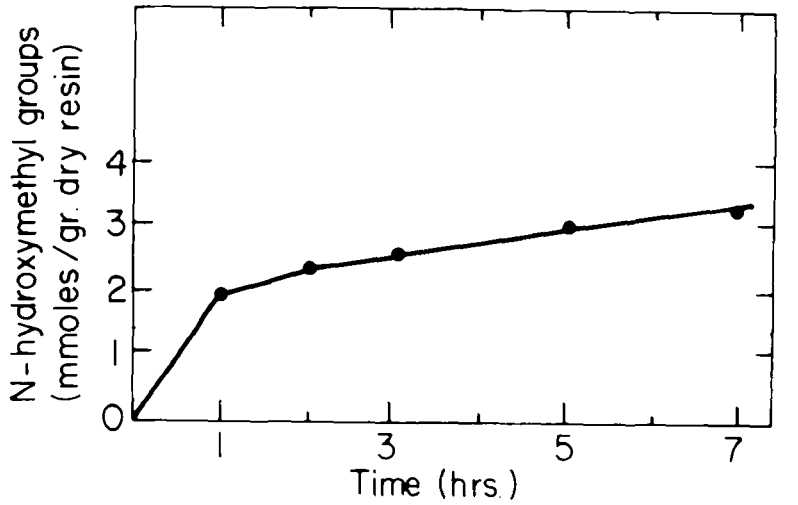

Fig. 3. *N*-hydroxymethylation of Biogel P-150, at pH 9.5 and 50°C.

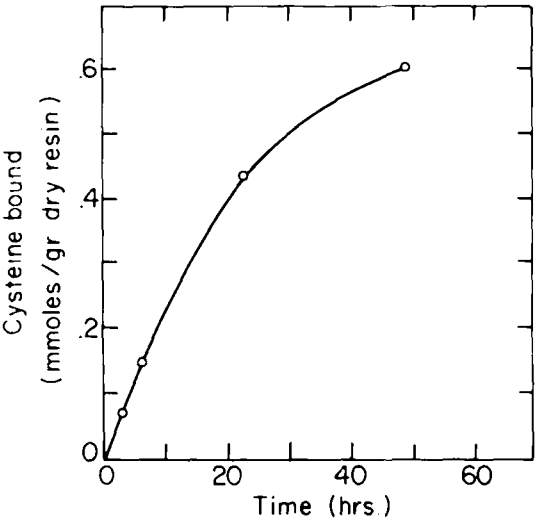

Fig. 4. Binding of cysteine to poly *N*-hydroxymethyl acrylamide. The reaction was carried out at pH 0.5 and its progress was followed by the determination of sulfur bound to the solid.

tide or of a protein (Table 1), suggesting strong steric hindrance by the polymer backbone. It became evident that poly *N*-hydroxymethyl acrylamide, similar to other polymers in which the functional group is near the polymeric backbone, is a poor carrier for the direct binding of peptides and proteins. On the other hand it has a very high capacity for the attachment of low molecular weight sulfhydryl compounds.

In the present investigation we reacted the poly *N*-hydroxymethyl resin with cysteamine, which then served as a part of a spacer arm carrying a phenacyl bromide group at its free end (Fig. 2). Unreacted *N*-hydroxymethyl groups were decomposed by reaction with sulfite (*21*). Starting with a polymer containing 2 meq *N*-hydroxymethyl groups/g dry weight, we obtained a product containing 1.2 meq mercaptoethylamine groups, and binding of the remainder of the side chain yielded a polymer containing 0.9 meq phenacyl bromide groups/g of resin.

Binding of Chicken Pepsin to the Phenacyl Bromide-Containing Carrier

Reaction of the single cysteinyl residue in chicken pepsin with phenacyl bromide has been previously shown (*3*) to be a fast reaction. It was therefore anticipated that the rate of attachment of the enzyme to the phenacyl bromide-containing carrier will be determined by the rates of mass transfer into and inside the carrier beads. To accelerate this process, the binding experiments were carried out by adding dry carrier to the enzyme solution, based on the assumption that swelling will create a flux of liquid into the beads and this will sweep the enzyme molecules into the polymer matrix. The results (Fig. 5) show that disappearance of chicken pepsin from the solution was indeed rapid and was virtually complete in 10–20 min. The solids were then washed extensively to remove physically adsorbed protein, and

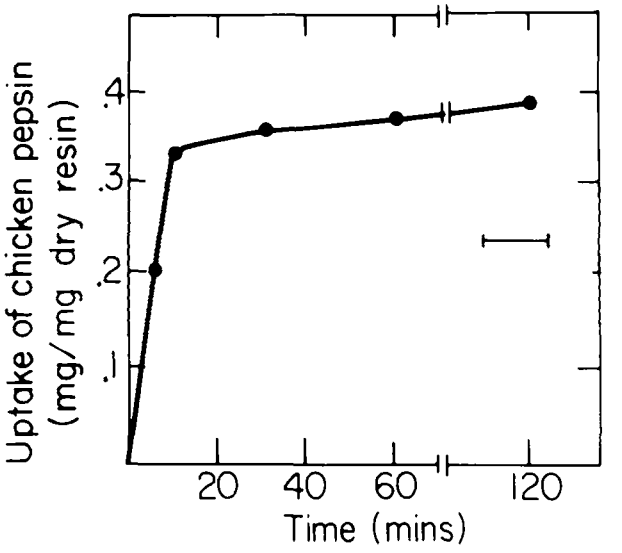

Fig. 5. Time course of the uptake of chicken pepsin by the carrier. Uptake was computed from the decrease of A280 of the solution. The value marked (|—|) was computed from amino acid content after extensive washing of the bound protein.

submitted to amino acid analysis after acid hydrolysis. It was found that about two thirds of the protein taken up by the resin were bound chemically to the matrix. To determine the binding capacity of the carrier, it was challenged with solutions containing increasing concentrations of enzyme, whereupon up to 360 mg of protein were bound chemically to 1 g of carrier (Fig. 6). It should be pointed out that the reaction conditions used here were designed to force a high extent and rapid binding, while sacrificing yield, and only 20–50% of the enzyme presented to the carrier

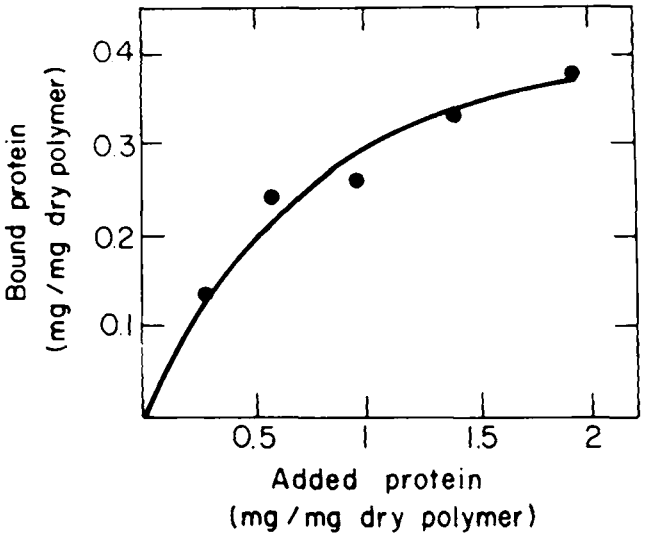

Fig. 6. Determination of the binding capacity of the carrier by challenging with increasing concentration of chicken pepsin. The amount of chemically bound protein was computed from the amino acid content after extensive washing.

was bound (Fig. 6). If the yield of bound enzyme is important, one could reuse the leftover enzyme for a second binding run.

The specificity of binding was tested by reacting the phenacyl bromide containing resin with an amino acid mixture (amino acid analyzer standard, not including cysteine) under conditions identical to those used for the binding of the enzyme. Analysis revealed that all the amino acids were fully (±5%) recovered by washing, indicating that none was bound chemically to the resin. To test recovery after acid hydrolysis the phenacyl bromide resin was reacted with reduced glutathione. Acid hydrolysis of the product yielded glycine and glutamic acid in amounts equimolar (±2%) to that of the glutathione which had been bound.

Studies of the Primary Structure of Bound Chicken Pepsin

Structural studies were carried out with preparations containing about 200 mg protein/g of solids, which swelled to a cake of about 10 mL. A higher loading density was avoided since it might make the particles impermeable to reagents and enzymes. Freshly immobilized chicken pepsin preparations were steeped in a solution of 0.5*M* mercaptoethanol in 8*M* urea to reduce the disulfide bridges of the enzyme and simultaneously to block unreacted phenacyl bromide groups on the polymer. The mercaptoethanol was washed out and the solid was reacted with 50 m*M* iodoacetic acid. The reduced carboxymethylated bound enzyme yielded on acid hydrolysis 5.8 (theory 6) mol carboxymethyl cysteine/mol bound protein, indicating that reduction and carboxymethylation were complete.

Immobilized reduced and carboxymethylated chicken pepsin was cleaved with cyanogen bromide and the fragments released were washed out. A peptide containing close to 80 amino acid residues remained attached to the carrier (Table 2). The peptide contained two carboxymethyl cysteine residues, raising the possibility that it included a disulfide loop of the protein. Cyanogen bromide cleavage was therefore carried out on a sample of unreduced bound protein. The amino acid composition and terminal residues of the fragment that remained bound to the carrier were identical to those obtained after reduction and carboxymethylation. Furthermore, when this fragment was reduced and carboxymethylated, it showed two carboxymethyl cysteine residues, but no other change in composition. It was thus established that the cyanogen bromide fragment of chicken pepsin that contains its free SH group also contains a disulfide loop.

Tryptic cleavage of the reduced and carboxymethylated immobilized protein left attached to the polymer a long fragment that contained over 117 amino acids residues, including two lysyl and two arginyl residues (Table 2). This was not changed by repeated digestion with trypsin. Taken together with the finding of single N- and C-terminal residues (Table 2), the data suggested that the product of tryptic cleavage is a single peptide, and not a mixture resulting from partial cleavages. The arginyl and lysyl residues in the interior of this tryptic peptide are presumably followed by prolyl residues. The tryptic peptide also contained two methionyl residues, indicating that it spans at least one cyanogen bromide cleavage point.

Consecutive cleavage by cyanogen bromide and trypsin was carried out in this and in reverse order. In both cases a peptide of about 40 amino acids remained

TABLE 2
Composition and Terminals of Peptides that Remain Bound to the Carrier after Cleavage of Immobilized Chicken Pepsin

	Cleavage method					
	Trypsin		CNBr		Combined	
Amino acid	Residue mole found	Integer	Residue mole found	Integer	Residue/mole found	Integer
Lys	2.4	2	2.3	2	0.9	1
Arg	1.7	2	1.2	1	1.1	1
CMC	1.1	1	1.9	2	0.7	1
Asp	13.4	13	11.4	11	4.3	4
Thr	9.8	10	8.2	8	3.8	4
Ser	14.6	15	11.0	11	4.8	5
Glu	10.9	11	6.1	6	3.3	3
Pro	6.6	7	4.8	5	2.9	3
Gly	13.6	13	9.4	9	4.4	4
Ala	7.7	8	3.9	4	2.2	2
Val	9.2	9	5.8	6	3.1	3
Met	2.3	2	-			—
Ile	8.2	8	4.2	4	2.6	3
Leu	10.4	10	5.1	5	3.4	3
Phe	6.2	6	4.4	4	1.9	2
Total		117		78		39
N-terminus	Leu		Asp-Trp-Val-		Asp	
C-terminus	Gly-Lys-OH		Glu-Met-OH		-Gly-Lys-OH	

bound to the carrier (Table 2). The products had the same amino acid composition and terminal residues irrespective of the order of cleavages.

The terminal amino acid residues of the bound proteins and peptides were determined by the dansyl methods (*22*) and with carboxypeptidases A and B (*14*). No problems were encountered in applying these methods to the immobilized substrates. The data obtained for the carrier-bound fragments are collected in Table 2.

It is pertinent to mention here some experiments that proved unproductive. Attempts to liberate the bound peptides by cleavage of the thioether bond, connecting the side arm to the polymer backbone, were carried out with various concentration of mercuric ions, in acetate buffer pH 4.5 (*1*) and in solutions of acetic acid from 0.1 to 5 *M*. The resin was then washed copiously and submitted to amino acid analysis. The results were erratic and it was found that the cleavage was partial (10–50%), and even that required high concentrations of mercuric ions of 50–100 m*M*. Furthermore, the released peptides could not be separated at any reasonable yield from the mercuric ions in solutions. Sequential Edman degradation was attempted using peptide of known sequence (chain II of monellin) bound to the phenacyl bromide resin. The chemical yield was only 50%, and the repetitive yield was only 70% with relatively high (30–40%) carryover. It was observed that the resin beads collapsed irreversibly in the organic solvent used in the Edman proce-

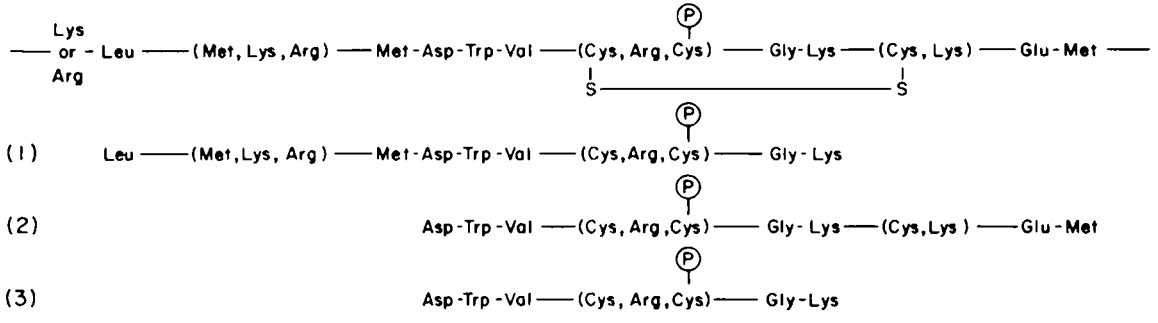

Fig. 7. Tentative structure of the cysteinyl-containing domain of the chicken pepsin molecule. (1) Product obtained on cleavage with trypsin; (2) product obtained on cleavage with CNBr (3); product obtained with trypsin and cyanogen bromide.

dure. This could block access of reagents to the entrapped peptides. Reliable results could thus be expected for no more than 2–3 cycles, and sequential degradation was carried out only on the cyanogen bromide cleavage product. The *N*-terminal sequence obtained Asp-Trp-Val- was in agreement with the finding of Asx as *N*-terminal by the dansyl method.

Figure 7 summarizes the information obtained in the present investigation on the structure of the region containing the single sulfhydryl group in chicken pepsin. The scheme was constructed from the amino acid compositions and terminal residues of all fragments that remained bound to the carrier. The ordering of the various fragmentation points is the only one that fits the experimental findings. The results show that the region of the chicken pepsin molecule investigated in this study is a stretch of about 160 amino acids comprising about a half of the polypeptide backbone of the enzyme. This part of the enzyme molecule contains one disulfide loop in addition to the free sulfydryl group, and it appears that the single cysteinyl residue is located either within this loop or in its vicinity.

The results presented above demonstrate the feasibility of structural studies on a protein immobilized through a specific amino acid residue. Though the techniques used so far failed to give the full sequences of the bound peptides, they readily provided data for the ordering of various cleavage products of the protein and enabled the construction of an overall picture of a large part of the chicken pepsin molecule.

A carrier designed to bind to SH groups was used in the present investigation. Obviously carriers can be designed to bind to active sites of enzymes or other unique points in a protein in order to study the structure of preselected regions of the protein molecule.

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