Fruton, J. S. (1970), *Advan. Enzymol. 33*, 401.
Hanson, K. R., Ling, R., and Havir, E. (1967), *Biochem. Biophys. Res. Commun. 29*, 194.
Hollands, T. R., and Fruton, J. S. (1968), *Biochemistry 7*, 2045.
Hollands, T. R., Voynick, I. M., and Fruton, J. S. (1969), *Biochemistry 8*, 575.
Ingles, D. W., and Knowles, J. R. (1967), *Biochem. J. 104*, 369.
Inouye, K., and Fruton, J. S. (1967), *Biochemistry 6*, 1765.
Jackson, W. T., Schlamowitz, M., and Shaw, A. (1965), *Biochemistry 4*, 1537.
Koshland, D. E., and Neet, K. E. (1968), *Annu. Rev. Biochem. 37*, 359.
Medzihradszky, K., Voynick, I. M., Medzihradszky-Schweiger, H., and Fruton, J. S. (1970), *Biochemistry 9*, 1154.
Ottesen, M., Johansen, J. T., and Svendsen, I. (1970), *in* Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, H., and Ottesen, M., Ed., Copenhagen, Munksgaard, p 175.
Sachdev, G. P., and Fruton, J. S. (1969), *Biochemistry 8*, 4231.

Fragmentation of Bovine Chymotrypsinogen A and Chymotrypsin Aα. Specific Cleavage at Arginine and Methionine Residues and Separation of Peptides, Including B and C Chains of Chymotrypsin*

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ABSTRACT: Chymotrypsinogen A and chymotrypsin Aα have been quantitatively degraded and separated into homogeneous peptide fragments utilizing simple aqueous reaction and solvent systems. Succinylation both solubilized the peptide derivatives and blocked the lysine residues to the action of trypsin. Disulfide bonds were cleaved by reduction with dithiothreitol and the newly formed cysteine residues alkylated with iodoacetamide. Specific trypsin cleavage at three arginine residues of the chymotrypsinogen derivative produced four pure peptides containing amino acid residues 1–15, 16–145, 146–230, and 231–245, respectively. Under the chosen conditions, no significant cleavage occurred at the fourth arginine residue, Arg 154. Size and solubility differences among the peptides allowed their complete separation and isolation in high yield by gel filtration on Sephadex columns in 0.01 M ammonium acetate. The succinylated, reduced, and carboxamidomethylated B and C chains (131 and 97 amino acids, respectively) of chymotrypsin Aα were isolated similarly except that no trypsin step was employed. Succinylated and carboxamidomethylated chymotrypsinogen was treated with cyanogen bromide to produce additional, and different, peptide fragments. By these methods many amino acid residues which have a role in enzyme function or which exhibit chemical reactivity can be readily segregated in unique peptide fragments.

Amino acid analyses of the individual peptides were in complete agreement with present knowledge of the sequences of chymotrypsinogen A and chymotrypsin Aα.

The characterization of chemically modified bovine chymotrypsinogen A and chymotrypsins A is hampered by the insolubility of the denatured protein and the tendency of large fragments to aggregate and precipitate. For example, upon digestion of the zymogen with trypsin over two-thirds of the protein is found in an insoluble aggregate, sometimes called the "core" (Hartley, 1964). For purposes of sequence analysis, various investigators have used methods of separation based on ion-exchange chromatography in 8 M urea after conversion of the protein into the oxidized, *S*-sulfo, *S*-carboxymethyl, or *S*-aminoethyl derivative (Hartley, 1963; van Hoang *et al.*, 1963; Meloun *et al.*, 1966, 1967). Separation of the B and C chains of *S*-sulfochymotrypsin Aα may also be achieved by fractional precipitation from solutions containing 7 M urea and 0.035 M sodium dodecyl sulfate (Richmond, 1966). Chymotryptic digestion of *S*-carboxymethylchymotrypsinogen leaves only 10% of the protein in the form of insoluble peptides (Kluh *et al.*, 1966). Most of the numerous soluble peptides are small, however, and quantitative recovery and identification would be exceedingly difficult. In all of these applications, difficulties resulting from aggregation have been only partially overcome and recovery of all portions of the protein has been far from quantitative. Burkhardt and Wilcox (1967) obtained a quantitative separation of fragments of the *S*-aminoethyl derivative of chymotrypsinogen

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on columns of Sephadex by using aqueous hexafluoroacetone as solvent.

This paper describes a different approach for the fragmentation and quantitative recovery of peptides from chymotrypsinogen A and chymotrypsins A. Soluble derivatives were achieved by exhaustive succinylation and conversion of the half-cystine residues to *S*-carboxamidomethylcysteine. Specific SCAM-peptides[1] could then be obtained by trypsin cleavage and these could be separated by Sephadex gel filtration in simple aqueous systems near pH 7.

Strategy employed for the formation of specific peptides is indicated in Figure 1. The primary structure (Hartley, 1964; Hartley and Kauffman, 1966) of the zymogen contains arginine residues at positions 15, 145, 154, and 230. Arginine 154 is resistant to tryptic attack and has therefore been omitted from Figure 1. Quantitative cleavage of peptide bonds at the susceptible positions and modification of the disulfide bridges yielded four specific peptides containing, respectively, 15, 130, 85, and 15 amino acid residues. These soluble peptides could then be separated by Sephadex filtration on the basis of molecular size and amino acid composition.[2] The lower portion of Figure 1 shows the three peptide chains which occur naturally in chymotrypsin Aα and Aγ.[3] The A, B, and C chains, respectively, contain 13, 131, and 97 residues. Upon disulfide modification these peptides could be readily separated on Sephadex provided that aggregation and insolubility were avoided.

Methods reported in this paper, and extensions of them, should facilitate chemical studies of bovine chymotrypsinogen A and chymotrypsins A. The approach may be particularly beneficial now that the structures of the enzymes (Aα, Sigler *et al.*, 1968; Aγ, Davies *et al.*, 1969) and of the zymogen (Freer *et al.*, 1970) have been determined at high resolution by X-ray crystallography.

Experimental Section

Materials. Five times recrystallized bovine pancreatic chymotrypsinogen A was a gift from Princeton Laboratories, Inc. (Princeton, N. J.). In order to reduce the chymotryptic activity to less than 0.2% the protein was recrystallized once more from ammonium sulfate containing phenylmethanesulfonyl fluoride.

Twice crystallized DIP-chymotrypsin Aα (Jansen *et al.*, 1949) was prepared from three times crystallized chymotrypsin Aα (Code CDI, Worthington Biochemical Corp.).

Twice crystallized trypsin (Code TRL, Worthington) was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone according to Carpenter (1967) and stored as a lyophilized powder.

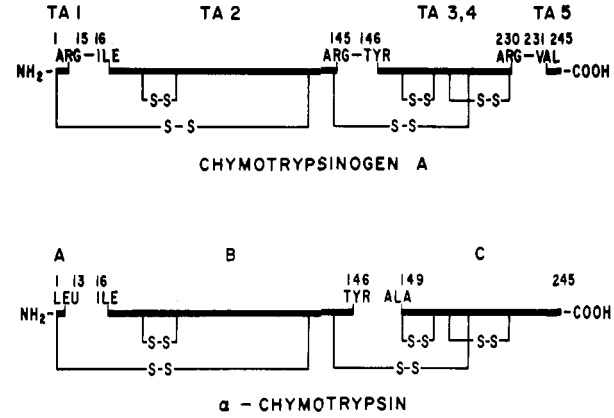

FIGURE 1: Representation of the primary structure of chymotrypsinogen A and chymotrypsin Aα. TA1, TA2, TA3,4, and TA5 refer to peptide fragments produced by trypsin hydrolysis of SCAM-chymotrypsinogen. The lower diagram shows the A, B, and C peptide chains naturally present in DIP-chymotrypsin Aα.

Soybean trypsin inhibitor (Code SI) and porcine carboxypeptidase B (Code COBC) were obtained from Worthington.

Succinic anhydride (Eastman), dithiothreitol (Cyclo Chemical Corp.), guanidine hydrochloride (Mann, ultra pure), and cyanogen bromide (Eastman) were used without further purification. Iodoacetamide (Mann) was recrystallized from cyclohexane and stored in the dark at −40° before use. Sephadex G-100 and G-25 were supplied by Pharmacia. A stock solution of DFP was prepared and stored as a 0.11 M solution in 2-propanol. All other chemicals were of reagent grade. Distilled and deionized water was used throughout.

Methods

Table I lists the steps followed for the preparation of peptide fragments from chymotrypsinogen and α-chymotrypsin. Two

TABLE I: Procedural Steps for the Preparation of Peptide Fragments from Chymotrypsinogen and DIP-chymotrypsin Aα.

	Scheme A	Scheme B
Step 1	Succinylation (6 M Gd·HCl) Dialysis Lyophilization	Succinylation (H_2O) Precipitation
Step 2	Reduction	Reduction
Step 3	Carboxamidomethylation Dialysis	Carboxamidomethylation Dialysis Lyophilization[b]
Step 4		Second succinylation[b,c] Dialysis
Step 5	Trypsin hydrolysis[a]	Trypsin hydrolysis[a]
Step 6	Gel filtration	Gel filtration

[a] Omit for DIP-chymotrypsin Aα. [b] The second succinylation is omitted if step 3 does not include lyophilization. [c] The second succinylation is obligatory with chymotrypsinogen in Scheme B.

[1] Abbreviations used are: SCAM, succinyl-*S*-carboxamidomethyl; CM-cysteine, *S*-carboxymethylcysteine; CPB, carboxypeptidase B; Gd·HCl, guanidine hydrochloride; DIP, diisopropylphosphoryl; DFP, diisopropyl phosphofluoridate; N-terminal, amino terminal; C-terminal, carboxyl terminal. The peptides produced by tryptic cleavage at arginine are designated TA1, TA2, etc., starting from the N-terminal of the molecule.

[2] Peptides TA1 and TA5, although of equal size, were separable on Sephadex due to the presence of tryptophan residue 237 in TA5. Peptide TA1 contained no aromatic residues (see Table IV).

[3] Although the conformation of Aγ is different from that of Aα, the covalent structures of the two forms are identical (Wright *et al.*, 1968; Matthews *et al.*, 1968).

general procedures were developed, the scheme of choice being determined primarily by the presence or absence of 6 M Gd·HCl in the initial succinylation reaction. All other reactions in both schemes were performed in water. The schemes are general and either may be used to produce fragments from chymotrypsinogen or α-chymotrypsin. Each step is described in detail in the following paragraphs.

Succinylation. STEP 1. Protein (500 mg, 20 μmoles) was dissolved in 30 ml of 3 mM HCl at 18°, or in 30 ml of 6 M Gd·HCl at pH 3.0. The temperature was maintained at 18° in the jacketed reaction vessel by means of a Haake circulating water bath. DFP (55 μmoles in 0.5 ml of 2-propanol) was added and the solution was titrated to pH 8 with 0.5 N NaOH. During the addition of each portion of powdered succinic anhydride (84 mg, 840 μmoles, threefold excess over lysine), the pH was held constant through automatic delivery of 5 N NaOH with a Radiometer pH-Stat type TTT1a in conjunction with an Ole Dich recorder. When the uptake of alkali stopped, another portion of succinic anhydride was added. A total of six additions were made. Thirty minutes after the last addition of succinic anhydride the succinylated protein was isolated according to Scheme A or Scheme B.

In Scheme A the reaction solution was dialyzed 24 hr in 10^{-4} M $NaHCO_3$ at 4° and stored in the freezer after lyophilization.

In Scheme B the succinylated protein was precipitated by the addition of 6 N HCl to pH 3. The preparation was centrifuged 10 min at 12,000 rpm in a Sorvall centrifuge equipped with an SS34 head. The pellet was washed once in 3 mM HCl and centrifuged a second time before proceeding to the next step.

Second Succinylation. STEP 4. A second succinylation step is called for in Scheme B. These reactions were carried out as above with the exception that one-half the total amount of succinic anhydride was used, that is, only three additions were made. In the case of chymotrypsinogen a second succinylation was always carried out on the reduced and alkylated derivative. The product was then dialyzed at 4° for 12 hr against 10^{-4} M $NaHCO_3$ and then for an equal time against water. The dialyzed solution was used directly in step 5. The protein concentration was 5–10 mg per ml.

A second succinylation of DIP-chymotrypsin Aα was carried out only if it had been lyophilized in the previous step. The product was dialyzed at 4° for 24–48 hr against 10^{-4} M $NaHCO_3$ and then against water. The dialyzed solution was used directly in step 6. The protein concentration was 5–10 mg per ml.

Tyrosine residues which reacted with the anhydride were allowed to revert spontaneously to free tyrosine during dialysis against 10^{-4} M $NaHCO_3$ (Riordan and Vallee, 1964; Gounaris and Perlmann, 1967). No other precautions were taken to avoid or reverse the succinylation of aromatic or aliphatic hydroxyl groups.

Completeness of the succinylation reaction was determined through amino acid analysis of C-terminal lysine released by CPB after trypsin hydrolysis (see below).

Reduction of Disulfide Bonds. STEP 2. Reduction of the five disulfide bonds in chymotrypsinogen to thiol groups was performed with dithiothreitol (Cleland, 1964). The succinylated protein was dissolved in 15 ml of water. In Scheme A the lyophilized protein was dissolved directly, and in Scheme B the dropwise addition of 0.5 N NaOH was used to dissolve the pellet. The pH of the dissolved protein was 5.5–6.5. Oxygen-free nitrogen was bubbled through the solution for 30 min prior to the addition of dithiothreitol. Two drops of octanol were added to prevent foaming. Dithiothreitol (154 mg, 1 mmole, tenfold excess over cystine) was added and the solution was manually titrated to pH 9.5 with 0.5 N sodium hydroxide. Nitrogen was passed through the solution for 5 additional minutes after which the container was sealed and stirred 12 hr at 20°.

Carboxyamidomethylation. STEP 3. Alkylation of the reduced protein was carried out in the same vessel in order to minimize exposure to the atmosphere. Iodoacetamide (3.70 g, 20.0 mmoles, 20-fold excess over dithiothreitol) was dissolved in 10 ml of 3 M Tris-HCl at pH 8.0. Care was taken to minimize exposure to light. The iodoacetamide solution was added to the reduced protein, the con ainer was resealed, and the solution was stirred at room temperature. After 10 min the reaction was quenched by the addition of β-mercaptoethanol (2.34 g, 30 mmoles), the pH being maintained near 8 by the manual addition of 5 N NaOH. Thirty minutes later the solution was transferred to dialysis tubing and dialyzed 24–48 hr at 4° against 10^{-4} M $NaHCO_3$. The dialyzed solution was normally used directly in the next step. Amino acid analysis for CM-cysteine, which formed from carboxyamidomethylcysteine during acid hydrolysis, was used to determine the extent of reduction and alkylation of the disulfide bonds. The method of quantitation is indicated below.

Trypsin Hydrolysis. STEP 5. The trypsin step was performed on SCAM-chymotrypsinogen only. A freshly dialyzed protein solution was treated with trypsin in a thermostatted reaction vessel at 10°. The pH was controlled at 7.8 through automatic addition of 0.05 N NaOH by the recording pH-Stat. Protein concentration was 5–10 mg per ml. Hydrolysis was started by the addition of 1% by weight of trypsin which had been freshly dissolved in a small amount of cold water. At 10–12 min, 2% by weight of soybean trypsin inhibitor dissolved in water was added to the reaction vessel. The solution was subjected to step 6 immediately. As described below, the extent and specificity of the trypsin cleavage were determined on aliquots removed from the reaction vessel.

Carboxypeptidase B Digestion. C-Terminal lysine and arginine were determined by amino acid analysis after release by CPB. CPB (0.05 mg) in 5 μl of 0.1 N NaCl was added to a 1-ml aliquot containing 4–8 mg of peptides at pH 7.8 and 37°. The reaction was stopped after 1 hr by addition of 1 ml of 0.1 M HCl, the precipitate was removed by centrifugation, and 1 ml of the supernatant was applied directly to the short column of the amino acid analyzer. The pH did not change during the CPB digestion.

N-Terminal Amino Acid Determination. N-Terminal amino acids in peptides which could be dialyzed without loss were determined using the potassium cyanate method (Stark, 1967). Analyses of small peptides were performed on 0.5-μmole samples in 2 ml of 0.2 N *N*-ethylmorpholine acetate buffer, pH 8.0 at 50°. Potassium cyanate (20 mg) was added and the reaction continued overnight. No urea was included. One milliliter of glacial acetic acid was added to decompose the excess cyanate, the sample was evaporated to dryness, and the carbamylated derivatives were cyclized in the usual manner. An internal standard of 0.5 μmole of norleucine was included. An equal amount of α-aminoisobutyric acid was added prior to the Dowex 50-X2 step in order to detect possible elution

TABLE II: Amino Acid Analyses of SCAM Derivatives of Chymotrypsinogen and DIP-chymotrypsin Aα[a]

Amino Acid	SCAM-Chymotrypsinogen		TA1		TA2		TA3,4		TA5		B Chain[b]		C Chain[b]		CB1		CB2	
	T	*F*	*T*	*F*	*T*	*F*	*T*	*F*	*T*	*F*	*T*	*F*	*T*	*F*	*T*	*F*	*T*	*F*
Lysine	14	13.4	0	0	8	7.5	6	5.6	0	0	8	7.9	6	6.0	12	12.0	2	1.7
Histidine	2	1.7	0	0	1	1.6	0	0	0	0	2	2.0	0	t	2	1.8	0	0
Arginine	4	3.8	1	1.0	1	0.9	2	1.6	0	0	1	0.9	2	1.9	3	2.5	1	0.8
CM-Cysteine	10	9.5	1	1.0	4	4.0	5	5.0	0	0	4	3.9	5	4.9	6	5.6	2	2.2
Aspartic	23	22.1	0	0	13	12.7	8	8.1	2	1.5	13	12.9	9	9.0	19	18.6	4	3.8
Threonine[c]	23	22.5	0	t	13	12.7	8	7.8	2	2.1	13	12.7	9	9.1	17	16.9	6	6.0
Serine	28	26.9	2	2.0	15	14.5	11	10.8	0	0	15	14.6	11	10.8	19	18.5	6	5.7
Glutamic	15	15.0	1	1.0	10	10.2	2	2.3	2	2.1	10	10.3	4	4.2	13	13.4	2	2.3
Proline	9	8.3	2	2.1	3	3.1	4	4.3	0	0	3	3.0	4	4.0	7	5.7	2	2.3
Glycine	23	22.2	2	1.9	11	10.7	10	*10.0*	0	0.1	11	10.7	10	10.1	14	14.1	7	*7.0*
Alanine	22	*22.0*	1	*1.0*	11	*11.0*	7	7.2	3	*3.0*	11	*11.0*	10	9.9	15	15.5	5	5.1
Half-cystine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Valine	23	20.4	2	2.0	13	11.1	5	4.9	3	3.1	13	13.0	8	7.9	15	14.2	7	6.0
Methionine	2	1.8	0	0	0	0	2	1.7	0	t	0	t	2	1.9	0	t	0	0
Isoleucine	10	8.4	1	1.0	6	5.3	3	2.6	0	t	6	5.8	3	3.1	8	7.6	1	1.0
Leucine	19	17.8	2	1.9	9	8.7	6	6.0	2	2.0	9	8.9	8	8.0	15	14.5	4	3.9
Tyrosine	4	3.6	0	0	1	1.1	3	2.7	0	t	2	1.9	2	2.0	3	3.2	1	1.0
Phenylalanine	6	5.6	0	0	6	5.7	0	0.1	0	t	6	6.0	0	0.1	6	6.2	0	0.2
Homoserine and lactone															1	0.8	0	t

[a] Analyses are normalized to an integral value for one of the amino acids, this value being shown in italics. *T* stands for theory based on published sequence data and *F* stands for results found. [b] Average of 12-, 24-, 48-, 72-, 96-hr hydrolyses, except for threonine and serine which were extrapolated to zero time hydrolysis, and isoleucine and valine which are averages from 72- and 96-hr hydrolysates. [c] All threonine and serine values corrected using destruction factors calculated from the analyses of B and C chains.

of free amino acids from the column. Controls were performed on noncarbamylated proteins and peptides.

Gel Filtration. STEP 6. Gel filtration experiments were done at room temperature on 2.5 × 95 cm Pharmacia columns of Sephadex G-100. The columns were eluted with a 20-cm hydrostatic head of 0.01 M ammonium acetate, pH 6.0. Flow rates were 20–30 ml/hr. Effluent absorption at 282 nm was determined on every other fraction with a Beckman DU spectrophotometer. Small peptides were separated on 2.0 × 100 cm columns of Sephadex G-25, medium, eluted with 0.1 M acetic acid. Precautionary measures were necessary, however, to minimize a marked tendency toward aggregation, particularly with the B and C chains of SCAM-DIP-chymotrypsin Aα (see below). After the peaks had been pooled, the peptides were harvested either by lyophilization or by lowering the pH to 3 with 6 N HCl and collecting the precipitate by centrifugation.

Cyanogen Bromide Reaction. CNBr reactions were carried out in 70% formic acid at 20° (Gross, 1967). Protein concentrations were 10 mg/ml. The succinylated protein derivative was first dissolved in 98% formic acid and then diluted to 70% with water. CNBr (180 molar excess over total methionine) was added as the crystalline solid. After 20 hr, 5 volumes of water were added and the resulting precipitate was collected by centrifugation for 15 min at 12,000 rpm. The pellet was redissolved in water with the required amount of 5 N NaOH and subjected to gel filtration on columns of Sephadex G-100.

Amino Acid Analysis. Amino acid analyses of the SCAM-peptides were carried out by the method of Spackman *et al.* (1958) with a Beckman/Spinco Model 120C analyzer equipped with an Infotronics Model CRS-10AB2 digital integrator. Samples weighing about 1 mg were hydrolyzed in 6 N HCl at 110°. A crystal of phenol was added to the sample tubes before they were evacuated and sealed. For analysis of the B and C chains of SCAM-DIP-chymotrypsin Aα, samples were hydrolyzed 12, 24, 48, 72, and 96 hr. Other samples were hydrolyzed in duplicate for 24 or 48 hr. The color value for CM-cysteine, which eluted 5 min previous to aspartic acid, was determined from a commercial sample (Mann) to be 97% that for aspartic acid. Homoserine and homoserine lactone were calculated according to Hoffman (1964).

Results

Modification Reactions. Application of the modification reactions listed in Schemes A and B of Table I resulted in the preparation of specific peptides from SCAM-chymotrypsinogen and SCAM-DIP-chymotrypsin Aα in high yield. The quantitative nature of the reactions was indicated by the specificity of the trypsin step (see below), and by amino acid analysis of the isolated proteins and peptides.

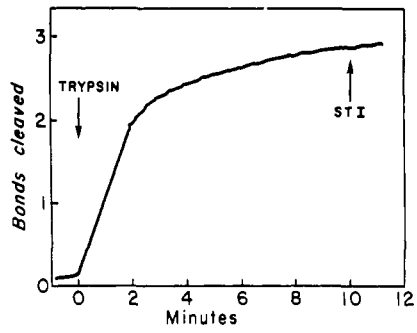

FIGURE 2: Action of trypsin (1:100, wt/wt) on SCAM-chymotrypsinogen (5 mg/ml) in water at 10° and pH 7.8. The ordinate scale is based on an average pK_a of 7.8 for the newly released α-amino groups; STI, soybean trypsin inhibitor.

The amino acid analysis of SCAM-chymotrypsinogen is shown in Table II. Half-cystine was totally absent in the analysis, whereas 9.5 residues of CM-cysteine were found, indicating complete modification of the 10 sulfhydryl groups produced from the reduction of the disulfide bonds. Histidine and methionine were recovered in expected yields suggesting that modification of these potentially reactive sites had been avoided (Gurd, 1967).

Upon amino acid analysis of the succinylated derivatives a new peak was observed which emerged after valine and near the buffer change front of the 60-cm column. The amount of amino acid in the unknown peak was calculated using a ninhydrin color value equal to that of valine. It was found that addition of the micromoles in the unknown peak to the micromoles in the lysine peak gave a sum equal to the amount of total lysine expected from the protein. The unknown peak represented about 15–20% of the total lysine after a 24-hr hydrolysis. Hydrolysis for 72 hr converted essentially 100% of the unknown compound into free lysine. Several experiments were performed with authentic ϵ-*N*-succinylated lysine (K. D. Hapner and R. A. Bradshaw, 1969, unpublished data). The results confirmed the similar observations of Gounaris and Ottesen (1965) which led them to propose that the unknown peak represented the ϵ-*N*-succinimide derivative of lysine. No other differences from analyses of nonsuccinylated proteins were observed. All other amino acids were found in expected yields.

Trypsin Cleavages. Specific cleavage at the arginine residues of SCAM-chymotrypsinogen was accomplished by the action of trypsin. In Figure 2 the course of the trypsin step is shown to result in a very rapid uptake of base upon addition of the enzyme to the modified protein. Approximately two bonds were cleaved during the initial phase of the reaction which lasted 2–3 min. Uptake of base then slowed and approached an equivalent of 3 bonds after 10–12 min. At this point further reaction was inhibited by the addition of soybean trypsin inhibitor.

Maximal specificity was attained by carrying out the reaction at 10°. If higher temperatures were used the extent of cleavage at arginine was proportionally greater; however, additional N-terminal amino acids were then detected suggesting that cleavage of peptide bonds was occurring at positions other than arginine. The effect of allowing the slower phase of the reaction to continue beyond 10–12 min was much the same. Hence, the restricted conditions of 10–12 min reaction time at 10° were selected in order to minimize cleavage at amino acids other than arginine.

TABLE III: New Terminal Amino Acids Detected after Trypsin Hydrolysis of SCAM-Chymotrypsinogen.[a]

Amino Acid	Expected	Found	Background	Adjusted Net Yield
N-Terminal[b]				
Isoleucine	1.0	0.88	.06	.96
Tyrosine	1.0	0.75	.03	.81
Leucine	1.0	0.12	.08	.05
Valine	1.0	0.88	.06	.93
Norleucine[c]	1.0	0.88	0.95	1.0
C-Terminal[d]				
Arginine	4.0	3.3		
Lysine	0	0.05		

[a] Results are expressed as moles of amino acid per mole of protein derivative. [b] N-terminals residues were determined as the difference between the untreated protein (Background) and the trypsin-treated protein (Found). The adjusted net yield was calculated using corrections based on the recovery of the internal standard and the inherent yield of the method (Stark, 1967). No pyrollidone corrections were made and all determinations contained 0.2–0.3 residue of glutamic acid. [c] Internal standard. [d] Carboxypeptidase B hydrolysis and amino acid analysis.

Table III lists the new N-terminal and C-terminal amino acids which were detected after the 10-min trypsin hydrolysis. Isoleucine, tyrosine, and valine were found in high yield, whereas only 5% of 1 mole of leucine was detected. When compared with the data of Figure 2 these results indicated that three peptide bonds had been cleaved, namely those on the carboxyl side of arginine residues 15, 145, and 230 (see Figure 1). The Arg 154–Leu 155 bond was almost totally resistant to the trypsin treatment. An additional check on the arginine specificity of the trypsin step was performed by CPB digestion of the reaction mixture. Since all of the expected N-terminal amino acids were also present in the sequence on the carboxyl side of lysine residues, the possibility existed that their appearance was at least in part the result of trypsin cleavage at lysine rather than at arginine. Results from the CPB digest are also shown in Table III. Very little lysine (0.05 residue per mole of protein) was detected, confirming the arginine specificity of the trypsin reaction. Although the data from N-terminal analysis would suggest about 3 moles of C-terminal arginine, a little more than this amount was found. That the Arg 154–Leu 155 bond was hydrolyzed slowly by trypsin was shown by the appearance of small amounts of N-terminal leucine. If the trypsin reaction was carried out at higher temperatures or for longer times, leucine, alanine, and glycine residues were detected as new N-terminal amino acids in approximately equal amounts. For example, at 10° and 25 min, 0.30 leucine, 0.20 alanine, and 0.25 glycine residues appeared as new N-terminals in addition to the expected isoleucine, tyrosine, and valine end groups.

TABLE IV: Characteristics of SCAM-peptide Fragments Derived from Chymotrypsinogen and DIP-chymotrypsin Aα.

Peptide	Length	ϵ_{282}[a]
TA1	15	0
TA2	130	23,100
TA3, 4	85	19,800
TA5	15	5,500
B chain	131	24,200
C chain	97	24,200
CB1[b]	180[c]	30,800
CB2	53	17,600

[a] Calculated from total tryptophan and tyrosine content assuming molar extinctions of 5500 and 1100, respectively, for the single residues (Edelhoch, 1967). [b] CB1 and CB2 designate CNBr fragments from SCAM-chymotrypsinogen. [c] About 50% of CB1 is peptide 1–192 due to lack of complete cleavage at Met 180 (see text).

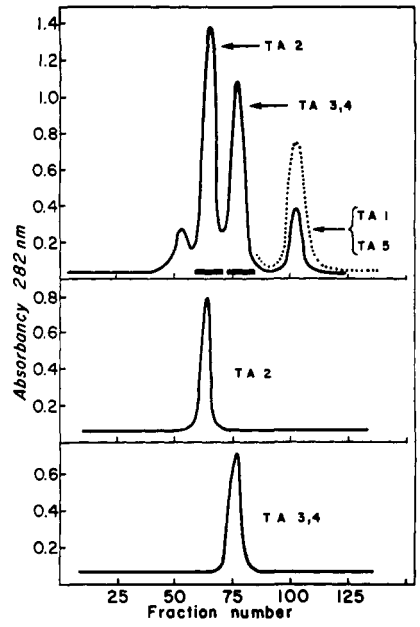

FIGURE 3: Typical Sephadex G-100 gel filtration of tryptic arginine peptides from SCAM-chymotrypsinogen. Columns 2.5 × 95 cm were eluted with 0.01 M ammonium acetate, pH 6.0, at 20 ml/hr and fractions of 3.7 ml were collected. The column load was 30 mg and total recovery of materials was greater than 95%. Absorbancy at 230 nm (---) was determined in the case of the overlapping TA1 and TA5 peptides (see text). The lower curves show the elution patterns observed when samples from the pooled peaks (black bars) of the initial separation were passed through the column a second time.

Gel Filtration of Tryptic–Arginine Peptides. Those peptide fragments which result from the trypsin hydrolysis of SCAM-chymotrypsinogen are presented in Table IV. The source of the peptide, its length, and calculated molar absorbancy are included in order to facilitate the description of results obtained from Sephadex gel filtration experiments.

The separation of the trypsin–arginine peptides on Sephadex G-100 is shown in Figure 3. The three main peaks appeared at the relative positions which would be predicted from their size (Table IV). Peptide TA2 emerged at approximately 220 ml followed by peptide TA3,4 at 275 ml. Near complete resolution was normally achieved with one pass through the column. The two remaining small peptides, TA1 and TA5, eluted as a single peak near the salt volume of the column. Peptides TA2 and TA3,4 were pooled and precipitated, respectively, in 95 and 89% yield after lowering the pH to about 3.0 with 6 N HCl. The pellets were redissolved in water by the dropwise addition of required amounts of 0.5 N NaOH, and upon a second gel filtration on the same column, each peptide was eluted as a single peak. Overall yields were 90 and 84%, respectively, for TA2 and TA3,4. The peptides were stored either as a frozen solution or a lyophilized powder.

Samples of the purified peptides were subjected to amino acid and N-terminal analysis. The only N-terminal amino acids detected in peptides TA2 and TA3,4 were isoleucine and tyrosine, respectively. The N-terminal of peptide TA2 therefore resulted from tryptic cleavage of the Arg 15–Ile 16 bond, and similarly TA3,4 was produced by cleavage of the Arg 145–Tyr 146 bond.

Amino acid analyses of the peptide fragments are contained in Table II. Since all of the histidine and phenylalanine residues in chymotrypsinogen are found in the B chain region, and the methionine residues are in the C chain region of the molecule, these amino acids serve as good indicators of the relative purity of the peptides. No methionine was detected in peptide TA2, which indicated lack of contamination by peptide TA3,4. Likewise, the amount of phenylalanine present in peptide TA3,4 indicated less than 2% contamination by TA2. The overall amino acid analysis confirmed the origin of peptides TA2 and TA3,4, and was in complete agreement with the compositions expected from the published data on the sequence of chymotrypsinogen.

Amino acid analysis of the final small peak which eluted from the G-100 Sephadex column (Figure 3) indicated that peptides TA1 and TA5 were present in nearly equimolar amounts. That the two peptides were superimposed was also indicated by spectrophotometry, since absorbancy at 230 nm at each point throughout this region of the chromatogram was proportional to absorbancy at 282 nm. Since TA1 contained no aromatic amino acids, its presence outside the peak containing TA5 could have gone undetected at 282 nm. Even though TA5 contained a tryptophan residue, the two 15-membered peptides were eluted in the same peak from the G-100 Sephadex column. The pooled peak containing both peptides was lyophilized.

When attempts were made to redissolve the lyophilized material in water or 0.1 N acetic acid in preparation for a second gel filtration an insoluble residue remained. The insoluble fraction was removed by centrifugation and the supernatant was applied to a column of Sephadex G-25, which gave an elution profile shown in Figure 4. Absorbancy measurements at 230 nm indicated the presence of a single large peak. After lyophilization of the pooled peak, samples were subjected to amino acid analysis. The insoluble fraction which was removed prior to gel filtration was also analyzed. Table II contains the results. It was found that the insoluble fraction was pure TA5 peptide. The single large peak isolated from the supernatant by gel filtration on the G-25 column

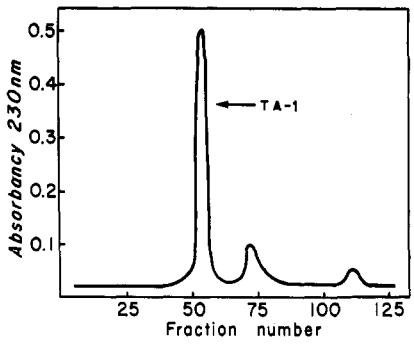

FIGURE 4: Gel filtration of peptide TA1 on a 2.0 × 100 cm column of Sephadex G-25, medium, after removal of TA5 by precipitation and centrifugation (see text). The column was eluted with 0.1 M acetic acid at 44 ml/hr and 3.7-ml fractions were collected. The column load was about 5 mg.

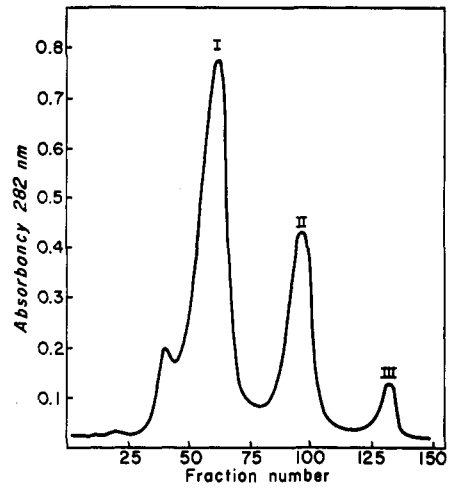

FIGURE 6: Sephadex G-100 gel filtration of cyanogen bromide treated SCAM-chymotrypsinogen. The column was 2.5 × 95 cm, and was eluted with 0.01 M ammonium acetate, pH 6.0, at 20 ml/hr. Fractions (3.4 ml) were collected and the absorbancy of every second tube was determined at 282 nm. Column load was 50 mg, of which 95% was recovered.

was pure TA1 peptide. Upon lyophilization, therefore, the initially soluble TA5 peptide became almost completely insoluble. Amino acid analyses of peptides TA1 and TA5 (Table II) were in excellent agreement with the published sequence. Both were isolated in overall yields of nearly 90%. Valine was found to be the only N-terminal amino acid in TA5, confirming that the peptide resulted from tryptic cleavage at the Arg 230–Val 231 bond. No N-terminal was found in TA1, presumably because the amino terminal of the protein had been succinylated (Habeeb *et al.*, 1958).

The small peak emerging just after TA1 (Figure 4) was found by amino acid analysis to be a 3:2 mixture composed of peptides TA5 (a small amount of which had remained in solution) and TA3 which was produced by cleavage at arginine 154 in less than 5% yield during the trypsin reaction. The third small peak contained no amino acids and presumably represented reagents. It was noted that peptide TA5, no doubt due to its one tryptophan residue, migrated more slowly on Sephadex G-25 than did peptide TA1, although they are similar in size. In fact, TA5 was eluted in the same volume as TA3 which contained only 9 residues.

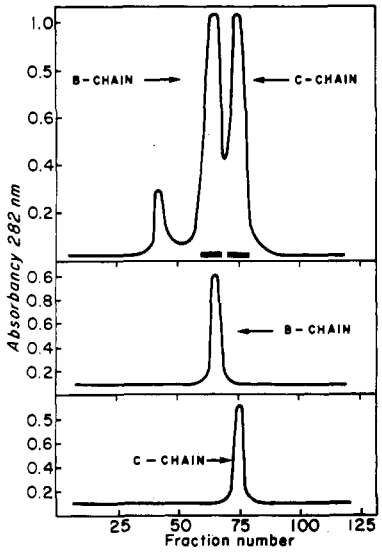

FIGURE 5: Typical Sephadex G-100 gel filtration of the B and C chains of SCAM-DIP-chymotrypsin Aα. The column load was 25 mg and 3.4-ml fractions were collected. Recovery from the column was greater than 95%. Other conditions were as described in Figure 3.

Gel Filtration of B and C Chains. Figure 5 shows the results of gel filtration of SCAM-DIP-chymotrypsin Aα on Sephadex G-100. The elution pattern consisted of two peaks of identical size as anticipated from knowledge of the molar extinction coefficients listed in Table IV. Each peak was harvested in 95% yield after precipitation at low pH and centrifugation. Purification of each fraction by a second gel filtration resulted in single peaks which were recovered in 90% yield. The SCAM derivative of the B chain was eluted first at about 220 ml of effluent. The C chain derivative emerged at 250 ml. As in the case of the corresponding TA2 and TA3,4 peptides, the B chain was nearly free of contamination by C chain, and the C chain had less than 2% contamination by B chain. Results of amino acid analyses are included in Table II. The amino acid composition of the chains was in excellent agreement with that determined from sequence studies. Dialysis of the SCAM-DIP-α-chymotrypsin prior to gel filtration resulted in loss of the A chain (13 residues). No attempt was made to retain the A chain since it was similar to peptide TA1 previously isolated.

Cyanogen Bromide Cleavage. Chymotrypsinogen contains two methionine residues located at positions 180 and 192. Reaction of CNBr with SCAM-chymotrypsinogen produced two major fragments which were separated on Sephadex G-100 as shown in Figure 6. Peak I eluted at 205 ml and was harvested in 93% yield by centrifugation after precipitation at pH 3. Peaks II and III were separately pooled and lyophilized. Amino acid analyses were performed on each fraction and the results are included in Table II. Peak I represented the N-terminal region as suggested by its histidine and phenylalanine content. Peak II resulted from cleavage of the molecule at methionine 192 and accounted for the C-terminal portion of the protein. The overall yield of peak II was about

90%. Peak III contained low amounts of various amino acids and could not be definitely related to the peptide sequence between the methionine residues.

Discussion

Succinylation. The utility of succinylation for the dispersal and solubilization of proteins has been demonstrated by other investigators (Freisheim *et al.*, 1967; Gounaris and Perlmann, 1967; Slobin and Singer, 1968). In this work succinylation was found to disperse and solubilize peptide and protein derivatives, in addition to restricting tryptic cleavage to the susceptible arginine peptide bonds.

Butler *et al.* (1967) earlier observed that the reaction of chymotrypsinogen with maleic anhydride resulted in products which were soluble at neutral and alkaline pH, presumably due to the large accumulation of the negative charges and the resulting electrostatic repulsion. Succinylation of the 14 lysine residues in chromotrypsinogen would result in a net negative change of at least 28 charges. If threonine and serine were modified, which is probable (Gounaris and Perlmann, 1967), the increase in negative charge could be considerably greater than 28. Our observation that repeated succinylation dispersed aggregated B and C chains of chymotrypsin Aα may be related to reaction at nonlysine sites. Such reactions would not significantly affect the separation of fragments on the basis of molecular size.

When succinylation was performed in 6 M Gd·HCl as in Scheme A (Table I), the reaction was complete and all lysine residues were immune to the action of trypsin. In Scheme B, however, when no denaturant was used in the reaction medium, complete blockage of lysine did not occur as shown by the presence of C-terminal lysine in the tryptic hydrolysate. The second succinylation, performed on the presumably less structured carboxamidomethylated derivative dissolved in water, resulted in complete masking of the lysine residues toward tryptic attack. Ten of the fourteen lysines of chymotrypsinogen are localized in two small segments, one midway in the B chain and one midway in the C chain regions of the molecule. Possibly steric or electrostatic effects influenced the extent of reaction at these clusters in the absence of denaturing solvents.

Reduction and Alkylation. Reduction of the half-cysteine residues to monothiol groups with dithiothreitol and subsequent alkylation with iodoacetamide converted them into ten *S*-carboxamidomethylcysteine residues. Quantitative conversion was achieved only when the alkylation step was performed in the presence of the reducing agent, dithiothreitol. Both the reduced and alkylated forms of the succinylated protein were water soluble, and consequently urea or Gd·HCl was not required. For complete reduction, the pH of the dithiothreitol reaction had to be adjusted to 9.5. At pH 8–9 the yield of CM-cysteine was about 80% of the theoretical, suggesting the presence of a particularly unreactive disulfide bond. Inclusion of 10^{-3} M EDTA was without effect.

During initial phases of this work iodoacetic acid was considered a more appropriate sulfhydryl-blocking reagent because of the increased total negative charge which would result upon its incorporation into the molecule. Use of the reagent was abandoned when it was observed that routine laboratory manipulations of the succinylated carboxymethylated protein and peptides resulted in losses of CM-cysteine and in the appearance of new N-terminal amino acids. That reactions leading to unpredictable fragmentation were occurring was also suggested by chromatographic peaks of lower molecular weight which appeared in filtrations through Sephadex. A satisfactory explanation of these observations was not available. SCAM derivatives did not suffer this manipulative degradation and iodoacetamide was therefore chosen for modification of the sulfhydryl groups.

Specificity of the Trypsin Cleavage. Complete trypsin specificity for arginine was observed only when the reaction conditions were limited to 10° and 10 min. Under these conditions arginine bonds 15, 145, and 230 were cleaved nearly 100%, whereas the Arg 154–Leu 155 bond cleaved only 5% (Table III). It was observed that the yield of leucine as an N-terminal amino acid could be increased if the reaction was carried out at higher temperatures, or for longer periods of time. This observation probably accounted for the fact that a greater than expected amount of arginine was liberated during the CPB digestion.

Hartley (1964) has determined the sequence around the Arg 154–Leu 155 bond to be Pro-Asp-Arg-Leu-Gln. It is well known that the rate of tryptic cleavage at arginine or lysine is decreased when the basic residue is preceded in the sequence by an acidic amino acid residue (Smyth, 1967). It was to be expected, therefore, that the rate of cleavage at the Arg 154–Leu 155 bond would be slow in comparison with that at Arg 15, Arg 145, or Arg 230 near which no acidic amino acids are present. A similar inhibition of cleavage was recently elucidated in studies dealing with the removal of the activation peptide, Val-Asp_4-Lys, in the autoactivation of trypsinogen (Abita *et al.*, 1969).

It was not possible to increase the extent of cleavage at the Arg 154–Leu 155 bond and still maintain complete specificity for arginine. When this was attempted, N-terminal glycine and alanine were detected in amounts approximately equal to that of leucine, suggesting that peptide bonds other than arginine were being cleaved. That these additional N-terminal residues were not the result of slow hydrolysis of a lysine bond was indicated by the CPB results which showed no increase in C-terminal lysine. Furthermore, no Lys-Ala or Lys-Gly sequences are present in the primary structure of the protein.

A better explanation for the origin of alanine and glycine during tryptic hydrolysis may be found in the published sequence data. Maroux and Rovery (1966) during investigations on the sequence of the *S*-sulfo C chain of chymotrypsin Aα found that highly purified preparations of trypsin cleaved the Tyr 228–Ala 229 bond in high yield. Similarly, Meloun *et al.* (1966) isolated from the tryptic digest of *S*-β-aminoethylated B chain of chymotrypsin Aα the tetrapeptide, Gly-Leu-Thr-Arg, which resulted from cleavage of the Trp 141–Gly 142 bond and the Arg 145–Tyr 146 bond. They also isolated the Ala-Arg dipeptide observed by Maroux and Rovery (1966). Possibly, since these bonds have extraordinary susceptibility to trypsin, the glycine and alanine which were liberated during the prolonged trypsin treatment represented hydrolysis of the Trp 141–Gly 142 bond and the Tyr 228–Ala 229 bond at a slow rate approximately equal to that of the resistant Arg 154–Leu 155 bond. Since each of the "nonspecific" bonds was sequentially near arginine residues which were rapidly hydrolyzed, *i.e.*, Arg 145 and 230, their limited cleavage would not significantly change the size of the expected peptides and, therefore, would not alter the appearance of the

subsequent gel filtration. In fact, no differences were observed in the gel filtration pattern of the peptides produced by reaction with trypsin at 10° for 10 min and at 15° for 25 min.

Solubility and Aggregation Characteristics of Peptide Fragments. The peptides prepared from SCAM derivatives of chymotrypsinogen and chymotrypsin Aα exhibited generally similar solubility behavior, as expected from their compositions (Figure 1).

The SCAM derivative of the C chain appeared to be slightly less soluble than peptide TA3,4, apparently the result of amino acid residues 231–245 which were not a part of peptide TA3,4. This C-terminal region of the zymogen molecule was isolated in peptide TA5, which, after initial separation, was insoluble in water. Meloun *et al.* (1966) observed nearly identical solubility characteristics when they isolated the same peptide from tryptic digests of *S*-β-aminoethylated C chain of DIP-chymotrypsin Aα.

In order to avoid aggregation it was necessary to employ dialysis against water at strategic steps in the procedure and to avoid lyophilization or freezing of solutions at critical points. The normally fast cleavage of SCAM chymotrypsinogen by trypsin was noticeably slower, and arginine specificity decreased, if the protein derivative was not dialyzed in water for several hours before addition of the trypsin. Additionally, in order to achieve acceptable separation and purification, it was imperative that the tryptic arginine peptides were subjected to gel filtration immediately after the enzyme had been inhibited with soybean trypsin inhibitor. If the peptides were lyophilized or frozen prior to gel filtration, considerable aggregation occurred. However, after their initial separation and precipitation, peptides TA2 and TA3,4 could be redissolved and purified by gel filtration directly without special handling. They showed no tendency toward further aggregation. Best results were always obtained when the protein concentration was kept below 10 mg/ml.

The B and C chains of SCAM-DIP-chymotrypsin Aα were initially more prone to aggregation than the tryptic arginine peptides, probably as a result of the retention of the insoluble C-terminal portion of the C chain. Satisfactory separation was achieved after exhaustive dialysis (24–48 hr) of the freshly prepared peptide chains against water. The protein concentration was usually about 5 mg/ml. For effective separation it was absolutely essential that the B and C chain derivatives were not frozen or lyophilized before gel filtration. If they were, a second succinylation step was necessary in order to achieve adequate separation. Once separated from the C chain, the B chain derivative no longer had a strong tendency toward further aggregation, a behavior similar to that of peptide TA2. In contrast, SCAM-C chain was very prone to aggregation. Before SCAM-C chain could be purified by gel filtration, it was necessary to dialyze the peptide in cold water several hours in order to completely disperse aggregated molecules.

Cyanogen Bromide Reaction. Cleavage of the two methionine residues at positions 180 and 192 would result in three peptides, a large N-terminal fragment of 180 residues, a C-terminal fragment containing 54 residues, and a short 12-membered peptide. Under conditions used here, the 12-membered peptide between the two methionine residues was not observed, and close examination of the amino acid composition of the large N-terminal CNBr fragment indicated that complete cleavage had not occurred at methionine 180. Initial attack by CNBr on both methionine residues must have occurred because only trace amounts of free methionine were detected by amino acid analysis.

End group data supported this hypothesis. Several experiments were done, not only with SCAM-chymotrypsinogen, but also with succinylated chymotrypsinogen and unmodified protein. Reactions (24 hr) in dilute HCl and in 70% formic acid, both at 30° and at 40°, with excesses of cyanogen bromide ranging from 100- to 400-fold, all resulted in the appearance of one residue of glycine and about 0.5 residue of isoleucine. This gave strong evidence that the Met 192–Gly 193 bond was being cleaved completely and that the Met 180–Ile 181 bond was resistant to cleavage (but not to reaction) with cyanogen bromide. The slow cleavage at the Met 180–Ile 181 bond remains unexplained. Similar results were observed by Burkhardt and Wilcox (1967) in a highly disruptive organic solvent, and by Moravek *et al.* (1966) with a 27-membered peptide containing the two methionine residues. The resistance most likely arises from the influence of the primary structure surrounding the methionine residue. The proximity of the two methionine residues and the absence of interesting residues between them rendered lack of complete cleavage at Met 180 inconsequential.

Peptides Obtainable through CNBr and Tryptic Arginine Cleavage. Table V summarizes peptide fragments which may be isolated by gel filtration from SCAM derivatives of chymotrypsinogen A and DIP-chymotrypsin Aα through application of methods described in this paper. Isolation of such fragments results in the segregation of potentially reactive amino acids into limited regions of the sequence and facilitates their identification after chemical modification of the protein.

The distribution of the tyrosine residues among the individual peptide fragments is included as an example in Table V. For chymotrypsin Aα, isolation of the B and C chains resulted in separation of the tyrosine residues into two groups. Tyr 94 is internal in the B chain, whereas Tyr 146 is C-terminal. Removal of Tyr 146 with carboxypeptidase A would result in the unambiguous isolation of Tyr 94. Similarly, Tyr 171 and Tyr 228 were isolated in the C chain. Trypsin cleavage of the C chain (fragment 4) would then place Tyr 228 two residues removed from the C-terminal and expose it to action of CPA after Arg 230 was removed by carboxypeptidase B, resulting in isolation of tyrosine 171.

Degradation of chymotrypsinogen similarly resulted in segregation of the tyrosine residues. Tyrosines 94 and 228 were isolated respectively in fragments 10 and 14. Peptide TA3,4 (fragment II) contained three tyrosine residues, of which tyrosine 146 was N-terminal and available for chemical analysis. Tyrosine 171 may then be identified by difference between fragments 11 and 14. Utilizing these approaches it is possible to unambiguously identify each of the four tyrosines in chymotrypsinogen and chymotrypsin Aα.

Similar schemes may be derived for the quantitative separation into small segments of any amino acids of interest. Incorporation of CNBr cleavage into isolation schemes particularly broadens the possibilities. The eight tryptophan residues in chymotrypsinogen A, for example, may be segregated into individual peptides containing respectively four, two, one, and one tryptophan residues.

It remains to be pointed out in this discussion that the B chain portion of the molecule (residues 16–146) cannot be

TABLE V: Fragments of SCAM-chymotrypsin Aα and SCAM-chymotrypsinogen Which May be Isolated after Various Cleavages at Arginine Residues and at Methionine 192.[a]

Fragment		Preparation	Tyrosine Content
1	1–13	A Chain from SCAM-DIP-chymotrypsin Aα	
2	16–146	B Chain from SCAM-DIP-chymotrypsin Aα	94, 146[c]
3	149–245	C Chain from SCAM-DIP-chymotrypsin Aα	171, 228
4	149–230	Trypsin cleavage of fragment 3[b]	171, 228
5	231–245	Trypsin cleavage of fragment 3[b]	
6	149–192	CNBr cleavage of fragment 3[b]	171
7	193–245	CNBr cleavage of fragment 3[b]	228
8	193–230	Trypsin cleavage of fragment 7[b]	228
9	1–15	TA1 from SCAM-chymotrypsinogen	
10	16–145	TA2 from SCAM-chymotrypsinogen	94
11	146–230	TA3,4 from SCAM-chymotrypsinogen	146,[d] 171, 228
12	231–245	TA5 from SCAM-chymotrypsinogen	
13	1–192	CNBr cleavage of SCAM-chymotrypsinogen	94, 171, 146
14	193–245	CNBr cleavage of SCAM-chymotrypsinogen	228
15	146–192	Trypsin cleavage of fragment 13[b]	146,[d] 171
16	193–230	Trypsin cleavage of fragment 14[b]	228

[a] The table is incomplete. Other combinations of trypsin and CNBr cleavages may be used to produce additional fragments. [b] Fragment was not prepared in the present study. [c] Tyrosine 146 is C-terminal. [d] Tyrosine 146 is N-terminal.

further degraded by methods presented here. A likely method for additional fragmentation of this portion of the protein would be the conversion of half-cystine residues in the succinylated derivative into potential trypsin cleavage sites by alkylation with ethylenimine (Plapp *et al.*, 1967). It is doubtful, however, that the derived fragments could be separated in pure form by Sephadex gel filtration because several would be similar in size. Some more indirect procedure will be required.

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References

Abita, J. P., Delaage, M., and Lazdunski, M. (1969), *Eur. J. Biochem. 8*, 314.

Burkhardt, W. A., and Wilcox, P. E. (1967), *Biochem. Biophys. Res. Commun. 28*, 803.

Butler, P. J. G., Harris, J. I., Hartley, B. S., and Leberman, R. (1967), *Biochem. J. 103*, 78 p.

Carpenter, F. H. (1967), *Methods Enzymol. 11*, 237.

Cleland, W. W. (1964), *Biochemistry 3*, 480.

Davies, D. R., Cohen, G. H., Silverton, E. W., Braxton, H. P., and Matthews, B. W. (1969), *Acta Crystallogr. A25*, S182.

Edelhoch, H. (1967), *Biochemistry 6*, 1948.

Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T., and Xuong, Ng. H. (1970), *Biochemistry 9*, 1977.

Freisheim, J. H., Walsh, K. A., and Neurath, H. (1967), *Biochemistry 6*, 3010.

Gounaris, A., and Ottesen, M. (1965), *C. R. Acad. Sci. 35*, 37.

Gounaris, A. D., and Perlmann, G. E. (1967), *J. Biol. Chem. 242*, 2739.

Gross, E. (1967), *Methods Enzymol. 11*, 238.

Gurd, F, R. N. (1967), *Methods Enzymol. 11*, 523.

Habeeb, A. F. S. A., Cassidy, H. G., and Singer, S. J. (1958), *Biochim. Biophys. Acta 29*, 587.

Hartley, B. S. (1963), *Fifth Int. Congr. Biochem. 4*, 104.

Hartley, B. S. (1964), *Nature (London) 201*, 1284.

Hartley, B. S., and Kauffman, D. L. (1966), *Biochem. J. 100*, 229.

Hoffman, T. (1964), *Biochemistry 3*, 356.

Jansen, E. F., Nutting, M. D. F., Jang, R., and Balls, A. K. (1949), *J. Biol. Chem. 179*, 189,

Kluh, I., Morávek, L., Junge, J. M., Meloun, B., and Sǒrm, F. (1966), *Coll. Czech. Chem. Commun. 31*, 152.

Maroux, S., and Rovery, M. (1966), *Biochim. Biophys. Acta 113*, 126.

Matthews, B. W., Cohen, G. H., Silverton, E. W., Braxton, H., and Davies, D. R. (1968), *J. Mol. Biol. 36*, 179.

Meloun, B., Kostka, V., Vanecek, J., Kluh, I., and Sǒrm, F. (1966), *Collect. Czech. Chem. Commun. 31*, 321.

Meloun, B., Morevek, L., and Sǒrm, F. (1967), *Collect. Czech. Chem. Commun. 32*, 1947.

Moravek, L., Kluh, I., Junge, J. M., Meloun, B., and Sǒrm, F. (1966), *Collect. Czech. Chem. Commun. 31*, 1604.

Plapp, B. V., Raftery, M. A., and Cole, R. D. (1967), *J. Biol. Chem. 242*, 265.

Richmond, V. (1966), *Biochim. Biophys. Acta 127*, 499.

Riordan, J. F., and Vallee, B. L. (1964), *Biochemistry 3*, 1768.

Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R. (1968), *J. Mol. Biol. 35*, 143.

Slobin, L. I., and Singer, S. J. (1968), *J. Biol. Chem. 243*, 1777.
Smyth, D. G. (1967), *Methods Enzymol. 11*, 214.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stark, G. R. (1967), *Methods Enzymol. 11*, 125.
van Hoang, D., Rovery, M., Guidoni, A., and Desnuelle, P. (1963), *Biochim. Biophys. Acta 69*, 188.
Wright, H. T., Kraut, J., and Wilcox, P. E. (1968), *J. Mol. Biol. 37*, 363.

Peptide Sequences and Relative Reactivity of the Reactive Sulfhydryl Groups of Rabbit Muscle Phosphorylase*

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ABSTRACT: Rabbit muscle phosphorylase *b* contains four cysteine residues which are exceptionally reactive with 2,4-dinitrochlorobenzene. These have been characterized by preparing phosphorylase *b* with an average of three groups substituted as *S*-[^{14}C]DNP-cysteine, digesting with trypsin, and isolating two unique radioactive peptides. The following sequences were found: peptide A, Ile-DNP-Cys-Gly-Gly-Trp-Gln-Met-Glu-Glu-Ala-Asp-Asp-Trp-Leu-Arg; peptide B, Phe-Gly-DNP-Cys-Arg-Asp-Pro-Val-Arg. Peptide B does not overlap with the sequence Lys-Phe, which is one of the points of attachment of pyridoxal phosphate in native phosphorylase. Phosphorylase *a* also yields these two peptides. The reactive sulfhydryl groups were found to react with 2,4-dinitrochlorobenzene at nearly equal rates in both phosphorylases *a* and *b;* AMP, however, selectively protects the sulfhydryl group giving rise to peptide B in both forms. It has been found possible to maintain the reactive sulfhydryl groups of phosphorylase *b* in the fully reduced state by frequently recrystallizing the protein in the presence of dithiothreitol. Otherwise, the number of reactive sulfhydryl groups is less than four, even in freshly prepared enzyme, and gradually decreases over a period of weeks.

Dinitrophenylating agents, such as FDNB[1] and CDNB, appear to react rapidly with four sulfhydryl groups of phosphorylase *b* (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) from rabbit muscle at pH 8.0 (Gold, 1968). The precise number of reactive groups observed varies with different preparations of enzyme up to a maximum of 3.6, but is significantly lower in many cases. Since the enzyme is a dimer of apparently identical subunits of 92,500 molecular weight, it is probable that each subunit contains two especially reactive cysteine residues out of a total of nine cysteine residues (Zarkadas *et al.*, 1968). Dinitrophenylation of the four reactive sulfhydryl groups results in an increase in the kinetic constants of the enzyme without greatly changing the maximum velocity or causing dissociation of subunits. The remaining sulfhydryl groups react slowly with FDNB and CDNB.

Kleppe and Damjanovich (1969) have reported that phosphorylase *b* contains two sulfhydryl groups that are highly reactive with DTNB in disulfide interchange at pH 6.8, and another four cysteine residues that react more slowly. Substitution of the reactive groups results in no loss of activity, measured in a standard assay, while substitution of the less reactive sulfhydryl groups produces inactivation with concomitant dissociation into subunits. In investigating the reaction of phosphorylase *b* with DTNB, Kastenschmidt *et al.* (1968) isolated a derivative containing up to 3.2 mixed disulfide groups which retained dimeric structure and essentially full enzymic activity at high substrate concentration.

Another reagent that has proven valuable in the study of phosphorylase is iodoacetamide. Battell *et al.* (1968a) found that two sulfhydryl groups of phosphorylase *b* react rapidly with iodoacetamide at pH 7.5 without inactivation, while another four sulfhydryls react more slowly and their substitution results in inactivation and dissociation into subunits. The two classes of cysteine residues were identified by isolation of three unique peptides containing alkylated cysteine (Battell *et al.*, 1968b); the reactive groups occur in the sequence Gly-Cys-Arg-Asp, while the four less reactive sulfhydryls occur in the sequences Ala-Cys-Ala-Phe and Asn-Ala-Cys-Asp. More recently this group reported that freshly prepared phosphorylase *b* contains 3.1 highly reactive sulfhydryl groups (Zarkadas *et al.*, 1970). They also succeeded in isolating a new alkylated cysteine peptide, Asn-Gln-Lys-Ile-Cys-Gly-Gly-Trp-Gln-Ser, and extending the sequence of the other reactive cysteine residue to Gly-Cys-Arg-Asp-Pro-Val-Arg-Thr-Asn-Phe.

In the present work we have isolated two tryptic peptides

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[1] Abbreviations used are: FDNB, 2,4-dinitrofluorobenzene; CDNB, 2,4-dinitrochlorobenzene; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PTH, phenylthiohydantoin; DNS or dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.