The Promotion of Activation of Bovine Trypsinogen by Specific Modification of Aspartyl Residues*

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ABSTRACT: Modification of the carboxylate groups of trypsinogen under mild conditions resulted in the incorporation of about 2.5 glycinamide residues in the protein. The modified zymogen could be activated in the absence of calcium ions. Active trypsin was isolated from the activation mixture and found to be unmodified. Peptides isolated from the activation mixture were found to be a mixture of mono- and diglycinamide derivatives of the activation peptide Val-$(Asp)_4$-Lys. Edman degradation of the substituted peptides indicated that each of the four carboxyls is modified in a rather random manner.

The N-terminal undecapeptide of trypsinogen was isolated and it was found that Ca^{2+} accelerated the tryptic hydrolysis of the Lys^6–Ile^7 bond. Since modification of the carboxylate groups in the N-terminal region of trypsinogen eliminates the requirement of calcium ions in the activation, it is suggested that the role of calcium ions in the autocatalytic activation of trypsinogen is to bind these carboxylate groups thus enhancing the susceptibility of the Lys^6–Ile^7 bond for cleavage by trypsin.

It has been established by MacDonald and Kunitz (1941) that calcium ions are essential for the complete tryptic activation of bovine trypsinogen. The activation involves the hydrolysis of the Lys^6–Ile^7 bond, resulting in the cleavage of the anionic hexapeptide, Val-$(Asp)_4$-Lys, from the N-terminal region of the molecule (Davie and Neurath, 1955; Desnuelle and Fabre, 1955). In the absence of calcium, slow and nonspecific cleavage of trypsinogen results in the formation of inert protein (Gabeloteau and Desnuelle, 1957). While the exact function of calcium and other divalent cations in the activation reaction is unknown, the presence of a cluster of four β-carboxylate groups adjacent to Lys^6 may provide a calcium binding site (Delaage and Lazdunski, 1967). Shielding of these negative charges by chelation with calcium thus could direct and enhance tryptic hydrolysis of the Lys^6–Ile^7 bond and promote activation. To test this hypothesis we have modified the carboxylate groups of trypsinogen with amines in the presence of a water-soluble carbodiimide.

In preliminary studies (Radhakrishnan *et al.*, 1967) it was found that amidation of trypsinogen with glycine ethyl ester eliminated the requirement of calcium in the activation process. In the present experiments glycinamide was used as the amine and the site of modification which relieves the calcium requirement for the activation of trypsinogen was located.

Materials

Crystalline bovine trypsinogen (Lot No. TG6423), twice-crystallized bovine trypsin (Lot No. TR 6EA) containing approximately 50% $MgSO_4$, and crystalline soybean trypsin inhibitor were purchased from Worthington Biochemical Corp. Trypsinogen and trypsin were dialyzed exhaustively against 10^{-3} M HCl at 5° and stored at −20°.

EDPC[1] and glycinamide hydrochloride were purchased from Ott Chemical Co. and Cyclochemical Corp., respectively. All other reagents used were analytical grade.

Methods

Trypsin assays and protein concentrations were determined as described by Radhakrishnan *et al.* (1969). Amino acid analysis was performed on a Beckman-Spinco Model 120B amino acid analyzer according to Spackman *et al.* (1958). Unless stated otherwise, all samples were hydrolyzed with glass-distilled, constant-boiling HCl in evacuated and sealed Pyrex tubes at 110° for 24 hr.

Ninhydrin Analysis. After development of the chromatograms, the separation of the components was examined in 0.05–0.1-ml aliquots of effluent fractions by color reaction with ninhydrin after alkaline hydrolysis as described by Schroeder and Robberson (1965).

Modification of the Carboxylate Groups of Trypsin and Trypsinogen with Glycinamide in the Presence of EDPC. Trypsin or trypsinogen (0.25 mM) was treated for 15 min at pH 4.5, 25°, with a 20-fold molar excess of EDPC in 1.0 M glycinamide. The reaction was terminated by the addition of an equal volume of 1.0 M sodium acetate (pH 3.6). The experimental conditions employed were the same as described previously (Radhakrishnan *et al.*, 1967), except that glycinamide was used in the reaction mixture instead of glycine ethyl ester. After exhaustive dialysis, the modified protein

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[1] Abbreviations used are: EDPC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; NEMO, *N*-ethylmorpholine; HEPES, *N*-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid; STI, soybean trypsin inhibitor; GE-cellulose, guanidinoethylcellulose; PTC, phenylthiocarbamyl.

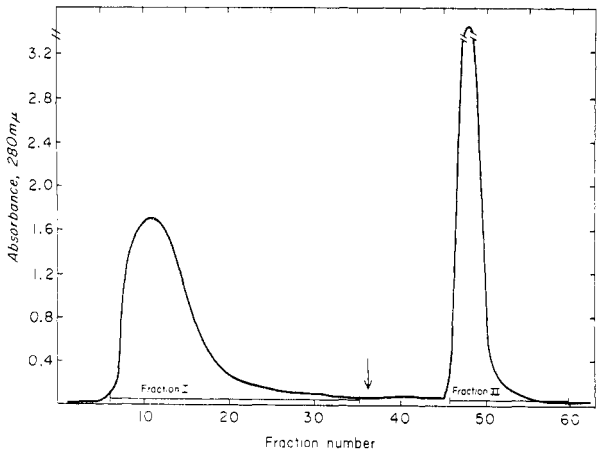

FIGURE 1: Chromatography on a GE-cellulose column (1.5 × 25 cm) of the protein portion of the activation mixture of modified trypsinogen after treatment with STI. The column was equilibrated at 4° with 0.07 M Tris-Cl buffer (pH 7.8) containing 0.01 M $CaCl_2$ and the chromatogram was developed at a flow rate of 60 ml/hr; 4-ml fractions were collected. After collecting 36 fractions (indicated by an arrow), 0.75 M NaCl was included in the eluting buffer. The fractions were monitored by measuring the absorbance at 280 mμ and pooled as indicated by solid bars.

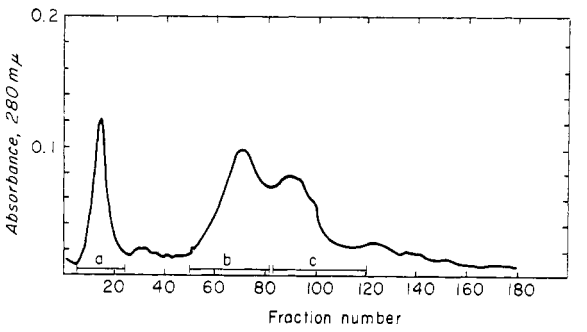

FIGURE 2: Chromatography on a SE-Sephadex column (1.5 × 25 cm) of the inactive component, fraction I, isolated from the activation mixture of modified trypsinogen. The column was equilibrated at 4° with 5 × 10^{-3} M sodium citrate buffer (pH 3.0) containing 0.3 M NaCl and developed at a flow rate of 10 ml/hr. Fractions of 4 ml were collected and they were monitored by measuring the absorbance at 280 mμ. The fractions indicated by the solid bars were pooled.

samples were concentrated in a Diaflo (American Instrument Co.) pressure cell at 5° to about 1–2 mM.

Activation of the Modified Trypsinogen with Trypsin at pH 7.0 in the Absence of $CaCl_2$ and Isolation of the Activation Peptides. The activation was carried out for 90 min with 5 ml of the mixture containing 14 mg of modified trypsinogen/ml and 0.6 mg of trypsin/ml of 0.04 M NEMO–acetate (pH 7.0). Aliquots were withdrawn from the activation mixture at definite time intervals and assayed for trypsin activity. After activation, the pH of the mixture was adjusted to 4.0 with 1.0 N HCl and loaded on a column (2.5 × 40 cm) of Sephadex G-25 (fine) which was previously equilibrated at 5° with 2.0 M ammonium acetate (pH 4.0). The chromatogram was developed at flow rate of 10 ml/hr. The protein emerged with the breakthrough peak but a peptide fraction was retarded. The peptide fraction was pooled and lyophilized. To remove traces of ammonium acetate, the material was lyophilized twice after dissolving in distilled water.

The lyophilized powder was dissolved in 0.5 ml of distilled water and streaked in a 10-cm line in the center of Whatman No. 3 MM paper (20 × 57 cm). Electrophoresis was carried out using pyridine–acetate buffer (pH 6.5) at 2000 V for 2 hr. After drying at room temperature a guide strip (representing 25 μl of the original solution) was stained with ninhydrin. Corresponding peptide-containing zones were eluted with pyridine–acetate buffer (pH 6.5). The solvent was evaporated and each peptide was dissolved in 2 ml of distilled water, 0.5 ml of each peptide solution was hydrolyzed, and its amino acid composition was determined.

Purification of the Protein Fractions from the Activation Mixture of the Modified Trypsinogen. This fractionation was based on the procedure employed by Beeley and Neurath (1968), for the separation of inactive and active trypsin.

The breakthrough fraction obtained above from chromatography of the activation mixture on Sephadex G-25 was pooled and dialyzed against 10^{-3} M HCl at 5°. After lyophilization, an aliquot was assayed for trypsin activity. The dry powder (138.5 mg by pooling the material from two identical experiments) was dissolved in 20 ml of a solution containing 30.2 mg of STI in 0.07 M Tris-HCl buffer (pH 7.8) containing 0.01 M $CaCl_2$. The solution was readjusted to pH 7.8 (measured at 5°) with 1 M Tris and applied to a GE-cellulose column and developed as illustrated in Figure 1. The material which was eluted by the starting buffer is referred to as fraction I and contained largely inactive trypsin; the material which was eluted at the higher salt concentration was designated as fraction II and consisted of active trypsin in a complex with STI. Fractions I and II were pooled separately as indicated in Figure 1, dialyzed against 10^{-3} M HCl at 5°, and lyophilized.

Fraction I was dissolved in 5 ml of 0.005 M sodium citrate buffer (pH 3.0) containing 0.3 M NaCl and applied to an SE-Sephadex C-50 column (1.5 × 25 cm) previously equilibrated with the same buffer. The column was developed at 5° at 10 ml/hr and fractions of 4 ml were collected (Figure 2).

Fraction II was separately chromatographed under similar conditions employed for fraction I except that after collecting 185 fractions the NaCl concentration of the eluting buffer was increased from 0.3 to 0.35 M to elute STI completely from the column (Figure 3).

After chromatography of fractions I and II, pooled fractions shown in Figures 2 and 3 were dialyzed aginst 10^{-3} M HCl at 5° and lyophilized. The yield (by weight) from fraction I of largely inactive trypsin was 4.1 mg and from fraction II of active trypsin was 18.7 mg.

Formation and Carbamylation of a Copper Complex of Val-$(Asp)_4$-Lys. A solution containing 1 μmole of the unmodified or modified N-terminal hexapeptide, Val-$(Asp)_4$-Lys, isolated from a tryptic digest of either native or modified trypsinogen was evaporated to dryness in a test tube. It was dissolved in 2 ml of a solution containing 0.1 M HEPES–0.1 M KCl (pH 8.5) and 1 ml of a solution containing 1 μmole of $CuCl_2$ was added. The solution immediately turned pale pink indicating the formation of a copper complex. The absorption spectrum of the complex between 470 and 650 mμ was found to be similar to other copper complexes reported by Shearer *et al.* (1967), with an ε of 90 at 550 mμ.

The copper complex of the peptide in a volume of 3 ml was

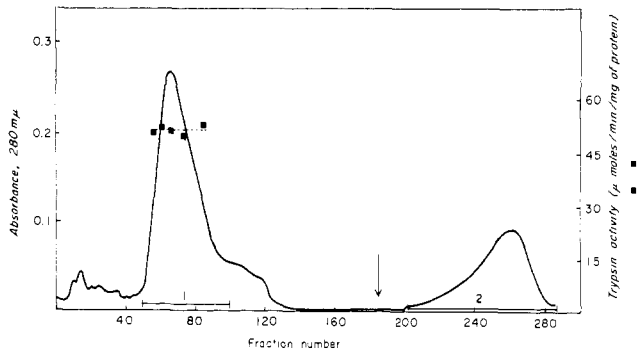

FIGURE 3: Chromatography on a SE-Sephadex column (1.5 × 25 cm) of the active component, fraction II in Figure 1, isolated from the activation mixture of modified trypsinogen after complexing with STI. The column was equilibrated at 4° with 5 × 10^{-3} M sodium citrate buffer (pH 3.0) containing 0.3 M NaCl and developed at a flow rate of 10 ml/hr. Fractions of 4 ml were collected and after collecting 185 fractions (shown by an arrow), the salt concentration of the eluting buffer was increased to 0.35 M. Separation was monitored by measuring the absorbance at 280 mμ and the trypsin activity of the fractions across the first peak (filled squares) was determined. The fractions indicated by the solid bars were pooled.

carbamylated on the ε-amino group of lysine by treatment with 100 mg of potassium cyanate at 50° for 6 hr (Stark and Smyth, 1963). The absorbance of the complex did not change. This reaction mixture was desalted on a Sephadex G-10 column (1.5 × 85 cm) previously equilibrated with 0.1 M NEMO–acetate buffer (pH 7.0). Fractions of 1.0 ml were collected at a flow rate of 10 ml/hr. Aliquots of 50 μl from alternate fractions were taken for ninhydrin analysis. Fractions containing the modified peptide in the breakthrough peak were pooled and dried in a rotary evaporator.

This desalted carbamyl peptide–copper complex was dissolved in 2 ml of distilled water and extracted three times with 1.0-ml aliquots of 8-hydroxyquinoline in chloroform (6.5 mg/ml) to remove copper by the method of Tommel

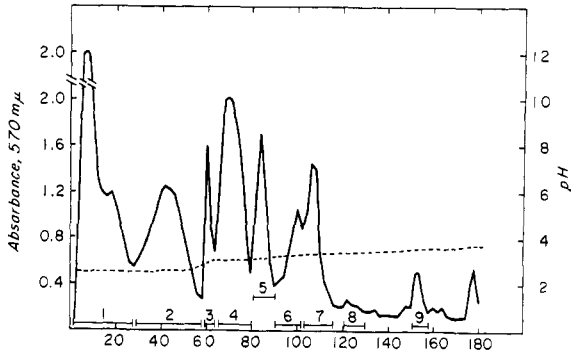

FIGURE 4: Chromatography on a Dowex 50-X8 column (2 × 24 cm) of the α-chymotryptic digest of *S*-sulfotrypsinogen. The column was equilibrated at 55° with 0.05 M pyridine–acetate buffer (pH 2.5) at 80 ml/hr and was developed with a linear gradient from 500 ml of 0.05 M pyridine (pH 2.5 with acetic acid) to 500 ml of 0.5 M pyridine (pH 3.75 with acetic acid); 6-ml fractions were collected; 100-μl aliquots of alternate fractions were monitored by ninhydrin analysis and the absorbance at 570 mμ shown is in the ordinate. The dashed lines indicate the pH profile of the gradient and the solid bars, the fractions pooled.

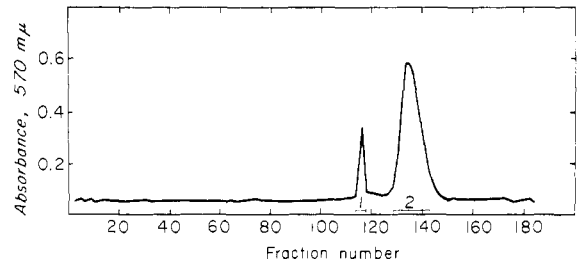

FIGURE 5: Further fractionation on a Dowex 50-X8 column (2 × 24 cm) of the N-terminal undecapeptide obtained from fractions pooled across peak 5 illustrated in Figure 4. Pyridine–acetate buffer (0.05 M, pH 2.5) was pumped onto the column for 1 hr at 80 ml/hr and the chromatogram was then developed with a linear gradient of 500 ml of 0.05 M pyridine–acetate (pH 2.5) to 500 ml of 0.2 M pyridine–acetate (pH 3.1). Fractions of 6 ml were collected; 50-μl aliquots of alternate fractions were monitored as outlined in Figure 4. The solid bars indicate the fractions pooled.

et al. (1968). The aqueous layer was washed three times with 1.0-ml aliquots of chloroform to remove traces of 8-hydroxyquinoline and finally taken to dryness in a rotary evaporator.

Edman Degradation. The carbamylated peptide was subjected to subtractive Edman degradation (Konigsberg and Hill, 1962), as modified by Shearer *et al.* (1967). However, for amino acid analysis the modified peptide was hydrolyzed with 5 N NaOH in evacuated and sealed Pyrex tubes at 110° for 24 hr to reconvert homocitrulline into lysine. After hydrolysis the NaOH was neutralized with HCl and the solution was evaporated to dryness. The residue was stirred with 3.0 ml of citrate buffer (pH 2.2) (Moore and Stein, 1954) and insoluble silicates were removed by centrifugation; 2.5 ml of the clear supernatant solution was taken for amino acid analysis.

Isolation of the Peptide Val-(Asp)$_4$-Lys-Ile-Val-Gly-Gly-Tyr from the Chymotryptic Digest of S-Sulfotrypsinogen. *S*-Sulfotrypsinogen was prepared from about 2.5 g of trypsinogen by the method described by Pechère *et al.* (1958). The chymotryptic digest of *S*-sulfotrypsinogen was prepared according to Walsh *et al.* (1962); 1.3 g of the lyophilized digest was dissolved in 20 ml of 0.05 M pyridine–acetate buffer (pH 2.5); the pH of the solution was adjusted to 2.2 with HCl and the solution was centrifuged. The clear supernatant solution was applied to a column of Dowex 50-X8 (200–400 mesh) and developed as described in Figure 4. The resulting peptide fractions were pooled as shown in Figure 4. Examination of the pools by electrophoresis at pH 6.5 revealed that peak 5 contained the most anionic peptide with a trace of contaminants. The amino acid composition corresponded to the N-terminal 11 amino acid residues of trypsinogen. The material obtained from peak 5 of two identical chromatograms was pooled and further purified on a column of Dowex 50-X8 (200–400 mesh) which was previously equilibrated with 0.05 M pyridine–acetate buffer (pH 2.5). The column was developed as illustrated in Figure 5 and the fractions containing the purified peptide were pooled as shown in Figure 5, lyophilized, and the amino acid composition was determined.

Tryptic Hydrolysis of Val-(Asp)$_4$-Lys-Ile-Val-Gly-Gly-Tyr. The rate of cleavage of the Lys–Ile bond by trypsin in the above peptide was followed by end-group analysis; 2.0

TABLE I: Amino Acid Compositions of Trypsinogen, Modified Trypsinogen, and the Fractions Isolated from the Tryptic Activation Mixture of Modified Trypsinogen.[a]

Amino Acid	Trypsinogen	Modified Trypsinogen	Active Fraction	Inactive Fraction
Aspartic acid	25.4	26.9	22.1	22.3
Threonine	9.1	9.5	9.2	9.1
Serine	27.7	31.2	30.9	31.6
Glutamic acid	14.5	14.8	13.9	13.9
Proline	9.1	9.0	9.0	9.5
Glycine	24.8	27.6	25.1	25.7
Alanine	14.0	14.0	14.0	14.0
Half-cystine	10.8	11.4	10.1	10.1
Valine	15.5	15.4	13.7	13.3
Methionine	1.6	1.9	1.5	1.4
Isoleucine	13.9	13.9	13.3	13.2
Leucine	13.8	14.2	13.4	13.5
Tyrosine	8.4	9.5	9.3	8.3
Phenylalanine	3.3	3.3	2.9	2.9
Lysine	15.0	14.4	14.0	14.0
Histidine	3.0	3.0	3.0	3.0
Arginine	2.2	2.0	1.9	1.7

[a] The data are expressed as amino acid residues per molecule, assuming 14 residues of alanine and 3 residues of histidine per molecular weight of 24,500. The values represent 24-hr hydrolysate values and are not corrected for hydrolytic destruction.

μmoles of the peptide was dissolved in 0.85 ml of NEMO–acetate at pH 8.0, with or without 0.1 M $CaCl_2$, mixed with 0.15 ml of trypsin solution (16.5 mg/ml), and incubated for 2 hr at 0°. At regular intervals 0.1-ml aliquots were withdrawn into each of a series of tubes containing 1.0 ml of 0.0002 M benzamidine hydrochloride. The amino-terminal residues in these aliquots were determined by the cyanate method of Stark and Smyth (1963).

Results

Modification of Trypsin and Trypsinogen with Glycinamide in the Presence of EDPC. The influence of modification of the carboxylate groups of trypsin at pH 4.5 with glycinamide in the presence of EDPC is shown in Figure 6. After 1 hr, 60% of the esterase activity toward α-*N*-Bz-L-Arg ethyl ester was retained in spite of the incorporation of five residues of glycinamide. However when the reaction was carried out for only 15 min, about 75% of the activity of trypsin was retained with the incorporation of only two residues of glycine. The shorter treatment was adopted for trypsinogen modification since preliminary experiments indicated that these were conditions for modification of the zymogen with minimum loss of activatability. Table I compares the amino acid composition of native trypsinogen with that of modified trypsinogen. It is evident that the modified zymogen contained an excess of about 2.5 glycine residues compared with the native protein.

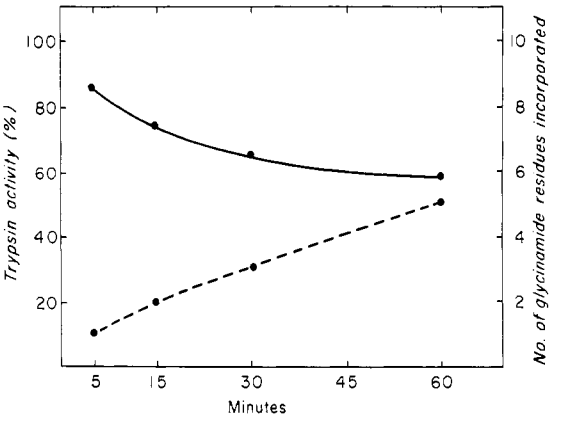

FIGURE 6: Modification of trypsin with glycinamide and EDPC at pH 4.5, 25°. The activity toward α-*N*-Bz-L-Arg ethyl ester (solid line) of the unmodified trypsin was taken as 100%. The broken line traces the incorporation of glycinamide residues.

Activation of Modified Trypsinogen. The course of activation of unmodified and modified trypsinogens, shown in Figure 7, reveals that the modified zymogen is activated to the extent of about 76% of that of the unmodified trypsinogen (control) with or without added calcium ions. Clearly, in contrast to the unmodified trypsinogen (control), calcium does not have any appreciable effect on the modified system.

Separation of Peptides from the Activation Mixture of Modified Trypsinogen. Modified trypsinogen was activated in the absence of calcium for only 90 min at pH 7.0 to minimize autolytic side reactions; 36.4% of zymogen was converted into active trypsin. The peptide fraction obtained by gel filtration of the activation mixture was found to be heterogeneous. As shown in Figure 8, high-voltage paper electrophoresis yields at least four peptides. The composition of the activation peptides is shown in Table II. Peptides A, C, E, and F were found to contain Val, Asp_4, and Lys with glycine residues ranging from 0 to 2 equiv. Peptides B

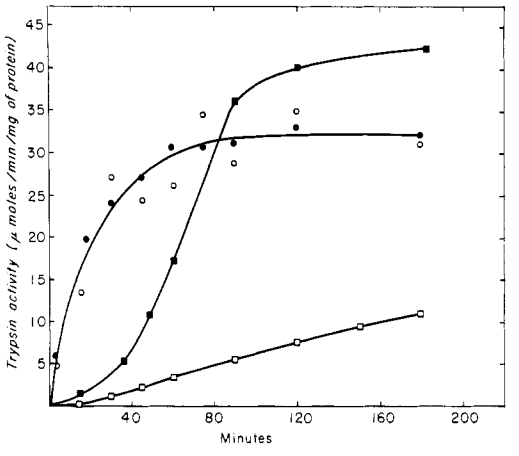

FIGURE 7: The course of activation of native (squares) and modified (circles) trypsinogen in the presence (filled symbols) and absence (open symbols) of 0.05 M $CaCl_2$. The zymogen concentration was 4.2 mg/ml in 0.05 M NEMO–acetate (pH 8.1) at 0°. At time zero, 0.15 mg of trypsin (in 25 μl of 10^{-3} M HCl) was added per ml of activation mixture.

TABLE II: Peptides Derived from the Activation of Trypsinogen Modified with Glycinamide in the Presence of EDPC.[a]

Peptide	Residues of Amino Acids Found (g)				Yield of Peptide (μmoles)	Rel Yield (%)
	Aspartic Acid	Valine	Lysine	Glycine		
A	4.0	0.97	0.99		0.12	6.8
C	4.0	0.98	0.80	0.96	0.71	40.5
E	4.0	1.0	0.83	1.9	0.81	46.3
F	4.0	0.98	1.0	2.2	0.11	6.4
Total					1.75	100

[a] Modified trypsinogen had been activated to the extent of 36.4% and the peptides were separated as in Figure 8. After correcting for analytical losses during the isolation procedures, the maximum theroetical peptide yield is 1.76 μmoles. The actual aggregate yield of activation peptides is 1.75 μmoles.

and D were present in trace amounts and their composition did not correspond to that of the activation peptide. Unlike the results obtained earlier with glycine ethyl ester substituted trypsinogen (Radhakrishnan *et al.*, 1967), in the present experiments, the activation peptides contained integral amounts of glycine. Peptide A corresponds to the unmodified activation peptide (6.8% of total) and is probably derived from small amounts of unmodified trypsinogen. Peptide C has a single glycinamide (yield 40.5%) whereas peptides E and F (yields 46.3 and 6.4%, respectively) each contained two residues of glycinamide and were distinctly separable from each other by high-voltage electrophoresis indicating that they are probably positional isomers. All attempts to fractionate the glycinamide-substituted activation peptide by ion-exchange chromatography were unsuccessful.

Characterization of the Protein Fraction from the Activation Mixture of Modified Trypsinogen. Fully active trypsin was separated from a largely inactive fraction as described in Methods. The inactive fraction was clearly heterogeneous (Figure 2). Only pooled fraction "b" showed esterase activity toward α-*N*-Bz-L-Arg ethyl ester, *i.e.*, 19 μmoles/min per mg of protein compared with 57.6 for native trypsin. The amino acid composition of pooled fraction "b" is given in Table I and reveals a slight elevation of the glycine content over native trypsin.

The active fraction, derived from fraction II (Figure 1) and purified as shown in Figure 3, had an amino acid composition identical with that of trypsin (Table I) and showed a specific activity toward α-*N*-Bz-L-Arg ethyl ester of 51.2 μmoles/min per mg of protein.

Sequence Analysis of the Substituted Val-(Asp)$_4$-Lys Peptides. Subtractive Edman degradation of mono- and diglycinamide-substituted activation peptides were carried out to attempt to locate the positions of glycyl substitution on the aspartyl residues. However, after the first cycle of Edman degradation, calculation of the residues removed in subsequent degradations became difficult because ε-PTC-lysine is not quantitatively recovered as lysine after acid hydrolysis. This difficulty was overcome by reversible conversion of the lysine residue with cyanate (Stark and Smyth, 1963) into homocitrulline while the α-amino group of the peptide was quantitatively protected by chelation with copper according to Bradshaw *et al.* (1968). After removing the copper from the carbamylated peptide, subtractive Edman degradation was carried out. The samples were hydrolyzed with 5 N NaOH to convert homocitrulline quantitatively into lysine and the amino acid composition was determined.

Preliminary experiments with unmodified Val-(Asp)$_4$-Lys substantiated the validity of this approach. The results obtained by sequence analysis of the two major activation peptides are shown in Tables III and IV. In the first step the valyl residues were removed in 86% yield. However, in the subsequent steps only fractional equivalents of aspartyl and

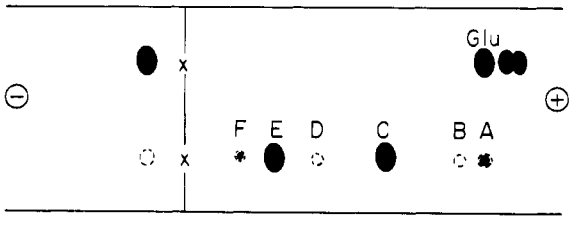

FIGURE 8: Sketch of the electropherogram of the peptide fraction isolated from the activation mixture of modified trypsinogen. Paper electrophoresis was carried out for 2 hr at 35 V/cm on Whatman No. 3 MM paper in pyridine–acetic acid–water (100:4:900, v/v) (pH 6.5). A mixture of amino acids serves as a marker for comparison. The peptides located after dipping in ninhydrin solution were identified as A–F in decreasing order of their mobilities.

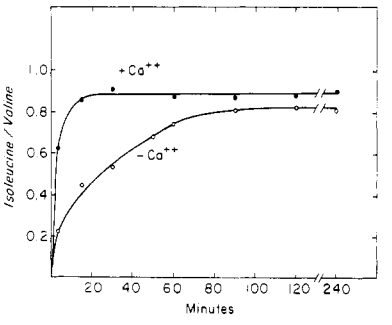

FIGURE 9: The course of hydrolysis of Lys^6–Ile^7 bond in the N-terminal undecapeptide of trypsinogen by trypsin in the presence (filled circles) and absence (open circles) of 0.085 M $CaCl_2$. The peptide-bond cleavage was followed by the cyanate method (Stark and Smyth, 1963) with aliquots withdrawn at regular intervals and the ratios of Ile/Val were plotted against time.

TABLE III: Subtractive Edman Degradation of Monoglycyl-Val(Asp)$_4$-homocitrulline (Peptide C).

	Number of Cycles of Edman Degradation									
	1		2		3		4		5	
Amino Acid	Ratio of Residues	Residues Lost	Ratio of Residues	Residues Lost	Ratio of Residues	Residues Lost	Ratio of Residues	Residues Lost	Ratio of Residues	Residues Lost
Lysine	1.00	0	1.00	0	1.0	0	1.00	0	1.00	0
Aspartic acid	4.31	0	3.56	0.75	2.82	0.74	1.98	0.84	1.59	0.39
Glycine	1.09	0	1.01	0.08	0.91	0.10	0.72	0.19	0.56	0.16
Valine	0.15	0.85								

TABLE IV: Subtractive Edman Degradation of Diglycyl-Val-(Asp)$_4$-homocitrulline (Peptide E).

	Number of Cycles of Edman Degradation									
	1		2		3		4		5	
Amino Acid	Ratio of Residues	Residues Lost	Ratio of Residues	Residues Lost	Ratio of Residues	Residues Lost	Ratio of Residues	Residues Lost	Ratio of Residues	Residues Lost
Lysine	1.00	0	1.00	0	1.00	0	1.00	0	1.00	0
Aspartic acid	4.19	0	3.44	0.75	2.86	0.58	2.10	0.76	1.39	0.71
Glycine	2.00	0	1.88	0.12	1.62	0.26	1.39	0.23	0.93	0.46
Valine	0.13	0.87								

glycyl residues were removed and hence it is difficult to assign exact positions to the glycyl residues along the sequence of aspartyl residues. The data seem to indicate that all aspartyl residues are modified to some degree in a rather random manner.

Studies with the Peptide Val-(Asp)$_4$-Lys-Ile-Val-Gly-Gly-Tyr. The N-terminal undecapeptide was isolated from the chymotryptic digest of *S*-sulfotrypsinogen (Figures 4 and 5). Peak 2 of the elution pattern illustrated in Figure 5 had the composition, Val (1.7), Asp (4.0), Lys (1.1), Ile (0.7), Gly (2.0), and Tyr (0.8), corresponding to the sequence 1–11 in trypsinogen (Walsh *et al.*, 1962). The low values for isoleucine and valine are expected because of the incomplete cleavage of the Ile–Val bond during 24-hr hydrolysis.

Figure 9 illustrates the course of tryptic hydrolysis of this peptide at Lys^6–Ile^7 in the presence and absence of calcium chloride at pH 8.0 and reveals that calcium ions promote hydrolysis of this peptide by trypsin.

Discussion

A characteristic structural feature of bovine trypsinogen, shared by trypsinogens of other species such as pig (Charles *et al.*, 1963), sheep (Schyns *et al.*, 1969), and goat (Bricteux-Grègoire *et al.*, 1968) is the acidic tetraaspartyl sequence in the amino-terminal peptide which is released during activation. Another, functional feature common to these zymogens, is the enhancement of tryptic activation by calcium and other divalent ions of the alkaline earth series which seemingly direct trypsin to the Lys^6–Ile^7 bond in bovine trypsinogen and suppress the formation of "inert protein" (MacDonald and Kunitz, 1941). It has long been suspected that these two phenomena, one structural and the other functional, are related to each other and the present investigation was therefore undertaken to clarify this relationship. The problem is of more general significance since it has been recently observed that calcium ions also promote the activation of certain other zymogens, such as bovine (Cox *et al.*, 1964) and dogfish (Lacko and Neurath, 1967) procarboxypeptidase A. Preliminary structural studies suggest that at least in one of these instances, *i.e.*, bovine procarboxypeptidase A, the activation peptide also contains an excess of negatively charged carboxylate side chains (Freisheim *et al.*, 1967).

The results of the present investigation clearly demonstrate that partial abolishment of the negative charges of the aspartyl residues by introduction of glycinamide groups eliminates the calcium effect. Both the rate and extent of activation are independent of the presence of calcium ions in the glycinamide-substituted zymogen. Analysis of the peptide cleaved during the activation reaction revealed that modification of but 1 equiv of aspartyl residues eliminates the calcium requirements. The modification reaction is specific in the sense that under minimal conditions none of the carboxylate groups on the trypsin moiety of the zymogen becomes substituted. The present experiments also demonstrate that calcium affects in a similar manner the hydrolysis of this bond in the

isolated undecapeptide fragment of trypsinogen, and earlier studies from this laboratory (Pechère *et al.*, 1958) have shown the same effect with *S*-sulfotrypsinogen. In a qualitative sense, therefore, the enhancement of tryptic cleavage of the Lys^6–Ile^7 bond by calcium or by modification by glycinamide is independent of any role of the remainder of the zymogen.

A mechanistic explanation of these phenomena requires consideration of the possible sites of interaction of calcium ions with trypsinogen and the products of activation, trypsin, and the activation peptide. By necessity, such deductions must also involve the most fundamental aspect of the process of zymogen activation, *i.e.*, the specificity of limited peptide-bond hydrolysis, and the role of the activation peptide in this reaction.

It has been proposed by Delaage and Lazdunski (1967) that trypsinogen possesses two binding sites for calcium, one residing in the trypsin moiety of the zymogen, and the other, weaker one, in the activation peptide. Model studies involving analogs of the N-terminal region of trypsinogen, have demonstrated that the affinity of these peptides for trypsin is greater when aspartyl residues are replaced by uncharged alanyl residues and that the calcium effect is abolished at the same time (Delaage *et al.*, 1967). The results of the present investigation are clearly in accord with these latter observations and support the conclusion that one of the sites of interaction of trypsinogen with calcium must be the carboxylate side chains of the activation peptide.

A quantitative correlation between the number and site of the aspartyl residues that are modified and the abolishment of the calcium requirements cannot be made from the present observations because partial substitution of the aspartyl residues occurs more or less randomly thus precluding identification of the specific groups that are involved in calcium binding. The consequences of the calcium requirements have been recently discussed by Abita *et al.* (1969) in a paper which came to our attention as the present manuscript was nearing completion. The main conclusions derived by these investigators from kinetic study of the activation of bovine trypsinogen and other related zymogens, and involving also model peptides, are clearly in accord with the views expressed here and in preceding publications from this laboratory. These authors have shown that the presence of but two aspartyl residues in model peptides lowers the affinity to trypsin, in a manner that can be reversed by calcium but has no effect on the rate constant, k_{cat}, of the hydrolysis of the Lys^6–Ile^7 bond. The complete set of four aspartyl residues reduces k_{cat} by two to three orders of magnitude both in the N-terminal nonapeptide fragment and in trypsinogen. The present findings that the introduction of but one to two glycinamide residues abolishes the calcium effect are in qualitative accord with the results obtained with the model peptides. Taken together, all these observations suggest the absence of any unique conformation of the N-terminal fragment in native trypsinogen that would be different from that of the same region in *S*-sulfotrypsinogen (Pechère *et al.*, 1958) or in model peptides. Thus the relatively slow hydrolysis rate of the Lys^6–Ile^7 bond even in the presence of calcium ions appears to be the consequence of the chemical character of the amino acid sequence. The phenomenon of limited hydrolysis that characterizes the activation of trypsinogen and other zymogens must therefore be related to the lack of reactivity of all other lysyl or arginyl bonds in the zymogen (Neurath, 1957) and their inert character at neutral pH is dependent upon the presence of calcium ions in a manner that may be different from the calcium effects that have been observed in the present study. The appearance of enzymatic activity concomitant to the cleavage of the Lys^6–Ile^7 bond also appears mechanistically unrelated to the character of the activation peptide but rather must be referred to aspects of the process which generates the conformation which is achieved after the activation peptide has been released.

Finally, it should be noted that carboxylate modification with glycinamide not only abolishes the calcium effect but greatly enhances the rate of activation (Figure 7). The present studies do not distinguish between an effect on K_m and on k_{cat} and hence a quantitative interpretation of these data must await further analyses. Contrary to our preliminary report (Radhakrishnan *et al.*, 1967), it is now certain calcium does not enhance the activation of the modified zymogen. The small enhancement previously noted appears to be the result of the lower stability of the glycine ethyl ester used in earlier work for substitution, as compared with glycinamide in the present investigation. Partial loss of the ester function would result in a new carboxylate group in an altered location and thus once again give rise to a binding site for calcium.

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Studies on Steroid Hydroxylase. Molecular Properties of Adrenal Iron–Sulfur Protein*

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ABSTRACT: The molecular weight of adrenal iron–sulfur protein (adrenodoxin) is approximately 12,000 as determined by sedimentation–diffusion, by sedimentation–equilibrium, and from amino acid composition of the protein. The partial specific volume is 0.70 ml/g at 20°, and the diffusion coefficient, $D_{20,w}$, is 11.2 × 10^{-7} cm^2 sec^{-1}. As described previously, the sedimentation coefficient, $s_{20,w}$, is 1.55 S. The frictional ratio, f/f_0, was calculated to be 1.29. The intrinsic viscosity is 3.0 ml/g at 22°. Therefore, the protein is a globular protein with the value of a/b (for major to minor axis of an ellipsoid) = about 5.

The adrenal iron–sulfur protein contains approximately 100 amino acid residues with an abundance of acidic amino acids and a paucity of aromatic amino acids. From the ultraviolet optical rotatory dispersion, circular dichroism, and infrared absorption measurements, a gross difference in protein conformation between adrenal and spinach iron–sulfur proteins was found.

Adrenal iron–sulfur protein (adrenodoxin)[1] containing both nonheme iron and labile sulfur has been isolated from mitochondria of the adrenal cortex and found to serve as an oxidation–reduction component in the electron transport system of steroid hydroxylation reactions at the 11β, 18, 20, and 22 positions (Suzuki and Kimura, 1965; Omura *et al.*, 1965; Nakamura *et al.*, 1966; Ichii *et al.*, 1967).

Investigation of those physical and chemical properties of adrenal iron–sulfur protein which are influenced by the nonheme iron and its environment revealed marked similarities to plant ferredoxins and distinct difference from bacterial ferredoxins. Thus, both adrenal iron–sulfur protein and plant ferredoxins appear to have an identical iron–sulfur linkage as an essential entity for catalysis, as judged from optical absorption (Kimura and Suzuki, 1967), optical rotatory dispersion (Kimura and Suzuki, 1967), circular dichroism (Palmer *et al.*, 1967; Kimura and Ohno, 1968), electron paramagnetism (Watari and Kimura, 1966), and magnetic susceptibility[2] measurements. However, in spite of fundamentally identical features of the iron coordination structure of both adrenal and plant proteins, spinach ferredoxin cannot substitute for adrenal iron–sulfur protein in the steroid 11β-hydroxylation reaction, and conversely, adrenal iron–sulfur protein cannot replace spinach ferredoxin in the photosynthetic pyridine nucleotide reduction mediated by chloroplasts (Suzuki and Kimura, 1965; Kimura and Ohno, 1968). These experimental facts led us to investigate the detailed properties besides the iron coordination structure of adrenal iron–sulfur protein in comparison with those of plant ferredoxins.

Experimental Procedure

Materials. Adrenal iron–sulfur protein was prepared from beef adrenal cortex tissue by the previously described pro-

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[1] A trivial name for adrenal iron–sulfur protein in the steroid hydroxylases.

[2] These data were presented by T. Kimura at the 3rd International Conference of Magnetic Resonance, Va., Oct 1968.