Preparation and Characterization of Guanidinated Trypsinogen and ε-Guanidinated Trypsin†

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ABSTRACT: Reaction of bovine trypsinogen with 1-guanyl-3,5-dimethylpyrazole converted all 15 lysine residues to homoarginyl residues. Trypsin could not activate guanidinated trypsinogen but an acid protease from *Aspergillus oryzae* generated active ε-guanidinated trypsin by cleaving the Har_6–Ile_7 bond in the modified zymogen and releasing the activation peptide Val-$(Asp)_4$-Har. After purification by affinity chromatography, the ε-guanidinated enzyme contained 0.94–0.98 active site/molecule and exhibited the same specific activity toward benzoyl-L-arginine ethyl ester as β-trypsin.

The α-amino group of bovine trypsin (Scrimger and Hofmann, 1967) and of other serine proteases (Ghelis *et al.*, 1970; Hess, 1971) has been implicated in enzymatic function. According to X-ray analyses this amino group forms a salt bridge with an aspartic acid residue which is adjacent to the serine residue of the active site (Blow *et al.*, 1969; Blow, 1971; Stroud *et al.*, 1972). In order to test the involvement of the α-amino group by chemical means it would be necessary to eliminate the reactivity of all other amino groups toward the reagent used for chemical modification. Since the differential reactivity of α- and ε-amino groups toward most reagents is not absolute, we have chosen to modify the zymogen and to convert it to a modified enzyme in which the α-amino group alone is free.

This approach was successfully applied to chymotrypsin by acetylating the zymogen and converting it to ε-acetylated δ-chymotrypsin with a free α-amino group (Ghelis *et al.*, 1970). The same procedure cannot be applied to trypsinogen since activation involves the cleavage of a Lys–Ile bond instead of an Arg–Ile bond and N-substituted lysyl bonds are resistant to tryptic hydrolysis. A number of other procedures for the chemical modification of amino groups were explored and the products tested for their ability to be activated by trypsin or other proteolytic enzymes. Amidination and guanidination were successful in this regard since the resulting derivatives could be activated by a mold protease. Guanidination was chosen for this study, and the preparation and characterization of guanidinated trypsinogen and the products of its activation are described in this communication.

Experimental Section

Materials

Bovine trypsinogen (once crystallized) and bovine trypsin (twice crystallized) were obtained from Worthington Biochemical Corp. Stock solutions were dialyzed against 1 mM HCl. "Rhozyme 41 Conc.," a proteolytic enzyme preparation from *Aspergillus oryzae*, was obtained from Rohm and Haas.

Ethyl acetimidate-HCl was synthesized by the method of McElvain and Nelson (1942). 1-Guanyl-3,5-dimethylpyrazole nitrate (GDMP)[1] was synthesized from 2,4-pentanedione and aminoguanidine by the method of Bannard *et al.* (1958).

DEAE-cellulose (Selectacel DEAE, 0.89 mequiv/g) was obtained from the Brown Co. Various Sephadex products were purchased from Pharmacia Fine Chemicals and prepared for use according to the manufacturer's recommendations.

N-α-Benzoyl-L-arginine ethyl ester (BAEE) was obtained from Cyclo Chemical Co. *p*-Nitrophenyl-*p'*-guanidinobenzoic acid (NPGB) was prepared in our laboratory by Dr. R. A. Kenner using the method of Chase and Shaw (1967).

Hydroxylamine (Baker Chemical Co.) was recrystallized from methanol. *N*-Ethylmorpholine (Eastman Organic Co.) was redistilled from ninhydrin and stored at −20° to prevent decomposition. α-Aminobutyric hydantoin was a gift of Dr. P. H. Petra. Norleucine was obtained from Calbiochem Corp.; sodium dodecyl sulfate from Eastman Kodak Co.; and benzamidine hydrochloride from Aldrich Chemical Co.

Methods

Preparation of Chemically Modified Trypsinogens. Trypsinogen (1.4×10^{-4} M) was *guanidinated* with 0.7 M GDMP by a modification of the method of Habeeb (1959, 1960). The reagent was dissolved in 2 M NaOH and the pH adjusted to 9.9 at room temperature. After the solution was cooled to 4°, trypsinogen was added and the pH was readjusted to 9.9. After 72 hr at 4° the pH was adjusted to 3 with HCl or formic acid and the excess reagent removed by dialysis against 1 mM HCl. Insoluble protein was removed by centrifugation.

Trypsinogen (1×10^{-4} M) was *acetylated* with acetic anhydride by the method of Fraenkel-Conrat *et al.* (1949) or *succinylated* by the method of Habeeb *et al.* (1958). The zymogen was *amidinated* by a modification of the methods of Wofsy and Singer (1963) and Hunter and Ludwig (1962). A 200-fold molar excess of ethyl acetimidate-HCl was added to trypsinogen (2×10^{-4} M) in 0.1 M *N*-ethylmorpholine–0.02 M $CaCl_2$, 4°, and the pH was adjusted to 9.0. In each case the reaction

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[1] Abbreviations used are: AOAP, *Aspergillus oryzae* acid protease; BAEE, *N*-α-benzoyl-L-arginine ethyl ester; CHOM, chicken ovomucoid; GDMP, 1-guanyl-3,5-dimethylpyrazole nitrate; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoic acid.

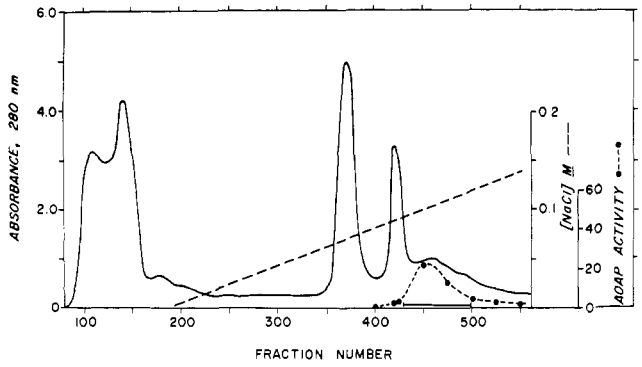

FIGURE 1: Chromatography of a water extract of Rhozyme 41 on a 5 × 80 cm column of DEAE-cellulose equilibrated with 0.01 M sodium acetate (pH 4.50). After sample application, the column was washed with 2 l. of equilibrating buffer and developed with 14 l. of a linear gradient reaching 0.2 M NaCl in 0.01 M sodium acetate buffer at pH 4.50. Fractions of 25 ml were collected at a flow rate of 1 l./hr and the active fractions pooled as indicated. AOAP activity was determined by adding 0.050 ml of a diluted (1:20) sample to 0.50 ml of trypsinogen (2 mg/ml) in 0.05 M sodium formate (pH 3.5) at 0° and measuring tryptic activity after 30 min.

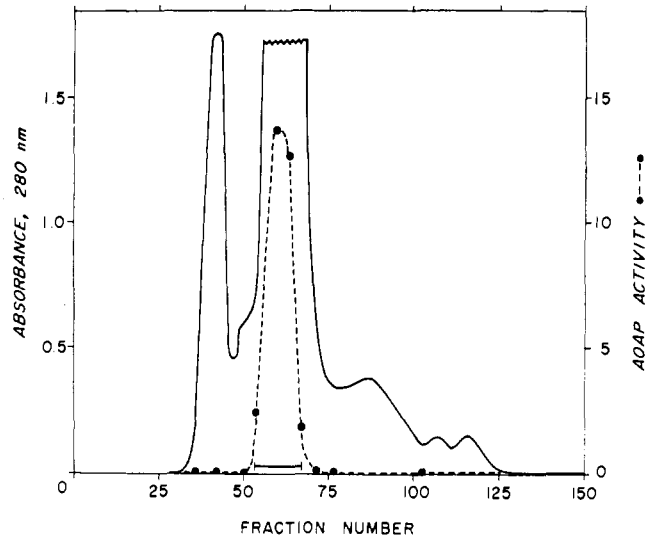

FIGURE 2: Gel filtration of the pooled active fractions of Figure 1 on Sephadex G-100 (see text for details). The flow rate was 24 ml/hr; the fraction size was 4.8 ml. AOAP activity was measured as described in Figure 1 and is given in relative units after 10 min of activation.

was stopped by adjusting the pH to 3 with HCl and the product was dialyzed against 1 mM HCl to remove excess reagent.

Purification of A. oryzae Acid Protease (AOAP).[2] AOAP was extracted from Rhozyme 41 by stirring 200 g in 2 l. of water at 4° for 2 hr. Subsequent operations were also performed at 4°. The insoluble material was removed by centrifugation at 16,000*g* for 20 min. The resulting solution was lyophilized, redissolved in 800 ml of 0.01 M sodium acetate buffer, and the pH was adjusted to 4.5. Precipitated protein was removed by centrifugation at 18,000*g* for 40 min.

The supernatant solution (A_{280} 1.5) was applied to a DEAE-cellulose column at pH 4.50 and the protein was eluted as described in the legend to Figure 1. Fractions containing the acid protease were pooled and concentrated to 9 ml by ultrafiltration. The concentrate was applied to a 2.5 × 110 cm column of Sephadex G-100 which had been previously equilibrated with 0.01 M sodium acetate (pH 4.50) containing 0.06 M NaCl. The elution of fractions containing AOAP activity is shown in Figure 2.

The pooled fractions were applied to a 2.5 × 40 cm column of DEAE-Sephadex A-50 equilibrated with 0.01 M sodium acetate (pH 4.50) containing 0.06 M NaCl. A linear gradient of 0.06–0.26 M NaCl was developed in a total volume of 2 l. at 25 ml/hr. Fractions containing enzymatic activity coincided with those absorbing at 280 nm, indicating no further purification was necessary. These fractions were pooled and stored either in solution or frozen in 0.01 M sodium acetate buffer (pH 4.50) containing 0.06 M NaCl. The protein appeared to be homogeneous by disc gel electrophoresis at pH 4.5 and 8.9.

Chromatographic Methods. Trypsinogen was separated from trypsin by chromatography on SE-Sephadex C-50 at 4°. The eluting buffer contained 0.05 M sodium acetate–0.20 M NaCl (pH 4.20). For the separation of guanidinated trypsinogen and ε-guanidinated trypsin, the NaCl concentration was raised to 0.3 M. Alternatively the guanidinated proteins were separated by chromatography on SE-Sephadex C-50 at pH 3.00 (Sanders, 1969). The eluting buffer containing 0.005 M sodium citrate–0.31 M NaCl (pH 3.00). In some cases trypsin and ε-guanidinated trypsin were further purified by affinity chromatography on CHOM-Sepharose (Robinson *et al.*, 1971). Dilute protein solutions were concentrated by ultrafiltration at 4° in an Amicon cell under nitrogen pressure using a UM-10 membrane.

Activation of Trypsinogen and Its Derivatives. Trypsinogen (5–10 mg/ml) was activated by trypsin at pH 8.1 in 0.1 M Tris buffer containing 0.05 M $CaCl_2$ at 0° (Pechère and Neurath, 1957). The initial ratio of trypsin to trypsinogen was 1:20. Using these conditions, maximum activation of about 75% of the protein occurred in 90–120 min.

Alternatively trypsinogen or its derivatives (1.0–2.0 mg/ml) were activated by AOAP at 0° in 0.05 M sodium formate buffer (pH 3.5) (the pH optimum), by adding 25 units[3] of AOAP/ml of solution. Maximum activation of 75% of the protein occurred in 90–120 min. The percentage activation of trypsinogen was independent of the concentrations of either AOAP (5–125 units/ml) or trypsinogen (1–10 mg/ml).

Enzymatic Assays. Esterase activity toward BAEE was measured in a pH-Stat as described by Walsh and Wilcox (1970). Pure trypsin catalyzes the hydrolysis of 67 μmol of BAEE/min per mg of enzyme (Robinson *et al.*, 1971).

The concentration of active sites was determined with *p*-nitrophenyl-*p'*-guanidinobenzoic acid (NPGB) in a Cary Model 16 spectrophotometer as described by Chase and Shaw (1967).

Amino-Terminal Analysis. Amino-terminal residues were determined by the cyanate method of Stark and Smyth (1963) using the denaturation procedure and internal standards suggested by Gertler and Hofmann (1967). Amino-terminal sequences were determined with a Beckman Model 890A Sequencer according to the method of Edman and Begg (1967) as modified by Hermodson *et al.* (1972).

Amino acid analysis was performed on a Beckman/Spinco Model 120B analyzer using the procedure of Spackman (1967).

[2] This acid protease was referred to as "trypsinogen kinase" by Nakanishi (1959) who partially purified the enzyme from *A. oryzae*.

[3] One unit of AOAP represents 1 μl of the purified preparation with an absorbance at 280 nm of 1.00 cm^{-1}.

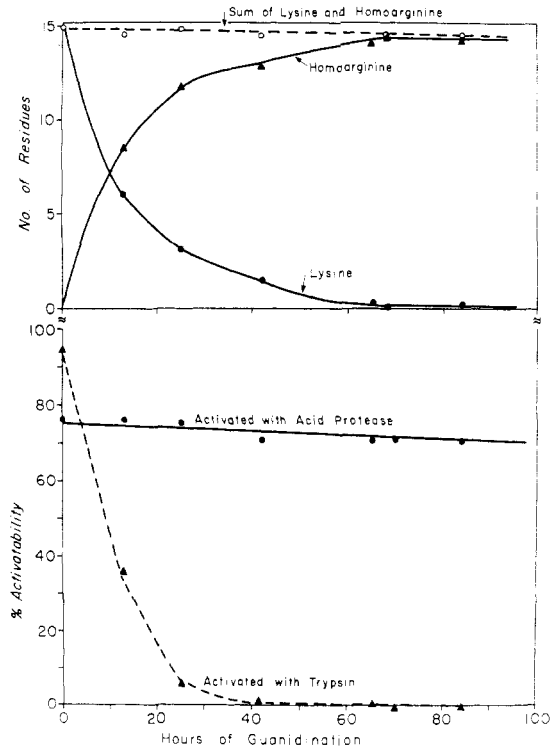

FIGURE 3: Time course of the guanidination of trypsinogen (see text). The upper diagram indicates the conversion of lysyl residues to homoarginyl residues measured after acid hydrolysis. In the lower diagram the per cent activatability was measured by assaying for tryptic activity after a standard activation of the modified trypsinogen with either AOAP or trypsin. (●) Activation by AOAP; (▲) activation by trypsin (90 min).

The ninhydrin color yield of homoarginine was identical with that of arginine.

Extinction coefficients were determined with a Cary Model 16 spectrophotometer and a differential refractometer (Phoenix Precision Instrument Co.) as described previously (Robinson *et al.*, 1971). In 1 mM HCl, $A_{280}^{1\%}$ was 15.0 cm^{-1} for trypsinogen and guanidinated trypsinogen, and 15.4 cm^{-1} for trypsin and ε-guanidinated trypsin.

Difference Spectra. The effect of NH_2OH on guanidinated trypsinogen was examined by measuring difference spectra in a Cary Model 15 dual-beam spectrophotometer. Solutions were prepared containing 4 M guanidine hydrochloride, 0.0625 M morpholinopropanesulfonic acid (pH 7.5), and either 0.625 M NH_2OH or 0.625 M NaCl. One milliliter of a protein solution (10 mg/ml in 1 mM HCl) was mixed with 5 ml of the guanidine hydrochloride–NaCl solution and allowed to denature for 2 hr. Equal volumes of each solution were placed in adjacent sectors of a two-sector difference spectra cell and a base line was established against a control cell prepared in an identical manner. The two solutions were mixed in the sample cell and spectra were recorded at 10-min intervals.

Results

Exploratory experiments established that acetylation, succinylation, amidination, or guanidination of the amino groups of trypsinogen prevents subsequent activation by trypsin. Amidinated and guanidinated trypsinogen can, however, be activated by AOAP. The guanidinated derivative was selected for further study because the extent of conversion of lysyl to homoarginyl residues can be quantitatively determined by amino acid analysis whereas amidinated lysyl residues are partially destroyed by acid hydrolysis.

TABLE I: Amino Acid Compositions of Trypsinogen and Guanidinated Trypsinogen.

Amino Acid	Trypsinogen[a]	Guanidinated Trypsinogen[a]	Lit. Values[b]
Aspartic acid	26.0	26.1	26
Threonine	9.3	9.3	10
Serine	32.2	32.3	33
Glutamic acid	14.4	14.1	14
Proline	9.4	9.2	9
Glycine	25.0	24.5	25
Alanine	14.0	14.0	14
Half-cystine[c]	9.5	9.8	12
Valine	13.2	12.8	18
Methionine	2.0	2.0	2
Isoleucine	12.7	12.4	15
Leucine	13.7	13.8	14
Tyrosine	9.7	9.6	10
Phenylalanine	3.1	3.1	3
Lysine	14.7	0.1	15
Histidine	3.0	3.1	3
Arginine	2.0	2.0	2
Homoarginine	0.0	14.8	0

[a] Average of duplicate analyses after 18-hr hydrolysis; 14.0 alanyl residues/molecule are assumed. [b] Walsh and Neurath (1964). [c] Half-cystine was not oxidized to cysteic acid.

Guanidinated Trypsinogen. The time course of guanidination was established by allowing 1.4×10^{-4} M trypsinogen to react with 0.7 M GDMP (pH 9.9) at 4°. At various times aliquots of the reaction mixture were adjusted to pH 3 and dialyzed against 1 mM HCl to remove excess reagent. Insoluble protein was removed by centrifugation and the soluble zymogens were activated by either trypsin or AOAP. The results of these analyses are shown in Figure 3. After 40 hr, activation by trypsin was completely abolished whereas activation by AOAP[4] was only slightly diminished. Complete guanidination required about 72 hr.

Comparison of the amino acid compositions of trypsinogen and guanidinated trypsinogen (Table I) indicated that the conversion of lysine to homoarginine was the only acid-stable modification. The extent of modification of the amino-terminal valyl residue could not be determined in this manner since several valyl peptides are slowly hydrolyzed in trypsinogen and fractional modification of one valine would not have been detected. Amino-terminal analysis by the cyanate method yielded 0.87 mol of valine and 0.14 mol of aspartic acid per mol of guanidinated trypsinogen, and 0.98 mol of valine and 0.11 mol of aspartic acid per mol of trypsinogen. Thus about 10% of the α-amino groups of Val_1 became guanidinated.

Chromatographic Purity of Guanidinated Trypsinogen. The chromatographic behavior of guanidinated trypsinogen on a column of Sephadex G-100 at pH 4.40 was compared with that of trypsinogen. Both proteins eluted at the same position

[4] Only 75% of trypsinogen is activated by AOAP. The remainder is largely inactivated by proteolysis.

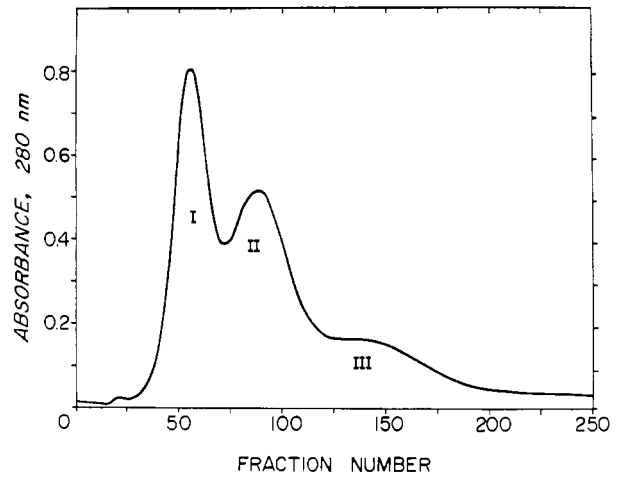

FIGURE 4: Equilibrium ion exchange chromatography of guanidinated trypsinogen (61 mg) on a 1.5 × 26 cm column of SE-Sephadex C-50 equilibrated with 0.05 M sodium acetate–0.30 M NaCl (pH 4.20). The flow rate was 14.6 ml/hr and eight fractions were collected per hr. Three different fractions are identified by their chromatographic mobility.

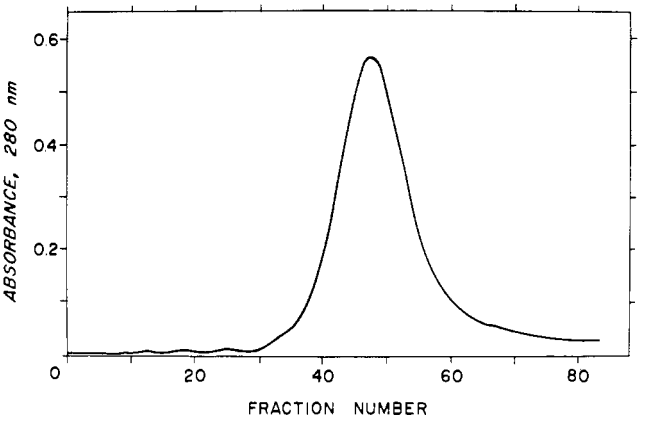

FIGURE 5: Chromatography of guanidinated trypsinogen on a 2.5 × 40 cm column of SE-Sephadex at pH 4.20 after treatment with 1 M NH_2OH for 20 min at pH 7.5. Treated guanidinated trypsinogen (57 mg) in 7.6 ml was applied to the column which had been previously equilibrated with 0.05 M sodium acetate (pH 4.20) containing 0.30 M NaCl. The flow rate was 43 ml/hr and the fraction size was 8.6 ml.

as single, symmetrical peaks, without any evidence of aggregation induced by guanidination.

The purity of guanidinated trypsinogen was also analyzed by equilibrium ion-exchange chromatography on SE-Sephadex at pH 3.00 and 4.20. At each pH at least three components were observed (Figure 4). Rechromatography of each component under the same conditions yielded single homogeneous peaks at the original positions. Amino acid analysis revealed that in each fraction all of the lysyl residues had been guanidinated.

In a control experiment, trypsinogen (in 1 mM benzamidine) was exposed to the same guanidinating conditions for 72 hr in the presence of 0.1 M *N*-ethylmorpholine and 0.7 M KCl in place of 0.7 M GDMP. The product was indistinguishable from untreated trypsinogen during equilibrium chromatography on SE-Sephadex at pH 4.2. It was concluded that the multiple peaks of guanidinated trypsinogen were caused by interaction of the protein with the reagent rather than by prolonged exposure to high pH.

Effect of Hydroxylamine on Guanidinated Trypsinogen. After treatment of unfractionated guanidinated trypsinogen with 1.0 M NH_2OH at pH 7.5 for 20 min at room temperature, the protein chromatographed as a single symmetrical peak at pH 4.20 (Figure 5) and pH 3.00. When individual fractions were similarly treated with NH_2OH, fraction II (Figure 4) was converted to a species chromatographically indistinguishable from fraction I, whereas fraction I was unaffected.

Treatment of fraction II with NH_2OH also produced changes in the absorption spectrum. The molar extinction coefficient at 280 nm increased by 300 ± 100 cm^{-1}. Treatment of native trypsinogen or of fraction I produced no changes.

The effect of NH_2OH was also evident in the chromatographic behavior of the products of zymogen activation. ε-Guanidinated trypsin was chromatographically homogeneous only if the guanidinated zymogen or enzyme had been treated with NH_2OH (Figure 6).

Rate of Activation by AOAP. The conversion of trypsinogen and of guanidinated trypsinogen by AOAP at pH 3.5 followed pseudo-first-order kinetics (Figure 7). The activation rate of guanidinated trypsinogen was about five times greater than that of trypsinogen.

Isolation of the Activation Peptide. In order to isolate the activation peptide, guanidinated trypsinogen (50 mg) was activated with AOAP in 0.05 M pyridine formate buffer (pH 3.5). After various time intervals, the reaction was stopped by quickly raising the pH of aliquots to 5 with pyridine and freezing the solutions. The samples were lyophilized, dissolved in water, and applied to Whatman No. 3MM paper. After 90 min of electrophoresis at pH 6.5 and 40 V/cm (Naughton *et al.*,

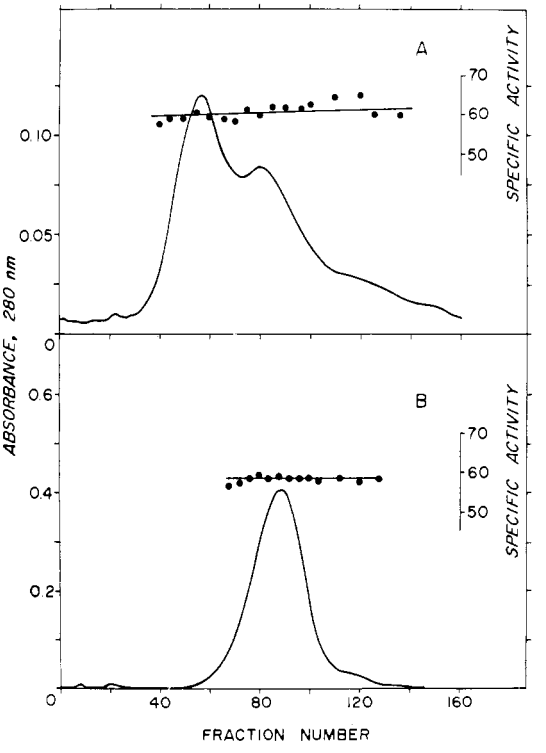

FIGURE 6: Equilibrium ion-exchange chromatography of guanidinated trypsin on SE-Sephadex at pH 3.00 in 0.005 M sodium citrate–0.38 M NaCl. The guanidinated trypsin had been generated from guanidinated trypsinogen before (A) and after (B) treatment with NH_2OH (see text). (A) Column size 1.5 × 15 cm; flow rate 20 ml/hr; ten fractions were collected per hour. (B) Column size 1.5 × 25 cm; flow rate 12 ml/hr; six fractions were collected per hour.

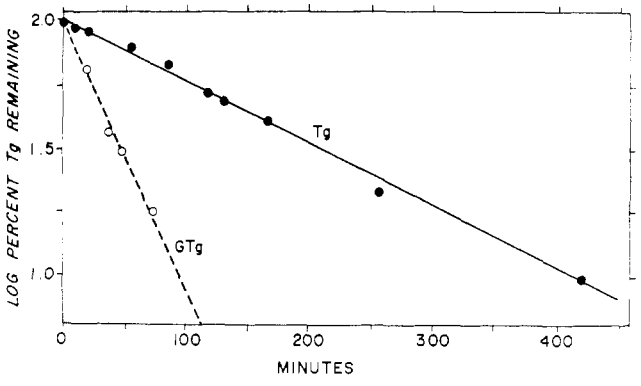

FIGURE 7: First-order rate plots of the activation of trypsinogen (Tg) and guanidinated trypsinogen (GTg) by AOAP. The activation conditions were: 7.65 mg of zymogen/ml; 5.5 units of AOAP/ml; 0.03 M sodium formate (pH 3.5), 0°.

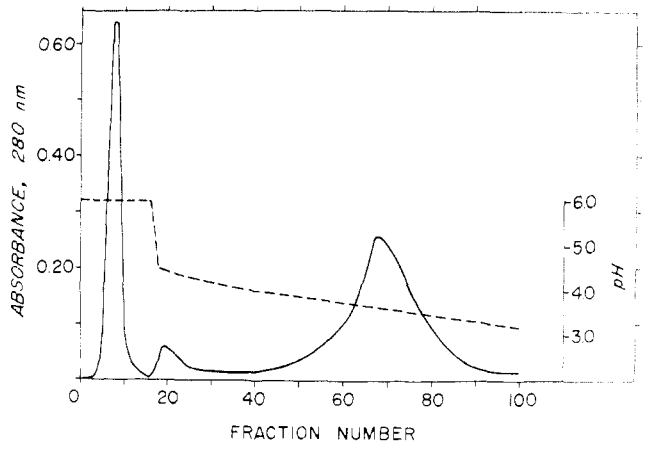

FIGURE 8: Chromatography at 4° of AOAP-activated guanidinated trypsinogen on a 0.9 × 66 cm column of CHOM-Sepharose. The column was equilibrated at pH 6.0 and a pH gradient (dashed line) from pH 4.50 to 2.75 was applied. To form the gradient two identical chambers were used. The first contained 0.1 M formic acid–0.50 M KCl, adjusted at *room temperature* to pH 4.50 with KOH; the second chamber contained 0.1 M formic acid–0.50 M KCl, adjusted to pH 2.75. Fractions of 14 ml were collected at a flow rate of 70 ml/hr. Tryptic activity was found only in fractions 50–90. The first two chromatographic peaks are inactive components.

1960), peptides were located on guide strips with ninhydrin and by the Sakaguchi reaction. After activation the zymogen yielded a single major peptide which moved toward the anode with a mobility of 0.67 relative to cysteic acid. Three anionic peptides with mobilities of 0.47, 0.78, and 0.89 relative to cysteic acid were ignored since they represented less than 10% yields. The peptide from guanidinated trypsinogen was eluted with water and analyzed. Its amino acid composition corresponded to that of the peptide released during tryptic activation of trypsinogen (Davie and Neurath, 1955) except that lysine was replaced by homoarginine (Table II). The small amounts of other amino acids corresponded to background levels eluted from paper blanks.

In similar experiments the time course of release of peptide was compared with the time course of appearance of full enzymatic activity. Least-squares treatment of 20 measurements yielded a correlation of 1.0 ± 0.1. This correlation is somewhat illusory since quantitative elution of the activation peptide is unlikely and some peptides may have been released from inactive protein (about 30% of total protein). Activation of guanidinated trypsinogen led to a progressive replacement of amino-terminal valine by isoleucine (Table III).

Purification and Characterization of ε-Guanidinated Trypsin. Activation of guanidinated trypsinogen by AOAP resulted in a 70% yield of ε-guanidinated trypsin (Figure 3). The active enzyme was separated from inert protein by chromatography on a CHOM-Sepharose column (Robinson *et al.*, 1971). Figure 8 illustrates the elution pattern obtained when a linear gradient known to separate α- and β-trypsin was applied. The purified enzyme eluted as a single peak at the same position as β-trypsin (fractions 50–90) whereas no protein eluted at the position of α-trypsin (fraction 43). Fractions across the chromatographic peak of purified ε-guanidinated trypsin had constant specific activity toward BAEE.

Amino-terminal analysis of the purified enzyme by the cyanate method gave isoleucine as the principal amino-terminal residue (Table IV). Sequenator analysis yielded the sequence Ile-Val-Gly which is characteristic of the single chain of β-trypsin.

ε-Guanidinated trypsin and β-trypsin which had been purified on a CHOM-Sepharose column had identical specific activities toward NPGB and BAEE. Both enzymes yielded 0.94–0.98 active site/mol of protein when titrated with NPGB and both hydrolyzed BAEE at a rate of 67–69 μmol/min per mg of protein. Both enzymes activated chymotrypsinogen at

TABLE II: Amino Acid Composition[a] of the Activation Peptide of Guanidinated Trypsinogen.

	Per Cent Activation		
Amino Acid	13	32	52
Major			
Valine	0.98	1.00	0.96
Aspartic acid	3.88	3.95	4.10
Homoarginine	*1.00*	*1.00*	*1.00*
Minor			
Serine	0.23	0.11	0.11
Glutamic acid	0.16	0.11	0.11
Glycine	0.17	0.11	0.20
Alanine	0.16	Trace	Trace

[a] Expressed as molar ratios of amino acids relative to homoarginine, taken as 1.00.

TABLE III: Amino-Terminal Amino Acids[a] Found after Activation of Guanidinated Trypsinogen by AOAP.

	% Activation			
	0	60	72	69
		Time (hr)		
	0	0.12	2.5	24
Aspartic acid	0.15	0.14	0.05	0.05
Valine	0.89	0.04	0.15	0.07
Isoleucine	0.00	0.97	0.92	1.09

[a] The cyanate procedure of Stark and Smyth (1963) was used. Yields are corrected for losses during base hydrolysis.

TABLE IV: Amino-Terminal Residues[a] of the Products of AOAP Treatment of Guanidinated Trypsinogen.

Amino Acid	Residues/Protein Molecule	
	Active Protein[b]	Inactive Protein[b]
Aspartic acid	0.05	0.38
Threonine	0.04	0.15
Serine	0.16	0.47
Glutamic acid	0.04	0.16
Glycine	0.07	0.23
Alanine	0.03	0.12
Valine	0.10	0.28
Isoleucine	0.85	0.29
Leucine	0.05	0.07

[a] Yields are corrected for losses during base hydrolysis using the values of Stark and Smyth (1963). [b] Active ε-guanidinated trypsin was separated from inactive products by affinity chromatography (Figure 8).

the same rate and both were inhibited by 1 equiv of either soybean trypsin inhibitor or CHOM.

Discussion

It was the purpose of this investigation to prepare and characterize ε-guanidinated trypsin without modifying the α-amino groups of the amino-terminal residue Ile_7. This modified enzyme can serve as a model to test the involvement of the α-amino group in the activity of the enzyme.

To this end trypsinogen was guanidinated by treatment with GDMP and converted to the active enzyme by an acid protease (AOAP) isolated from *A. oryzae*. According to amino acid analysis, all lysine but no other residues had been modified, yet the guanidinated zymogen separated into three chromatographically distinct components. Each was equally activated by AOAP and yielded enzymes having identical specific activities. Treatment with neutral NH_2OH converted two of the zymogen components to the third, suggesting that secondary sites had been guanidinated as well, yielding derivatives that are both acid and NH_2OH labile.

Activation of native bovine trypsinogen involves the cleavage of the Lys_6–Ile_7 bond in the amino-terminal sequence H·Val-$(Asp)_4$-Lys-Ile-Val-Gly- and the release of the hexapeptide Val-$(Asp)_4$-Lys (Davie and Neurath, 1955; Desnuelle and Fabre, 1955). Since homoarginyl bonds are slowly hydrolyzed by trypsin (Chauvet and Acher, 1971) if at all (Figure 3), it was important to discover that AOAP activated guanidinated trypsinogen by cleaving the Har_6–Ile_7 bond and releasing the peptide H·Val-$(Asp)_4$-Har. In fact, AOAP cleaved the homoarginyl peptide bond approximately five times more rapidly than the corresponding lysyl bond in native trypsinogen. Apparently the positive charge on residue 6 is essential for proteolysis since amidinated trypsinogen was also activated by AOAP at the same rate as native trypsinogen whereas the acetylated and succinylated zymogens were not.

ε-Guanidinated trypsin prepared as described herein consists of a single polypeptide chain, contains one active site per molecule, and has the same specific activity toward BAEE as β-trypsin. Guanidination of the ε-amino groups was complete, in contrast to the partial guanidination of trypsin reported by Nureddin and Inagami (1969). Unlike the ε-acetylated enzyme of Labouesse and Gervais (1967), the ε-guanidinated trypsin is soluble below pH 6. Since the guanidinated enzyme is also resistant to autolysis at neutral or basic pH, it lends itself to studies of the effects of modification of the α-amino group on enzymatic function (Robinson *et al.*, 1973).

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References

Bannard, R. A. B., Casselman, A. A., Cockburn, W. F., and Brown, G. M. (1958), *Can. J. Chem. 36*, 1541.
Blow, D. M. (1971), *Enzymes 3*, 185.
Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature (London) 221*, 337.
Chase, T., and Shaw, E. (1967), *Biochem. Biophys. Res. Commun. 29*, 508.
Chauvet, J., and Acher, R. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 18*, 265.
Davie, E. W., and Neurath, H. (1955), *J. Biol. Chem. 212*, 515.
Desnuelle, P., and Fabre, C. (1955), *Biochim. Biophys. Acta 18*, 49.
Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1*, 80.
Fraenkel-Conrat, H., Bean, R. S., and Lineweaver, H. (1949), *J. Biol. Chem. 177*, 385.
Gertler, A., and Hofmann, T. (1967), *J. Biol. Chem. 242*, 2522.
Ghelis, C., Garel, J., and Labouesse, J. (1970), *Biochemistry 9*, 3902.
Habeeb, A. F. S. A. (1959), *Biochim. Biophys. Acta 34*, 294.
Habeeb, A. F. S. A. (1960), *Can. J. Biochem. Physiol. 38*, 493.
Habeeb, A. F. S. A., Cassidy, H. G., and Singer, S. J. (1958), *Biochim. Biophys. Acta 29*, 587.
Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972), *Biochemistry 11*, 2427.
Hess, G. (1971), *Enzymes 3*, 213.
Hunter, M. J., and Ludwig, M. L. (1962), *J. Amer. Chem. Soc. 84*, 391.
Labouesse, J., and Gervais, M. (1967), *Eur. J. Biochem. 2*, 215.
McElvain, S. M., and Nelson, J. W. (1942), *J. Amer. Chem. Soc. 64*, 1825.
Nakanishi, K. (1959), *J. Biochem. (Tokyo) 46*, 1263.
Naughton, M. A., Sanger, F., Hartley, B. S., and Shaw, D. C. (1960), *Biochem. J. 77*, 149.
Nureddin, A., and Inagami, T. (1969), *Biochem. Biophys. Res. Commun. 36*, 999.
Pechère, J. F., and Neurath, H. (1957), *J. Biol. Chem. 229*, 389.
Robinson, N. C., Neurath, H., and Walsh, K. A. (1973), *Biochemistry 12*, 420.
Robinson, N. C., Tye, R. W., Neurath, H., and Walsh, K. A. (1971), *Biochemistry 10*, 2743.
Sanders, M. M. (1969), Ph.D. Thesis, University of Washington.
Scrimger, S., and Hofmann, T. (1967), *J. Biol. Chem. 242*, 2528.

Spackman, D. H. (1967), *Methods Enzymol. 11*, 3.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Stroud, R. M., Kay, L. M., and Dickerson, R. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36*, 125.
Walsh, K. A., and Neurath, H. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 884.
Walsh, K. A., and Wilcox, P. E. (1970), *Methods Enzymol. 19*, 37.
Wofsy, L., and Singer, S. J. (1963), *Biochemistry 2*, 102.

The Relation of the α-Amino Group of Trypsin to Enzyme Function and Zymogen Activation†

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ABSTRACT: Chemical modification of the α-amino group of ε-guanidinated trypsin by carbamylation, thiocarbamylation, or amidination renders the enzyme inactive toward specific ester and amide substrates. The carbamylated enzyme retains weak reactivity toward the pseudosubstrates diisopropyl phosphorofluoridate and *p*-nitrophenyl-*p'*-guanidinobenzoate. The modified enzyme resembles in this regard trypsinogen and guanidinated trypsinogen which react with these pseudosubstrates four to six orders of magnitude more slowly than trypsin. Since the common chemical characteristic of these weakly reactive enzyme derivatives is a blocked α-amino group of Ile_7, it follows that full enzymatic function requires that this group be free. It is proposed that during zymogen activation, *inter alia*, a latent enzymatic activity becomes very greatly enhanced rather than being generated *de novo*.

The conversion of trypsinogen to trypsin involves the obligatory cleavage of the peptide bond between Lys_6 and Ile_7 (Davie and Neurath, 1955; Desnuelle and Fabre, 1955) and results in the formation of a new α-amino group on Ile_7. The involvement of this newly formed α-amino group in enzyme function is suggested by its close proximity to the active site in the three-dimensional structures of trypsin and of homologous serine proteases (Stroud *et al.*, 1972; Blow *et al.*, 1969; Shotton and Watson, 1970). Deamination of the α-amino group of trypsin by nitrous acid (Scrimger and Hofmann, 1967) and similar modifications of chymotrypsin (Hess, 1971) cause inactivation of these enzymes.

In the present study the role of the α-amino group has been probed by determining the effects of chemical modification of this group on the activity of ε-guanidinated trypsin. This derivative is uniquely suited for this purpose since only the α-amino group of Ile_7 but none of the ε-amino groups is free (Robinson *et al.*, 1973). The amino group has been modified with various group-specific reagents and the activities of the products have been compared with those of the native enzyme and its parent zymogen. In the course of this study, catalytic activity of the zymogen as well as of the modified enzyme toward pseudosubstrates has been detected.

Experimental Section

Materials and Methods

Guanidinated trypsinogen and the acid protease of *Aspergillus oryzae* were prepared as described in the preceding paper (Robinson *et al.*, 1973). Trypsin was purified on a CHOM-Sepharose[1] affinity column (Robinson *et al.*, 1971) and dialyzed against 2 mM HCl.

N-α-Benzoyl-D,L-arginine-*p*-nitroanilide hydrochloride was obtained from Cyclo Chemical Co. Trifluoroacetic acid (Baker Chemical Co.) was distilled over chromic acid before use. Methyl isothiocyanate was purchased from Eastman Organic Chemical Co. and *N-tert*-butyloxycarbonyl-L-phenylalanine-*N*-hydroxysuccinimide ester from Fox Chemical Co.

Diisopropyl phosphorofluoridate (DFP) was obtained from Pierce Chemical Co. and [^{14}C]DFP from New England Nuclear. Guanidine hydrochloride was purchased from Heico Inc. (Ultra High Purity) and from Mann Research Laboratories (Mann Ultra Pure). Some lots of the Mann product contained ammonium ions which were removed as ammonia *in vacuo* after titration of saturated solutions to pH 10 with 8 M NaOH. The sources of other materials are listed in the preceding paper (Robinson *et al.*, 1973).

The methods of amino acid analysis, amino-terminal analysis, chromatography on SE-Sephadex and CHOM-Sepharose, active-site titrations with NPGB, and esterase

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[1] Abbreviations used are: BAEE, *N*-α-benzoyl-L-arginine ethyl ester hydrochloride; CHOM-Sepharose, chicken ovomucoid covalently coupled to Sepharose (Robinson *et al.*, 1971); DFP, diisopropyl phosphorofluoridate; DIP, diisopropylphosphoryl; Gdn·HCl, guanidine hydrochloride; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoic acid; *t*-Boc-Phe-OSu, *tert*-butyloxycarbonyl-L-phenylalanine-*N*-hydroxysuccinimide ester.