Chemical Modification of Amino Groups and Guanidino Groups of Trypsin

PREPARATION OF STABLE AND SOLUBLE DERIVATIVES

By AIDA NUREDDIN* and T. INAGAMI

Department of Biochemistry, Vanderbilt University School of Medicine,
Nashville, Tenn. 37232, U.S.A.

(Received 19 August 1974)

1. Isoionic chemical modification of amino groups of trypsin (EC 3.4.21.4) was studied for the purpose of obtaining a well-defined modified trypsin with minimum changes in physicochemical properties and with sufficient stability at neutral pH. Acetamidination with methyl acetimidate hydrochloride proceeded very rapidly at pH 9.8 and 5°C and all 14 ϵ -amino groups were modified in 2 h. The reaction was limited to ϵ -amino groups. The α -amino group of *N*-terminal isoleucine was modified only by repeated reactions in the presence of 5.5M-guanidine or 8M-urea. 2. The ϵ -acetamidinated derivative of β -trypsin retained enzymic activity at values comparable with those of native enzyme tested with α -*N*-benzoyl-L-arginine ethyl ester and α -*N*-benzoyl-L-arginine *p*-nitroanilide as substrates; it also showed substrate activation comparable with that of native enzyme. The acetamidination of α -trypsin resulted in approx. 50% decrease in its esterolytic activity. 3. The ϵ -acetamidinated β -trypsin was very stable at pH 8 and 25°C in the absence of Ca^{2+} . The activity of 0.04% (w/v) enzyme solution remained practically unchanged for 10 h, and after 24 h 90% of the activity was still retained. Possible autolytic cleavage of peptide bonds of acetamidinated enzymes was followed by *N*-terminal analysis by using automated Edman degradation. Only the Arg₍₁₀₅₎-Val₍₁₀₆₎ bond was found to be cleaved to an appreciable extent. Thus β -trypsin can be stabilized simply by complete acetamidination of ϵ -amino groups without modifying guanidino groups of arginine residues. Acetamidinated α -trypsin was unstable, but its inactivation at a neutral pH could not be attributed to the cleavage of a single specific peptide bond. 4. The acetamidination of the α -amino group of the *N*-terminal isoleucine results in the inactivation of esterolytic activity. However, this enzyme retained the ability to react with *p*-nitrophenyl *p'*-guanidinobenzoate. 5. It was concluded that acetamidination of β -trypsin is a convenient method for preparing a well-defined stable and soluble trypsin derivative without appreciable change in its physical properties.

Trypsin (EC 3.4.21.4) undergoes rapid autolytic inactivation at neutral pH presumably due to hydrolysis of lysine and arginine peptide bonds. This autolysis has hampered detailed studies at neutral pH of the enzyme molecule in its active form. Such autolysis may be prevented or minimized by chemical modification of the ϵ -amino group of lysine residues and the guanidino group of arginine residues. A number of experiments to modify the ϵ -amino group were carried out in the past in order to stabilize trypsin (Epstein *et al.*, 1962; Glazer *et al.*, 1962; Levin *et al.*, 1964; Labouesse & Gervais, 1967; Terminiello *et al.*, 1955; Trenholm *et al.*, 1969; Westman, 1969; Wong & Liener, 1960; Habeeb, 1967; Glassmeyer & Ogle, 1971). Two main types of reactions used were acylation and coupling to bulky polymers. These

reactions often introduce drastic changes in the net electrical charge and other surface properties of the enzyme molecule. Further, some reactions were not strictly specific for the ϵ -amino groups. Sometimes not all of the ϵ -amino groups were modified. Thus the products of the reactions were often not chemically homogeneous. Such products are not suitable for use in physical and enzymological studies as a model of native trypsin.

Lysine residues of proteins can be guanidinated by 3,5-dimethylpyrazole-1-carboximidate nitrate to homoarginine (Habeeb, 1960) or amidinated by imidoesters (Hunter & Ludwig, 1962) in highly specific reactions. These derivatives should be considerably more stable than the native enzyme against autolysis since the L-homoarginine and ϵ -acetamidino-L-lysine peptide bonds are generally more resistant to tryptic digestion (Hunter & Ludwig, 1962; Shield

* Present address: Department of Molecular Medicine, Mayo Clinic, Rochester, Minn. 55901, U.S.A.

et al., 1959), although even they seem to be susceptible to slow hydrolysis to some extent (Seidl & Liener, 1971; Chauvet & Acher, 1971). Since the positive electrical charge of the lysine side chain is conserved in these reactions, the effect of the modifications on the physicochemical properties of the enzyme molecule should be minimal.

Our preliminary studies (Nureddin & Inagami, 1969) suggested that the complete guanidination of all ϵ -amino groups of trypsin was not possible. Robinson *et al.* (1973a) were able to obtain by limited enzymic proteolysis, a fully guanidinated trypsin through the activation of completely guanidinated trypsinogen. In the work reported here we were able to obtain a stable completely acetamidinated trypsin by a fast and simple reaction, and some of its properties were investigated.

Materials and Methods

Materials

Trypsin, twice-recrystallized and freeze-dried, was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) and purified by column chromatography on sulphoethyl-Sephadex (Pharmacia, Piscataway, N.J., U.S.A.) as described by Schroeder & Shaw (1968). The molar concentration of trypsin was determined spectrophotometrically by using ϵ 25200 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ at 280 nm. Bovine pancreatic RNAase* A (EC 3.1.4.22), supplied as a solution in 0.1 M-sodium phosphate buffer by Worthington Biochemical Corp. was dialysed against glass-distilled water before use. The molar concentration of the RNAase solutions was determined by using ϵ 9900 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ at 275 nm (Nishi *et al.*, 1970).

Optically pure α -N-benzoyl-L-arginine *p*-nitroanilide hydrochloride was kindly made available for this work by Dr. N. Nishi of Hokkaido University, Japan. Bz-Arg-OEt was obtained from Mann Research Laboratories (New York, N.Y., U.S.A.). Benzamidine hydrochloride was purchased from K & K Laboratories (Plainview, N.Y., U.S.A.). Reagents and solvents for the automated Edman degradation were products of the Beckman Co. (Fullerton, Calif., U.S.A.). ϵ -Dinitrophenyl-L-lysine monohydrochloride and L-homoarginine hydrochloride were purchased from Calbiochem (La Jolla, Calif., U.S.A.). 1-Fluoro-2,4-dinitrobenzene and phenylthiohydantoin derivatives of amino acids were obtained from Pierce Chemical Co. (Rockford, Ill., U.S.A.).

The following compounds were synthesized by published methods: *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (Chase & Shaw, 1967); 1-propyl-

and 1-*n*-butyl-guanidine hydrochloride (Inagami & York, 1968); biacetyl trimer (Yankeelov *et al.*, 1968); methyl acetimidate hydrochloride (Hunter & Ludwig, 1962). Methyl [1-¹⁴C]acetimidate hydrochloride was prepared from [2-¹⁴C]acetonitrile. Its specific radioactivity was determined directly with preparations that attained a constant specific radioactivity after repeated recrystallization from anhydrous chloroform. The method of Reynolds (1968) in which RNAase A is used as a test material, was also used for the calibration of the radioactivity.

Methods

Modification of amino and guanidino groups. The acetamidination of the amino groups of trypsin was carried out essentially as described by Hunter & Ludwig (1962). To a 10 ml reaction mixture containing 0.1 % (w/v) trypsin was added 22 mg of methyl acetimidate hydrochloride every 20 min. The pH was adjusted first manually by the slow addition of 5 M-NaOH with rapid stirring to prevent denaturation of the enzyme by a high local concentration of the alkali, then it was maintained at a desired value with 1 M-HCl in a Radiometer pH-stat assembly model TTT1c/SBR2. Competitive inhibitors such as benzamidine (0.5 M), 1-*n*-butylguanidine (0.25 M), or 3,5-dimethylpyrazole-1-carboxamide nitrate (0.3 M) were used to prevent autolysis or to modulate the extent of the modification. The modification reaction was terminated by the acidification of the reaction mixture to pH 3.5 with HCl. After exhaustive dialysis against 0.7 M-HCl the enzyme protein was obtained as a dry powder by freeze-drying. In view of the instability of the amidine group in hot 6 M-HCl (Hunter & Ludwig, 1962), the extent of amidination was estimated either by using radioactive methyl acetimidate hydrochloride or by determining the unchanged lysine as its ϵ -dinitrophenyl derivative by using the short column of the Beckman Spinco model 120C amino acid analyser (Spackman *et al.*, 1958). The preservative phenol had to be eliminated from the eluting buffer to resolve ϵ -Dnp-lysine from arginine.

The guanidino group of arginine residues was modified essentially by the method of Yankeelov *et al.* (1968). A 0.1 % (w/v) protein solution in 0.5 M-sodium phosphate buffer, pH 6.0, was allowed to react with 0.4 M-biacetyl trimer at 25°C for 48 h. Since arginine residues modified by biacetyl trimer were destroyed by acid hydrolysis (Yankeelov *et al.*, 1968) the extent of modification was estimated by determining the remaining arginine residues by amino acid analysis after hydrolysis in 6 M-HCl.

Determination of N-terminal residues. The N-terminal residues were determined by the automated Edman degradation procedure by using a Beckman Sequencer 890B. This was followed by the quantifi-

* Abbreviations: RNAase, ribonuclease; Bz-Arg-OEt, α -N-benzoyl-L-arginine ethyl ester hydrochloride.

cation of phenylthiohydantoin derivatives by gas chromatography, on a Beckman GC45, as described in the Beckman Sequencer Manual (1969). Use of norleucine phenylthiohydantoin as internal standard permitted the determination of the amino terminal residue with a reproducibility higher than 95%.

Determination of enzyme activity. The enzyme activity of trypsin was determined either potentiometrically with 1mM-Bz-Arg-OEt as substrate (Schwert *et al.*, 1948) in a Radiometer pH-stat assembly model TTT1c/SBR2 and a glass pH electrode, or by an all-or-none-type assay by the use of the active-site titrant *p*-nitrophenyl *p'*-guanidinobenzoate by the method of Chase & Shaw (1967). The optically pure α -*N*-benzoyl-L-arginine *p*-nitroanilide (Nishi *et al.*, 1970) was also used as substrate for the comparison of kinetic parameters of modified and native enzymes.

Stability of enzyme solutions. The stability of trypsin was studied essentially as described by Kunitz & Northrop (1933). An enzyme solution in 0.10M-Tris-HCl buffer, pH8.0, was incubated at 25°C. Portions taken at intervals and diluted to an appropriate volume with 0.5mM-HCl containing 0.05M-CaCl₂ were used immediately for the potentiometric assay of the enzyme activity with Bz-Arg-OEt as substrate (Schwert *et al.*, 1948).

Results

Acetamidation of α - and β -trypsin with methyl acetimidate

The reaction of methyl acetimidate with functional groups of protein is exclusively directed to primary amino groups and the reaction proceeds much

more rapidly than guanidination reaction by 3,5-dimethylpyrazole-1-carboxamide. Further, the positive electrical charge of the primary group is retained in the acetamidated product at neutral pH and the acetamidated lysine peptide bond seems to be very resistant to tryptic digestion (Hunter & Ludwig, 1962).

By using methyl acetimidate, it was possible to achieve complete acetamidation of all 14 ϵ -amino groups of β -trypsin in 1 h at pH9.5 and 25°C in the presence of the competitive inhibitors, benzamidine (Mares-Guia & Shaw, 1965), or 1-*n*-butylguanidine (Inagami & York, 1968), added to prevent the autolysis of trypsin. Several reaction conditions given in Table 1 were tested to obtain optimum conditions for various different purposes. The extent of the modifications was not appreciably affected by lowering the reaction temperature from 25°C if sufficient time is allowed, nor by the competitive inhibitors benzamidine and 1-*n*-butylguanidine. On the other hand, 3,5-dimethylpyrazole-1-carboxamide seemed to restrict the amidation reaction to 13 lysine residues. This is consistent with previous observations in our and other laboratories (Nureddin & Inagami, 1969; Robinson *et al.*, 1973a), that 3,5-dimethylpyrazole-1-carboxamide interferes with the reactivity of one or two amino groups.

β -Trypsin has 15 potentially reactive groups: 1 α -amino group of *N*-terminal isoleucine and 14 ϵ -amino groups of lysine residues (Walsh & Neurath, 1964). In some experiments shown in Table 1 the number of the incorporated ¹⁴C-labelled acetamidino group is higher than 14. Aside from experimental errors, this may be explained by two possibilities, namely, that the α -amino group of the terminal isoleucine residue has been partly acetamidated

Table 1. Extent of acetamidation of β -trypsin with methyl acetimidate under different conditions

Reactions were carried out by the method described in the Materials and Methods section with successive additions of methyl acetimidate. Acetamidation was allowed to proceed in the presence of the competitive inhibitors indicated. The values are mol of lysine/mol of enzyme acetamidated determined with [1-¹⁴C]methyl acetimidate with the mol of lysine remaining/mol of enzyme (determined as ϵ -Dnp-lysine by amino acid analysis) given in parentheses.

Acetamidation in presence of:					
pH	Temperature (°C)	Duration (h)	0.5M-Benzamidine	0.25M-Butylguanidine	0.30M-3,5-Dimethylpyrazole-1-carboxamide
β -Trypsin					
9.8	5	2	14.1 (0)		
9.5	25	2.5	14.2 (0)		
9.5	25	1	14.5	14.2	
9.5	5	2	(0)		13.2 (0.9)
9.5	5	1	14.1 (0.5)	13.8	13.2
8.5	25	1	13.7	14.0	
8.5	5	2	13.0 (2.0)	12.5 (3.1)	
8.5	5	1		11.7	
α -Trypsin					
9.8	5	2	(0)		

and/or that a slight autolysis during the modification has resulted in formation of new α -amino groups providing sites for further amidination. The analysis of amino terminal residues, described below, seems to support the latter possibility. Analysis of the amino terminal residue of the thoroughly acetamidinated performic acid-oxidized β -trypsin was performed by automated Edman degradation followed by gas chromatography of the phenylthiohydantoin derivatives of terminal and subterminal amino acids (Edman & Begg, 1967; Beckman Sequencer Manual, 1969). Only one species was observed on the gas chromatogram of the terminal residue. It was identified as isoleucine phenylthiohydantoin from its position in reference to the position of norleucine phenylthiohydantoin used as internal standard. This was further confirmed by gas-chromatographic analysis of trimethylsilylated derivatives of the sample phenylthiohydantoin and by amino acid analysis after its acid hydrolysis. The phenylthiohydantoin amino acid from the second cycle of Edman degradation was similarly identified as that of valine. The use of norleucine phenylthiohydantoin as internal standard permitted the quantitative determination of the sample phenylthiohydantoin derivatives. Results of such analyses (Table 2) for trypsin acetamidinated under various conditions, indicate that the α -amino group of the *N*-terminal isoleucine residue is not modified under

a variety of conditions including pH 8.5 (Expt. 3) which favours the acetamidination of the α -amino group (Hunter & Ludwig, 1962). The presence or the absence of benzamidine has little effect. Repeated modification for a total of 4 h has failed to modify the terminal group (Expts. 3 and 4). Only the presence of denaturants such as 5.5M-guanidine or 8M-urea renders this amino group susceptible to the reaction (Expts. 5 and 6).

Guanidination of β -trypsin with 3,5-dimethylpyrazole-1-carboxamide

Guanidination of amino groups is generally much slower and less complete than acetamidination. Our preliminary results (Nureddin & Inagami, 1969) indicated that only 11 of 14 ϵ -amino groups in β -trypsin could be guanidinated by 0.3M-dimethylpyrazole-1-carboxamide in a reaction lasting for 204 h at pH 9.5 and 0°C in the presence of 0.05M-CaCl₂. To increase the extent of the modification, experiments were performed under various conditions. When 0.5M-benzamidine was substituted for CaCl₂ as stabilizer of trypsin, a twelfth residue was guanidinated. However, no further improvement was obtained by changing the reaction conditions. It was suspected that, in addition to the sluggish reactivity of 3,5-dimethylpyrazole-1-carboxamide, this molecule

Table 2. *N*-Terminal residues of trypsin acetamidinated under different conditions

Modified and freeze-dried trypsin, obtained by acetamidination by the procedure given in the text, was dissolved in a small volume of 0.7M-HCl and a sample containing 0.1 μ mol was applied to the reaction cup of an automatic protein sequencer. Norleucine phenylthiohydantoin (0.1 μ mol) dissolved in 100 μ l of ethyl acetate was placed in each of the fraction collector tubes of the sequencer. A thiazolinone sample from the *N*-terminal or subsequent residues was converted into its phenylthiohydantoin derivatives and then quantitatively analysed by gas chromatography with reference to the position and the peak height of the internal standard norleucine phenylthiohydantoin. Isoleucine was confirmed by gas chromatography of a silylated sample and amino acid analysis of the hydrolysate of the phenylthiohydantoin derivatives by the method given in the Beckman Sequencer Manual (1969). N.D., Not determined.

Enzyme	Conditions of acetamidination					Concn. (mol/mol of protein)	
	pH	Temperature (°C)	Duration (h)	Benzamidine (M)	Others	<i>N</i> -Terminal residue Ile	Sub-terminal residue Val
1. Performic acid-oxidized β -trypsin						0.97	0.94
2. Acetamidinated β -trypsin	9.8	5	2	0.5		0.94	0.90
3. Re-acetamidination of preparation 2	9.8	5	2	0		0.92	0.91
4. Re-acetamidination of preparation 2	8.5	5	2	0		1.01	0.95
						Val:0.52	Ala:0.47
5. β -Trypsin twice-acetamidinated in guanidine*	9.8	5	3 twice†	0	5.5M-Guanidine	0.05	N.D.
6. β -Trypsin in urea*	9.8	5	2	0	8M-Urea	0.14	N.D.
7. α -Trypsin acetamidinated	9.8	5	2	0.5		0.96	0.90
						Val:0.15	Ala:0.10

* Enzyme was first dissolved in the denaturant at pH 3 followed by the addition of methyl acetimidate hydrochloride to 0.22%. The pH was then raised to 9.8 with 3M-NaOH to start the reaction. The regular method of acetamidination was followed thereafter.

† Enzyme modified in the first reaction was isolated as a freeze-dried powder after exhaustive dialysis against 0.7M-HCl, then the same reaction was repeated once more.

may be bound to protein to render certain amino groups inaccessible since the structure of this reagent, consisting of an amidino group and a ring structure, suggests that it can be a competitive inhibitor of trypsin. Kinetic studies on the inhibition of the hydrolysis of α -*N*-benzoyl-L-arginine *p*-nitroanilide by 3,5-dimethylpyrazole-1-carboxamidine has supported such an hypothesis. A competitive inhibition with a K_i value of 3.1 mM was observed in reactions carried out in 5% (v/v) acetone at 25°C and pH 8.2 (0.05 M-sodium veronal buffer). These results indicated that 3,5-dimethylpyrazole-1-carboxamidine renders 2 ϵ -amino groups inaccessible presumably due to its binding to the active site of trypsin. However, binding to other sites cannot be excluded. The enzyme activity of β -trypsin remained practically at the value of the native β enzyme throughout the guanidination reaction as assayed with Bz-Arg-OEt and *p*-nitrophenyl *p'*-guanidinobenzoate used as substrates.

Enzymic activity of acetamidinated trypsins

The esterase activity of the acetamidinated product was determined by the titrimetric method (pH-stat) with 1 mM-Bz-Arg-OEt as substrate (Schwert *et al.*, 1948). As indicated in Table 3, little loss in activity occurred during acetamidination of β -trypsin in the presence of benzamidine ($K_i = 18 \mu\text{M}$; Mares-Guia & Shaw, 1965). Protection by 1-*n*-butylguanidine, which has a K_i of 1.3 mM and is a weaker inhibitor (Inagami & York, 1968), was less satisfactory. 3,5-Dimethylpyrazole-1-carboxamidine with a K_i of 3.1 mM is the weakest of the three

inhibitors used and was least effective in protecting the enzyme. α -Trypsin lost as much as 49% of the activity after 1 h of acetamidination at pH 9.8 even in the presence of benzamidine.

The steady-state kinetic parameters of the completely acetamidinated enzyme were compared with native enzyme by using the optically pure α -*N*-benzoyl-L-arginine *p*-nitroanilide (Nishi *et al.*, 1970) as substrate. This substrate was chosen for the present purpose because of its rather high K_m value which can be determined with a higher degree of accuracy compared with the very low K_m value of the commonly used Bz-Arg-OEt. As indicated in Fig. 1, the steady-state kinetics of the native and completely acetamidinated β -trypsin are essentially identical. The substrate activation observed in the hydrolysis of α -*N*-benzoyl-L-arginine *p*-nitroanilide by native trypsin by Nakata & Ishii (1972) is also observed to a similar extent with the ϵ -acetamidinated trypsins. By extrapolating the linear portion of the Eadie-Hofstee plots of Fig. 1, the completely acetamidinated β -trypsin was found to have a k_{cat} of 2.9 s^{-1} and K_m of 1.7 mM whereas the native β -trypsin has a k_{cat} of 3.0 s^{-1} and a K_m of 1.2 mM under the assay conditions of 25°C in 0.05 M-sodium veronal buffer, pH 8.2, containing 0.02 M- CaCl_2 and 4.5% (v/v) acetone. Thus the effect of acetamidination on the steady-state kinetic parameters of β -trypsin catalysis is very small, increasing the K_m value only by approximately 40%. On the other hand, the completely acetamidinated α -trypsin had a drastically decreased k_{cat} of 1.2 s^{-1} from that of the native form which has a k_{cat} of 2.2 s^{-1} .

Table 3. Enzyme activity of trypsin acetamidinated under different conditions

Acetamidinated β -trypsin was prepared in the presence of the various competitive inhibitors used as protector against autolysis by the method given in the Materials and Methods section. The enzyme activity of the trypsin derivatives (0.01–0.1 mg) was determined with 1 mM-Bz-Arg-OEt used as substrate in a 2 ml assay solution containing 0.05 M- CaCl_2 at 25°C and pH 8 maintained by a Radiometer pH-stat assembly TTT1a/SBR2.

Reaction conditions						
Catalytic activity (% of native enzyme) in the presence of:						
pH	Temperature (°C)	Duration (h)	0.5M-Benzamidine	0.25M-Butylguanidine	0.3M-3,5-Dimethylpyrazole-1-carboxamidine	5.5M-Guanidine
β -Trypsin						
9.8	5	2	97.2			
9.5	25	1	96.0	74.5		
9.5	5	1	98.1	90.5	68.8	
9.5	5	2			65.0	
9.8	5	3 twice*				Negligible
α -Trypsin						
9.8	5	2	51.0			

* Reaction conditions for the preparation of this derivative are given in footnotes of Table 2.

Fig. 1. Eadie-Hofstee plots for the hydrolysis of α -N-benzoyl-L-arginine *p*-nitroanilide

Native β -trypsin (●), completely acetamidated β -trypsin (○), and completely acetamidated α -trypsin (□). The acetamidated enzymes were prepared at pH 9.8 and 5°C by a reaction lasting for 2 h. $[E_0]$, Initial enzyme concentration; $[s]$, substrate concentration.

The acetamidation of the α -amino group of the *N*-terminal isoleucine residue caused a considerable decrease in the esterolytic activity. The β -trypsin modified at pH 9.8 and 5°C for 3 h in the presence of 5.5M-guanidine hydrochloride retained only 12% of the original esterolytic activity determined with Bz-Arg-OEt and α -N-benzoyl-L-arginine *p*-nitroanilide. When this enzyme protein was acetamidated for the second time under identical conditions, the esterolytic activity was decreased to 4% of the original value. Similar results were obtained with the anilide substrate. Since 5% of the *N*-terminal isoleucine remained intact even after these two steps of acetamidation, the 4% residual esterolytic activity could be attributed to the enzyme with an unreacted α -amino group of the *N*-terminal isoleucine. Control experiments performed under identical conditions in 5.5M-guanidine in the absence of methyl acetimidate caused only 6–8% decrease in the enzyme activity. Thus the acetamidation of the α -amino group of β -trypsin seems to abolish its enzyme activity as examined with Bz-Arg-OEt. However, most of the enzyme modified at the α -amino group still seems to react with *p*-nitrophenyl *p*'-guanidinobenzoate. As Fig. 2 (curve *a*) shows, 70% of the active site could be titrated with this active-site-directed reagent. The rate of the reaction, however, seems to be considerably diminished as indicated by a slowly rising curve (*a*) in contrast with the instantaneous rise of the β -trypsin modified only on the ϵ -amino group in the absence of guanidine hydrochloride (curve *b*). A control preparation (curve *c*) identical with that used for curve (*b*) but treated with 5.5M-guanidine

Fig. 2. Release of *p*-nitrophenyl *p*'-guanidinobenzoate by modified trypsins

Curve (*a*) was obtained with β -trypsin acetamidated twice for 3 h in 5.5M-guanidine at 5°C and pH 9.8 (preparation 5 of Table 2); curve (*b*), β -trypsin, acetamidated only at ϵ -amino groups by a 2 h reaction at pH 9.8 and 5°C in 0.5M-benzamidine (preparation 2 of Table 2); curve (*c*), as control, β -trypsin, acetamidated only at ϵ -amino groups was then treated, in the absence of methyl acetimidate, with 5.5M-guanidine twice for 3 h at pH 9.8 and 5°C. The titration was carried out by the method of Chase & Shaw (1967).

hydrochloride under the conditions of curve (*a*) behaved almost identically.

Stability of the modified trypsins

The modifications of lysine and arginine residues to forms resistant to the tryptic attack is expected to make the enzyme more stable. The stability of the modified enzymes was determined by following the time-course of the loss of enzyme activity during the incubation of a 0.04% (w/v) solution in the absence of Ca^{2+} at 25°C and pH 8. Results of such experiments with various derivatives of trypsin are shown in Fig. 3, where remaining activity is given as percentage of the original activity of native β -trypsin. The completely acetamidated β -trypsin with all 14 ϵ -amino groups modified showed remarkable resistance to the autolytic inactivation. The complete acetamidation, however, did not seem to be the absolute requirement for the stabilization. In fact, acetamidation of only 11.7 ϵ -amino groups was sufficient to obtain a stability comparable with the completely acetamidated enzyme. In contrast with these derivatives, the guanidinated derivatives, in which 10 lysine residues had been converted into homo-arginine (Nureddin & Inagami, 1969), were less stable than the partially acetamidated derivatives.

The native α -trypsin is less stable compared with the native β -trypsin as can readily be seen by comparing Figs. 3 and 4. As Fig. 4 indicates, the complete acetamidation of α -trypsin not only caused a drastic

decrease in enzyme activity, but it failed to stabilize the modified enzyme.

Autolysis of peptide bonds

The complete acetamidination of ϵ -amino groups still leaves two arginine peptide bonds potentially susceptible to tryptic cleavage plus some unknown number of sites for non-specific tryptic hydrolysis. We have investigated cleavage of these sites by allowing the autolysis of 0.04% (w/v) enzyme solution to proceed in 0.1M-Tris-HCl buffer, pH8.0, under various conditions (Table 4). Newly formed *N*-terminal residues were analysed by subjecting the autolysed protein to automated Edman degradation followed by gas-chromatographic quantitative analysis of phenylthiohydantoin-derivatives with reference to an internal standard norleucine phenylthiohydantoin. The completely acetamidinated β -trypsin incubated for a 2h period, during which practically no inactivation took place, had 0.28mol of new terminal sequence Val-Ala per mol of enzyme protein in addition to the original *N*-terminal Ile-Val sequence. The new sequence can be identified uniquely with the new *N*-terminus resulting from the cleavage of Arg₍₁₀₅₎-Val₍₁₀₆₎ bond. This cleavage does not seem to affect the enzyme activity, which agrees with the observation of Maroux & Desnuelle (1969). In

Fig. 3. Effect of modification of ϵ -amino groups of β -trypsin on the stability of the enzyme

Enzyme solutions (0.04%) were incubated in 0.2M-Tris-HCl buffer, pH8.0, at 25°C in the absence of Ca²⁺. Portions of the solutions were assayed for the remaining enzyme activity with 1 mM-Bz-Arg-OEt used as substrate in 0.1M-KCl at pH8.0 in a pH-stat. ●, Native β -trypsin; ○, β -trypsin with all 14 ϵ -amino groups acetamidinated with methyl acetimidate by a 2h reaction at pH9.8 and 5°C in 0.5M-benzamidine; □, β -trypsin with 11.7 ϵ -amino groups acetamidinated in 1h at pH8.5 and 5°C in 0.25M-1-*n*-butylguanidine; △, β -trypsin with 11 ϵ -amino groups guanidinated with 0.3M-3,5-dimethylpyrazole-1-carboxamide in 120h at pH9.5 and 0°C in the presence of 0.05M-CaCl₂ and 0.5M-benzamidine.

Table 4. Cleavage of peptide bonds during the autolysis of acetamidinated trypsins

Modified enzyme dissolved to 0.04% in 0.1 M-Tris-HCl buffer, pH8.0, left at 25°C for the specified duration. The autolysis reaction was stopped by lowering the pH to 3.5 with 6M-HCl. After dialysis against 0.7M-HCl the protein was recovered by freeze-drying. The protein was dissolved in a small volume of 0.7M-HCl and a sample containing 0.1 μ mol was applied to a Beckman Sequencer. Norleucine phenylthiohydantoin (0.1 μ mol) dissolved in 100 μ l of ethyl acetate was placed in each of the fraction collector tubes. The sample thiazolinone from the *N*-terminal and subterminal residues was converted into its phenylthiohydantoin derivative and then quantitatively analysed by gas chromatography with reference to the internal standard norleucine phenylthiohydantoin.

	Autolysis condition		Concn. (mol/mol of protein)		
	Reaction medium	Duration (h)	<i>N</i> -Terminal residues	2nd residues	3rd residues
Acetamidinated β -trypsin	0.2M-KCl	0	Ile 0.93	Val 0.90	
		2	Ile 0.93 Val 0.28	Val 0.98 Ala 0.27	
			Several peaks in small quantities*		
Acetamidinated α -trypsin	0.05M-CaCl ₂	0	Ile 0.96 Val 0.15	Val 0.90 Ala 0.10	
		2	Ile 0.98 Val 0.35	Val 0.88 Ala 0.20	
	0.2M-KCl	2	Ile 0.97 Val 0.74	Val 0.92 Ala 0.64	Gly 0.19 Ser†
		6	Ile 0.93 Val 0.85	Val 0.83 Ala 0.86	Gly 0.14 Ser†
			Several peaks in small quantities*		

* Details are given in the text.

† Not quantitatively determinable.

Fig. 4. Stability of α -trypsin and acetamidated α -trypsin

The acetamidated enzyme was prepared by a 2 h reaction at pH9.8 and 5°C in 0.5M-benzamidine by the method given in the Materials and Methods section. The stability was tested by following the remaining enzyme activity during incubation of native α -trypsin (●) and acetamidated α -trypsin (○), in 0.2M-Tris-HCl buffer, pH8.0, at 25°C in 0.2M-KCl buffer, pH8.0, at 25°C in 0.2M-KCl without Ca^{2+} . Bz-Arg-OEt at a concentration of 1mM was used as substrate.

addition to the major peaks of amino acid phenylthiohydantoin derivatives expected from the Ile-Val sequence, several minor peaks were also observed both in the first and second cycles of the Edman degradations. Although the size of these peaks prevented unequivocal identification of the new minor *N*-terminal residues, their positions indicated the presence of serine and glycine phenylthiohydantoin derivatives in the first cycle. The formation of the new minor *N*-terminal residues is presumably due to slow cleavage of ϵ -acetamidated lysine peptide bonds or non-specific cleavage of other bonds.

During the acetamidation of α -trypsin a partial (15%) cleavage of the peptide bonds $\text{Arg}_{(105)}\text{-Val}_{(106)}$ took place as indicated by the third entry in Table 4, and an extensive loss of the enzyme activity (49%) was observed (Table 3). This bond underwent further extensive cleavage during the incubation of the fully acetamidated α -trypsin under the conditions used for the stability test as indicated by the last three entries of Table 4. An extensive inactivation took place during this incubation in 0.2M-KCl as shown in Fig. 4. When 0.05M- CaCl_2 was substituted for KCl, no inactivation was observed although the cleavage of $\text{Arg}_{(105)}\text{-Val}_{(106)}$ bond did take place (Table 4). Thus the extent of the cleavage of this bond is not quantitatively related to the extent of the inactivation. Moreover, no other peptide bond seems to be cleaved to an extent which by itself can account for the degree of inactivation. For example, only 15%*

* The possibility of the acetamidation of newly formed α -amino groups exists, thus the actual value can be higher than this one.

of the $\text{Arg}_{(105)}\text{-Val}_{(106)}$ bond was cleaved during the acetamidation when 50% of the enzyme activity was lost. During the subsequent incubation of acetamidated α -trypsin for 6h at pH8, an additional 45% of the activity of the native enzyme was lost and 70% of the Arg-Val bond (estimated as the difference between the final 85% and the 15% cleaved during the acetamidation reaction) was hydrolysed. After the 6h incubation period new amino terminal amino acids were formed in small quantities (less than 20%). These minor components were serine, glycine and leucine. In the second cycle of the Edman degradation phenylthiohydantoin derivatives of serine and glycine were detected as minor components in addition to the major peaks of phenylthiohydantoin derivatives of alanine and valine. The third cycle produced small amounts of phenylthiohydantoin derivatives of glutamic acid, aspartic acid, serine and valine beside the major peaks for the glycine derivative. Since there were several minor components and since the small size of the peaks did not permit their quantification with a reasonable accuracy, it was not possible to identify all of the bonds partially cleaved during the 6h incubation period. Similar minor components were also identified in somewhat elevated quantities after 31h of incubation. The presence of glutamic acid in the third cycle after 6 and 31h of reaction time and the identification of leucine in the acid hydrolysate of amino acid phenylthiohydantoin obtained in the first cycle strongly suggest that a partial cleavage of the $\text{Arg}_{(55)}\text{-Leu}_{(56)}$ bond took place in the acetamidated α -trypsin. However, the partial cleavage of any one of these peptide bonds does not seem to be sufficiently extensive to account for the drastic inactivation of the acetamidated α -trypsin. The inactivation may have been the result of the cumulative effect of the partial multiple cleavages distributed in various portions of the enzyme molecule.

Modification of arginine residues

After complete acetamidation of all ϵ -amino groups of lysine, 2 arginine peptide bonds remain potentially susceptible to autolytic cleavage. The modification of the guanidino group of arginine residues was attempted with the hope that it may stabilize α -trypsin by making the $\text{Arg}_{(55)}\text{-Leu}_{(56)}$ bond refractory to autolysis. Treatment of completely acetamidated α -trypsin with a 0.4M solution of the biacetyl trimer (Yankeelov *et al.*, 1968) at pH6.0 at 25°C in the presence of 0.1M-benzamidine as inhibitor of autolysis, resulted in the almost complete modification of arginine residues of α -trypsin in 24h as judged from the disappearance of the arginine peak on an amino acid analysis chart. On the other hand, these residues of the acetamidated

β -trypsin were less reactive: one residue was completely modified in 12 h and 0.4 residue remained intact even after 48 h of reaction.

The complete modification of arginine residues of the acetamidinated α -trypsin produced an enzyme possessing only 35% of the activity of acetamidinated α -trypsin as determined with Bz-Arg-OEt used as substrate. On the other hand, a similar reaction with acetamidinated β -trypsin caused only a 26% loss in activity. The extensive inactivation of both isomers of trypsin may be at least partially due to the modification of histidine (Yankeelov *et al.*, 1968). In view of the extensive inactivation, the modification of arginine residues was not pursued further.

Discussion

The studies reported here were undertaken to obtain a preparation of trypsin which was stabilized against autolytic inactivation in the absence of an added stabilizer such as Ca^{2+} . Since the primary event in the inactivation should be the autocatalytic cleavage of lysine and arginine peptide bonds, attempts were made to modify these groups chemically to forms refractory to tryptic attack without introducing drastic changes in the physicochemical properties of the enzyme protein.

Amidination and guanidination are known to render lysine peptide bonds considerably resistant to tryptic attack without significantly perturbing the positive electric charge and hydration state of the lysine side chains (Hunter & Ludwig, 1962; Shield *et al.*, 1959). The present studies have indicated that the complete acetamidination of 14 ϵ -amino groups can be achieved readily by a very rapid reaction with methyl acetimidate (Table 1) and that a product with a satisfactory stability can be obtained (Fig. 3) without modifying arginine residues.

The resistance of the acetamidinated lysine peptide bonds to autolysis does not seem to be absolute. The modified lysine peptide bonds seems to be susceptible to slow tryptic hydrolysis as indicated by the appearance of small amounts of the new amino terminals after prolonged incubation at pH 8. These cannot be accounted for solely by the cleavage of arginine peptide bonds (Table 4). In view of the observation of Chauvet & Acher (1971) and Seidl & Liener (1971), the homoarginine peptide bonds can be hydrolysed slowly by trypsin, it is highly conceivable that its analogue ϵ -acetamidinated lysine peptide bonds are also susceptible to a slow tryptic cleavage.

It was surprising that the stabilization by acetamidination was achieved only with β -trypsin, which had a single unbroken chain. The α -trypsin, in which the peptide bond $\text{Lys}_{(131)}\text{-Ser}_{(132)}$ was broken (Schroeder & Shaw, 1968), was not converted into a stable derivative by complete acetamidination.

Moreover, a considerable loss in enzyme activity occurred during the modification (Fig. 4).

The diminished stability of α -trypsin and its acetamidinated derivative (Fig. 4) compared with the native and acetamidinated β -trypsin respectively may very well be due to the loosening of the folded structure caused by a single cleavage in the peptide chain. This explanation is supported by a difference in the reactivity of arginine residues, in the α - and β -trypsin, to the biacetyl trimer. Whereas both of the arginine residues of α -trypsin are modified by the reagent, only a partial modification can be obtained with β -trypsin after a prolonged reaction. In α -trypsin $\text{Lys}_{(131)}\text{-Ser}_{(132)}$ bond is cleaved (Schroeder & Shaw, 1968). Lysine-131 in trypsin (Walsh & Neurath, 1964; Mikeš *et al.*, 1967) is in the position corresponding to arginine-145 of chymotrypsin (Hartley, 1964). This arginine residue has been shown (Freer *et al.*, 1970) to undergo a displacement of 0.87 nm during the activation process of chymotrypsin and the resultant formation of the specificity site. The similar positioning of lysine-131 in trypsin seems to suggest that this residue and the adjacent peptide bond may play some role in maintaining the stable conformation of the binding site.

The completely acetamidinated β -trypsin contains two arginine peptide bonds potentially susceptible to tryptic digestion, yet the preparation is highly stable (Fig. 3). Maroux & Desnuelle (1969) have reported that the cleavage of the arginine peptide bond $\text{Arg}_{(105)}\text{-Val}_{(106)}$ has no effect on enzyme activity. The effect of the cleavage of the other arginine peptide is not known, to the authors' knowledge. However, on the basis of our observation that part of the arginine residue of the acetamidinated β -trypsin was difficult to modify, whereas in the α -trypsin it could be modified readily, it is more likely that the second arginine peptide bond $\text{Arg}_{(55)}\text{-Leu}_{(56)}$ of β -trypsin is not sufficiently exposed for tryptic attack. However, the present studies do not provide sufficient data to decide whether this peptide bond is essential for maintenance of the active conformation.

The results obtained with methyl acetimidate indicate that all 14 lysine residues are available for modification in agreement with the findings of Labouesse & Gervais (1967). However, two lysine residues were protected by the competitive inhibitor 3,5-dimethylpyrazole-1-carboxamide. This protection seems to be the cause of the initial failure in our attempt to achieve complete guanidination by this reagent (Nureddin & Inagami, 1969). Robinson *et al.* (1973a) have been able to obtain a fully guanidinated trypsin by activation of the completely modified zymogen. This difference in the reactivity of one or two lysine residues towards 3,5-dimethylpyrazole-1-carboxamide may be attributed to a subtle change in the conformation of trypsinogen

as it becomes transformed into its active state. One of the lysine residues protected by this reagent, which is a competitive inhibitor, may be in the immediate vicinity of the substrate-binding site which, in analogy to the chymotrypsinogen-chymotrypsin systems (Freer *et al.*, 1970), may be formed only as a result of the activation process. A likely candidate for such a location may be lysine-176 which is in the immediate vicinity of the anionic specificity-determining site provided by the carboxylate anion of aspartate-177 (Eyl & Inagami, 1971). However, it is difficult to explain the difference between 3,5-dimethylpyrazole-1-carboxamide and benzamidine since these compounds have similar molecular sizes, yet benzamidine allows the complete acetamidation whereas 3,5-dimethylpyrazole-1-carboxamide does not.

Under the conditions used in the present studies the α -amino group of the *N*-terminal isoleucine was not acetamidated as evidenced by the presence of the nearly stoichiometric amount of isoleucine phenylthiohydantoin after the Edman degradation of the completely acetamidated β -trypsin. The presence of a similar quantity of valine phenylthiohydantoin in the subterminal residue further confirms this conclusion. These observations indicate the tightness of the salt bridge between the terminal amino group and the β -carboxyl group of aspartate-182. Even by repeated acetamidation in the presence of 5.5M-guanidine hydrochloride only 95% of the *N*-terminal isoleucine could be modified. Since the esterolytic activity of this product was only 4% of the original preparation, it is safe to conclude that the acetamidation of the terminal α -amino group of the isoleucine residue destroys the esterolytic activity of trypsin. This modified enzyme, however, still seems to retain a part of the active-site conformation as indicated by its slow reaction with the active-site-directed chromogenic reagent *p*-nitrophenyl *p'*-guanidinobenzoate (Chase & Shaw, 1967). Almost 70% of the modified enzyme still seems to be reactive to this reagent. This is analogous to a similar slow reaction of α -carbamylated ϵ -guanidinated trypsin with *p*-nitrophenyl *p'*-guanidinobenzoate observed by Robinson *et al.* (1973b).

The steady-state kinetic parameters of the completely acetamidated β -trypsin and its native counterpart are practically identical. As discussed above, the acetamidated trypsin seems to be highly stable at room temperature and a neutral pH even in the absence of Ca^{2+} ions. Thus the acetamidated β -trypsin should be useful as a realistic model of the native β -trypsin in the study of its properties at a neutral pH.

This work was supported by National Science Foundation (U.S.A.) research grant GB 27583. We are greatly indebted to Dr. Nishi, Dr. Tokuta and Dr. Noguchi for the gift of α -*N*-benzoyl-L-arginine *p*-nitroanilide. We thank

Mrs. Alice Knapp and Mrs. Yuriko Murakami for their able technical assistance, and Mrs. Joanne Liddle for assistance in preparing the manuscript. Part of this paper is taken from the dissertation submitted by A. N. to the Graduate School of Vanderbilt University in partial fulfillment of the requirement of a Ph.D. degree.

References

- Beckman Sequencer Manual (1969) Spinco Division of Beckman Instruments Inc., Palo Alto, Calif.
- Chase, T., Jr. & Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* **29**, 508–514
- Chauvet, J. & Acher, R. (1971) *FEBS Lett.* **18**, 265–267
- Edman, P. & Begg, G. (1967) *Eur. J. Biochem.* **1**, 80–91
- Epstein, C. J., Anfinsen, C. B. & Sela, M. (1962) *J. Biol. Chem.* **237**, 3458–3463
- Eyl, A. W., Jr. & Inagami, T. (1971) *J. Biol. Chem.* **246**, 738–746
- Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T. & Xyong, Ng. H. (1970) *Biochemistry* **9**, 1997–2009
- Glassmeyer, C. K. & Ogle, J. D. (1971) *Biochemistry* **10**, 786–793
- Glazer, A. N., Bar-Eli, A. & Katchalski, E. (1962) *J. Biol. Chem.* **237**, 1832–1838
- Habeeb, A. F. S. A. (1960) *Can. J. Biochem. Physiol.* **38**, 493–501
- Habeeb, A. F. S. A. (1967) *Arch. Biochem. Biophys.* **119**, 264–268
- Hartley, B. S. (1964) *Nature (London)* **204**, 1284
- Hunter, M. J. & Ludwig, M. L. (1962) *J. Amer. Chem. Soc.* **84**, 3491–3504
- Inagami, T. & York, S. S. (1968) *Biochemistry* **7**, 4045–4052
- Kunitz, M. & Northrop, J. H. (1933) *J. Gen. Physiol.* **17**, 591–615
- Labouesse, J. & Gervais, M. (1967) *Eur. J. Biochem.* **2**, 215–223
- Levin, Y., Pechet, M., Goldstein, L. & Katchalski, E. (1964) *Biochemistry* **3**, 1905–1913
- Mares-Guia, M. & Shaw, E. (1965) *J. Biol. Chem.* **240**, 1579–1585
- Maroux, S. & Desnuelle, P. (1969) *Biochim. Biophys. Acta* **181**, 59–72
- Mikeš, O., Tomašek, V., Holeyšovský, V. & Šorm, F. (1967) *Collection Czech. Chem. Commun.* **32**, 655–667
- Nakata, H. & Ishii, S. (1972) *J. Biochem. (Tokyo)* **72**, 281–290
- Nishi, N., Tokura, S. & Noguchi, J. (1970) *Bull. Chem. Soc. Japan* **43**, 2900–2907
- Nureddin, A. & Inagami, T. (1969) *Biochem. Biophys. Res. Commun.* **36**, 999–1005
- Reynolds, J. H. (1968) *Biochemistry* **7**, 3131–3135
- Robinson, N. C., Neurath, H. & Walsh, K. A. (1973a) *Biochemistry* **12**, 414–420
- Robinson, N. C., Neurath, H. & Walsh, K. A. (1973b) *Biochemistry* **12**, 420–426
- Schroeder, D. D. & Shaw, E. (1968) *J. Biol. Chem.* **243**, 2943–2949
- Schwert, G. W., Neurath, H., Kaufman, S. & Snoke, J. E. (1948) *J. Biol. Chem.* **172**, 221–239
- Seidl, D. S. & Liener, I. E. (1971) *Biochem. Biophys. Res. Commun.* **42**, 1101–1107

- Shield, G. S., Hill, R. L. & Smith, E. L. (1959) *J. Biol. Chem.* **234**, 1747–1753
- Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1205
- Terminiello, L., Ram, J. S., Bier, M. & Nord, F. F. (1955) *Arch. Biochem. Biophys.* **57**, 252–261
- Trenholm, H. L., Spomer, W. E. & Wootton, J. F. (1969) *Biochemistry* **8**, 1741–1747
- Walsh, K. A. & Neurath, H. (1964) *Proc. Nat. Acad. Sci. U.S.* **52**, 884–889
- Westman, T. L. (1969) *Biochem. Biophys. Res. Commun.* **35**, 313–317
- Wong, R. C. & Liener, I. E. (1960) *Biochim. Biophys. Acta* **38**, 80–87
- Yankeelov, J. A., Jr., Mitchell, C. D. & Crawford, T. H. (1968) *J. Amer. Chem. Soc.* **90**, 1664–1666