CHEMICAL MODIFICATIONS OF AMINO GROUPS OF TRYPSIN*

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Received July 29, 1969

SUMMARY

Reactivity of amino groups of chromatographically homogenious β-trypsin was studied by chemical modification. Guanidination by 1-amidinyl-3,5-dimethyl pyrazole nitrate led to modification of 10 out of 14 lysyl residues per molecule of trypsin. The guanidinating reagent was found to be a competitive inhibitor of trypsin. Extent of acetamidination by methyl acetimidate hydrochloride was affected by pH, temperature and the presence of various competitive inhibitors. All 14 ε-amino groups of lysyl residues could be acetamidinated in the presence of the competitive inhibitors at pH 9.5 and 25° without loss of enzyme activity. When 11 or more lysyl residues are modified by acetamidination, the enzyme is stable against autolysis in the absence of Ca^{++}. The enzyme with 10 of its 14 lysyl residues guanidinated loses its enzyme activity by autolysis at neutral pH at a rate comparable with unmodified enzyme.

Studies of trypsin molecule at neutral pH have not been easy due to a very rapid autolysis. Splitting of lysyl and arginyl peptide bonds is thought to be the primary cause of the autolysis. A trypsin molecule contains 14 lysyl and 2 arginyl residues (1). Modification of these groups to a form not susceptible to the autolysis is expected to stabilize the enzyme. A guanidinated or amidinated lysyl peptide bond is not susceptible to tryptic digestion (2,3). As the first step of an attempt to stabilize trypsin, guanidination with ADMP** and amidination with MAI were studied.

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* This work was supported by NIH grant GM-14703.

** Abbreviations are: ADMP, 1-amidinyl-3,5-dimethyl pyrazole nitrate; MAI, methyl acetimidate hydrochloride.

Acylation was used to determine the importance of amino groups in trypsin (4-6). Since Labouesse (2) has observed that a complete acetylation does not cause a significant loss of enzyme activity, the ϵ-amino group is not essential for catalysis. However, the ϵ-amino groups are the most frequently occurring functional group in trypsin, and it is very likely that some of them may be located near the active site. If it is the case, substrate binding and subsequent conformational change can affect the reactivity of these groups. Reagents for amidination and guanidination are less reactive than acylating reagents, hence it is expected to detect subtle changes in the chemical reactivity of the amino groups. Furthermore, acylation is not specific to amino groups and causes a drastic change in the physical-chemical state of the protein by eliminating, at neutral pH, the positive charge of the amino group. Amination and guanidination, on the other hand, are specific to amino groups (3, 9) and, upon modification, leave unaltered the net charge on the enzyme. This communication describes application of such modification on trypsin and its effect on the stability of the enzyme.

MATERIALS AND METHOD

β-Trypsin, obtained by the chromatography of trypsin (Worthington) according to the procedure of Schroeder and Shaw (7) was used in all experiments. ADMP was prepared according to the method of Bannard et al. (8), and was recrystallized from ethanol (m.p., 167-168°). MAI-1-^{14}C was prepared from acetonitril-1-^{14}C and anhydrous methanol as described by Hunter and Ludwig (3).

The reaction with ADMP was carried out essentially as described

by Habeeb (9). Details are given in the legend to Fig. 1. The method of amidination with MAI-1-^{14}C is given in Table 1. Radioactivity was counted in a Packard Tri-Carb scintillation counter using a thixotropic gel (Packard). It was calibrated with internal standards. Protein concentration was determined spectrophotometrically at 280 mμ using $E^{1Cm}_{1\%}$ = 14.4 (10). Dinitrophenylation with fluorodinitrobenzene was carried out according to Wofsy and Singer (12). The dinitrophenylated protein was hydrolyzed in evacuated sealed tubes with 6 N HCl at 110°. Amino acid analysis was made according to Spackman et al. (13).

RESULTS AND DISCUSSION

Modification with ADMP: The modification reaction was carried out over a period of 205 hours. The extent of reaction was estimated by determining the content of lysine and homoarginine. Fig. 1

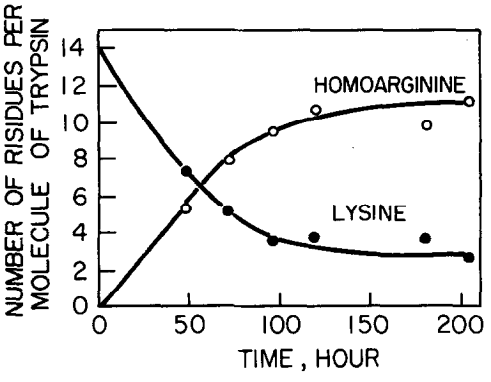

Fig. 1: Modification of ε-amino groups of β-trypsin with 1-amidinyl-3,5-dimethyl pyrazole.

The reaction was carried out with a mixture containing 0.3 M ADMP, 0.05 M $CaCl_2$, and 0.1% β-trypsin at pH 9.5 and 0°. Aliquots taken at intervals were acidified with 2 M sodium acetate buffer pH 3.5, dialyzed against 0.7 mM HCl for 3 days at 4° and lyophilized. (o——o) formation of homoarginine, (●——●) lysine remaining.

shows that 10 lysyl residues per molecule of trypsin are guanidinated in 120 hours, and an additional residue is modified in 205 hours by a slow ensuing reaction. The presence or absence of a competitive inhibitor, benzamidine (14), and Ca^{++} does not affect the rate and extent of the modification reaction. Thus 3 to 4 lysyl residues show a different reactivity to ADMP than others. The lack of reactivity of these residues to the modifying reagent can be due to their location

Table 1. Amidination of β-trypsin with methyl acetimidate-1-^{14}C

To a 10 ml reaction mixture containing 0.1% trypsin was added 22 mg of methyl acetimidate every 20 minutes. The pH was adjusted first manually with 5 N NaOH, then it was maintained at a desired value with 1 N HCl using a Radiometer pH-stat assembly.

			Moles of acetamidinyl-^{14}C incorporated and moles of lysyl residues remaining (in parenthes) per mole of trypsin			
			Competitive inhibitor			
pH	Temp. (°C)	Reaction period (hour)	None	Benzamidine[a]	Butylguanidine[b]	ADMP[c]
9.5	25	2 1/2		14.2 (0)*		
		1	14.0	14.5	14.2	
	5	2				13.2
		1	14.0	14.2 (0.5)*	13.8	13.2
8.5	25	1	14.2	13.7	14.0	
	5	2	13.2	13.0 (2.0)*	12.5 (3.1)*	
		1	11.2		11.7	

* Moles of lysyl residues remaining per mole of enzyme determined as ε-dinitrophenyl-L-lysine by amino acid analyzer

[a] 0.50 M, [b] 0.25 M, [c] 0.30 M

inside the enzyme. However, since ADMP has been found to be a competitive inhibitor of trypsin with K_i 1.4 mM, it seems more likely that the 4 lysyl residues are shielded from the reagent by the ADMP bound at the active site.

Modification with MAI-1-^{14}C: MAI is by far more reactive than ADMP, also this reagent is not a strong competitive inhibitor of trypsin. As shown in Table 1, it is possible to modify all 14 lysyl residues at pH 9.5 and 25°. It is interesting to note that competitive inhibitors do not exert their protective effect against the modification at pH 9.5. At pH 8.5 and 5°, however, only 12 lysyl residues are modified after 2 hours in the presence of benzamidine, and 11 residues can be reacted in the presence of another competitive inhibitor, 1-n-butylguanidine (14), as evidenced by the appearance of 2 and 3 moles of ε-dinitrophenyl-L-lysine per mole of protein respectively.* Thin-layer chromatography indicates that the amino terminal isoleucyl residue remains unmodified under this condition. This indicates that butyl guanidine protects one lysyl residue which benzamidine fails to do.

Stability of modified trypsin: The results shown in Fig. 2 indicate that the modification of 14 lysyl residues stabilizes trypsin against autolysis. In the absence of Ca^{++}, β-trypsin retains only 19% of its original activity after 24 hours of incubation at room temperature and pH 8.1, whereas the fully acetamidinated trypsin has still 90% of its activity after the same period of time. The stability afforded by the complete amidination is comparable to the effect of Ca^{++} on

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* J. H. Reynolds has modified 13 lysyl residues at pH 8.5 by MAI according to his personal communication.

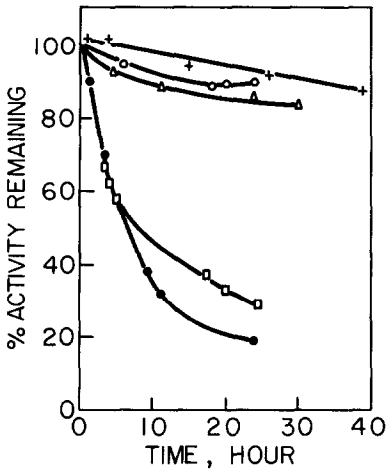

Fig. 2: Stability of modified and unmodified β-trypsin.

The loss of enzyme activity during incubation of a 0.04% solution of β-trypsin in 0.2 M Tris-HCl buffer pH 8.06 at 25° was followed by determining the activity of aliquots taken at intervals by a Radiometer pH-stat assembly using benzoyl-L-arginine ethyl ester as substrate (11).

(+———+) trypsin with all 14 lysyl residues acetamidinated, (o———o) unmodified trypsin incubated with 0.05 M $CaCl_2$, (Δ———Δ) trypsin with 11 lysyl residues acetamidinated by a reaction at pH 8.5, 5° for 2 hours in the presence of butylguanidine, (□———□) trypsin with 10 lysyl residues guanidinated to homoarginine, (•———•) unmodified trypsin incubated in the absence of Ca^{++}.

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unmodified β-trypsin. It is interesting to note that the modification of 10 lysyl residues does not significantly change the stability of trypsin towards autolysis. On the other hand, the modification of 11 lysyl residues stabilizes it to the same extent as the complete modification does. Thus there seems to be one critical lysyl peptide bond in trypsin which is essential for maintaining the integrity of the active site. Identification of this particular bond is under investigation in our laboratory.

ACKNOWLEDGEMENT

The authors express their thanks to Mr. J. D. Ford and Miss L. H. Clack for performing amino acid analyses and to Mrs. Alice Knapp for skillful assistance.

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