MODIFICATION OF CARBOXYL GROUPS IN THE ACTIVE SITE OF TRYPSIN*

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Summary - The chemical modification of carboxyl groups in chromatographically homogeneous bovine β-trypsin using the carbodiimide-nucleophile procedure resulted in complete loss of enzyme activity and the modification of 8 carboxyl groups. Under similar reaction conditions, the presence of a competitive inhibitor was found to decrease both the extent of modification and the loss of activity, giving a product with 7 carboxyls modified and 30% of the original activity. The carboxyl group protected to the largest extent was identified as Asp-177, with Asp-182 being protected to a lesser extent. Direct evidence is also presented indicating that residue 177 is indeed aspartic acid rather than asparagine.

Specific action of trypsin on linkages involving the carboxyl groups of lysine and arginine has long suggested an anionic site for the binding of substrate. Systematic studies of competitive inhibition (1, 2) also strongly suggested the presence of at least one carboxyl group in the specificity site. The development of a mild, specific method for the modification of carboxyl groups in proteins with a combination of a water-soluble carbodiimide and a nucleophile by Hoare and Koshland (3) led us to attempt identification of the carboxyls present in the binding site of trypsin. By using competitive inhibitors to protect the carboxyls in the binding site from the modification, it should be possible to subsequently identify those residues by using a radioactive label. This communication reports the identification of the carboxyls thus labeled. Effect of the modification on the enzyme activity is also described.

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MATERIALS AND METHODS

Chromatographically homogeneous β-trypsin obtained from commercial bovine trypsin (Worthington) by the procedure of Schroeder and Shaw (4) was used in all modification studies. The modification reaction was performed essentially as described by Hoare and Koshland (3) using a water-soluble carbodiimide EDC* in the presence of glycinamide as nucleophile. Details are given in the legend to Fig. 1. 1-^{14}C-Glycinamide was synthesized from 1-^{14}C-glycine (5). Edman degradation was performed in 3 stages essentially as outlined by Blombäck et al. (6) with conversion of the thiazolinone to the PTH-derivative carried out by the method of Edman and Begg (7). Purification of peptides was accomplished using column chromatography on Dowex 50-x8 (Beckman type 15A) with pyridine-acetate buffers, and DEAE-cellulose (Whatman DE-32) employing tris buffer. Protein and peptide concentrations were determined by amino acid analyses (8). Enzymatic activity was assayed by a pH-stat using benzoyl-L-arginine ethyl ester as substrate, or by active site titration using p-nitrophenyl p'-guanidino benzoate (9).

RESULTS AND DISCUSSION

The reaction of β-trypsin with EDC in the presence of glycinamide resulted in complete loss of enzyme activity within 3 hours, and modification of 8 carboxyl groups (Fig. 1). EDC is known to modify tyrosine phenolic groups as well as carboxyl groups (10). However, the loss of activity was entirely due to modification of carboxyl groups since the reacted tyrosines could be regenerated without significantly affecting

* Abbreviations: EDC, 1-ethyl-3-dimethylaminopropyl carbodiimide; PTH, phenylthiohydantoin; AECys, S-(β-aminoethyl)-L-cysteine; DFP, diisopropyl fluorophosphate.

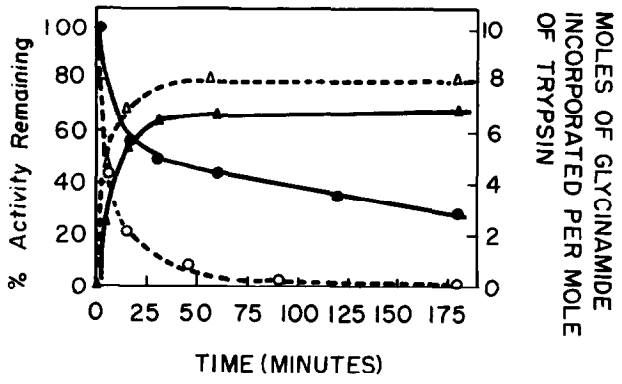

Fig. 1. Modification of trypsin with EDC and glycinamide in the presence and absence of benzamidine.

β-Trypsin (10 mg/ml) and 1-^{14}C-glycinamide (1.0 M) were dissolved at pH 3 in water, the pH readjusted to 4.75, and the reaction initiated by addition of EDC to a concentration of 0.1 M. Identical quantities of EDC were added at 1 and 2 hrs. pH was maintained at 4.75 with 0.5N HCl on a pH-stat at 25°C. Aliquots were removed at intervals, quenched in 2 M sodium-acetate pH 3.5, and dialyzed exhaustively against 0.7 mM HCl at 4°C. Radioactivity incorporated was determined by scintillation counting with the aid of a thixotropic gel, and enzyme activity of the aliquots assayed using benzoyl-L-arginine ethyl ester as substrate. Benzamidine·HCl when present was at a concentration of 0.4 M. Otherwise, 0.4M KCl was added to maintain ionic strength.

(△ --- △) Incorporation in absence of benzamidine
(o --- o) Enzyme activity in absence of benzamidine
(▲ — ▲) Incorporation in presence of benzamidine
(● — ●) Enzyme activity in presence of benzamidine

enzyme activity determined by active site titration (9). The observed complete inactivation of trypsin may reflect modification of the specificity site carboxyl group(s), as similar treatment of chymotrypsin (11, 12) resulted in only 60% inactivation. The modification reaction carried out in the presence of the competitive inhibitor of trypsin, benzamidine, decreased the rate and extent of the modification as well as the extent of activity loss. About 30% of the initial activity remained after 3 hours, and 7 carboxyls had been modified (Fig. 1).

Labeling of the residues protected by the inhibitor was achieved by

reaction carried out in two stages. β-Trypsin was first treated for 3 hours with EDC and unlabeled glycinamide in the presence of 0.4 M benzamidine. After removal of the inhibitor and excess reagents by dialysis, the lyophilized protein was incubated with EDC and 1-^{14}C-glycinamide for 3 hours in the absence of inhibitor. This resulted in the incorporation of 1.1 residues of labeled glycinamide per mole of trypsin. To avoid possible peptide heterogeneity due to partially reacted carboxyl sites, the labeled protein was treated once more with EDC and unlabeled glycinamide in 8 M urea for 1 hr.

The radioactive-labeled protein thus obtained was subjected to S-amino ethylation and tryptic digestion (13). The major radioactive peptide obtained by Dowex 50 chromatography contained 0.61 moles of ^{14}C-glycinamide and had the following amino acid composition: Asp_1, Gly_1, Ser_1, $AECys_1$. The composition uniquely identifies the peptide as arising from residues 177-179 in the trypsinogen sequence, with the labeled glycine attached to the β-carboxyl of Asp-177. A radioactive peptide corresponding to residues 180-189 was also found and contained 0.34 moles of ^{14}C-glycinamide per mole of peptide. These findings indicate that most of the radioactive glycinamide is attached to Asp-177 and Asp-182, with the former being the predominant site of labeling.

Since the proposed sequence for trypsinogen (14, 15) showed residue 177 as asparagine, it seemed desirable to obtain direct evidence that it is actually aspartic acid. For this purpose the "active serine" peptide comprising residues 177-192 was isolated from a tryptic digest of performic acid oxidized (16) Worthington trypsin and subjected to Edman degradation. The peptide was obtained in pure form by DEAE-cellulose chromatography of Dowex 50 break-through fractions. The disappearance

TABLE 1. Amino acid composition of "active serine" peptide obtained from a tryptic digest of oxidized Worthington trypsin before and after one Edman degradation.

Amino Acid	Before Edman (residues)	After Edman (residues)
Lys	1.00 (1)*	0.85
Asp	1.87 (2)	1.04
Ser	2.44 (3)	2.67
Glu	0.97 (1)	1.00
Pro	0.98 (1)	1.05
Gly	3.81 (4)	4.00
Val	1.29 (2)	1.41
Cysteic	2.02 (2)	1.93

* Numbers in parenthesis indicate expected ratios based on the sequence of residues 177-192 (13).

of aspartic acid after Edman degradation (Table 1) confirms results obtained using other methods (17, 18). However, thin-layer chromatography of the N-terminal PTH-derivative gave no trace of PTH-asparagine (Fig. 2). The conditions employed for conversion of the thiazolinone to the PTH-derivative result in some deamidation of PTH-asparagine (7) but to an extent of only 20-25% as determined in our laboratory. Similar results were obtained with peptides isolated from ^{3}H-DFP-treated and chromatographically purified trypsins, indicating that deamidation of residue 177 did not arise as an artifact in either procedure. We must conclude that residue 177 is indeed asparatic acid.*

The finding that Asp-177 is the major carboxyl residue protected from modification by the binding of a competitive inhibitor strongly suggests that this residue provides the ionic binding site which largely determines the specificity of trypsin. The recent discovery by Smith

* We recently became aware via a note added to the paper of Smith and Shaw (18) that both Hartley and Walsh have independently arrived at the same conclusion

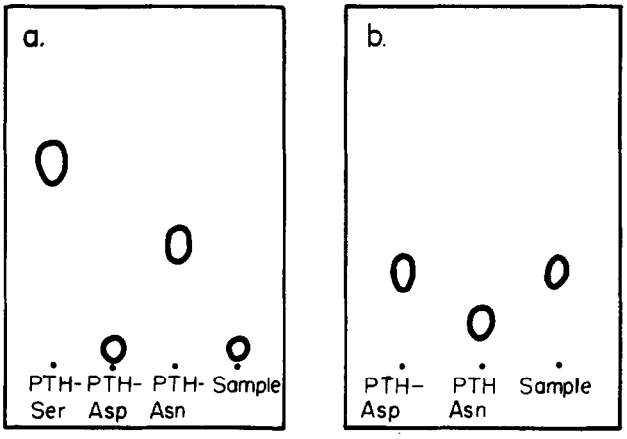

Fig. 2. Thin-layer chromatography of PTH derivative obtained from the N-terminal residue of "active serine" peptide isolated from oxidized Worthington trypsin. a. Silica gel G with chloroform/methanol, 9:1, as solvent. b. Silica gel G with chloroform/formic acid, 100:5, as solvent. Spots were localized with an Iodine-azide spray. N-terminal PTH-derivative was spotted at position marked "sample".

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and Shaw (19) that an autolytic split at residues 176-177 drastically reduces affinity of the enzyme for cationic substrates and inhibitors lends further support to this conclusion. Interestingly enough, Asp-177 occurs in a peptide sequence which is not homologous to chymotrypsin but is adjacent to the homologous sequence which contains the active seryl residue (Ser-183). Furthermore, this non-homologous sequence is longer by 2 amino acid residues than the corresponding structure of chymotrypsin as shown below (14, 15).

	170	177	182
Trypsin	-GLY-tyr-leu-glu-gly-gly-lys-asp-	SER-CYS-gln-GLY-ASP-	
Chymotrypsin	-GLY-ala-ser-gly-val-ser-	SER-CYS-met-GLY-ASP-	

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REFERENCES

1. Inagami, T., J. Biol. Chem., 239, 787 (1964).
2. Mares-Guia, M. and Shaw, E., J. Biol. Chem., 240, 1579 (1965).
3. Hoare, D.G. and Koshland, D.E., Jr., J. Biol. Chem , 242, 2447 (1967).
4. Schroeder, D.D. and Shaw, E.J., J. Biol. Chem., 243, 2943 (1968).
5. Anderson, G.W., Zimmerman, J.E., and Callahan, F.M., J. Am. Chem. Soc., 86, 1839 (1964).
6. Blombäck, B., Blombäck, M., Edman, P , and Hessel, B., Biochim. Biophys. Acta., 115, 371 (1966).
7. Edman, P., and Begg, G., Eur. J. Biochem., 1, 80 (1967).
8. Spackman, D.H., Stein, W.H., and Moore, S., Anal. Chem., 30, 1190 (1958).
9. Chase, T., Jr., and Shaw, E., Biochem. Biophys. Res. Comm. 29, 508 (1967).
10. Carraway, K.L. and Koshland, D.E., Jr., Biochim. Biophys. Acta., 160, 274 (1968).
11. Carraway, K.L., Spoerl, P., and Koshland, D.E., Jr., J. Mol. Biol., 42, 133 (1969).
12. Abita, J.P. and Lazdunski, M., Biochem. Biophys. Res. Comm. 35, 707 (1969).
13. Raftery, M.A. and Cole, D.R., Biochem. Biophys. Res. Comm., 10, 465 (1963).
14. Walsh, K.A. and Neurath, H., Proc. Nat. Acad. Sci., 52, 884 (1964)
15. Mikes, O., Holeysovsky, V., Tomasek, V., and Sorm, F., Biochem. Biophys. Res Comm., 24, 346 (1966).
16. Hirs, C.H.W., Meth. Enzymol. XI, 197 (1967).
17. Dixon, G.H., Kauffman, D.L., and Neurath, H., J. Biol. Chem., 233, 1373 (1958).
18. Holeysovsky, V., Alexijev, B., Tomasek, V., Mikes, O., and Sorm, F., Coll. Czech. Chem. Comm., 27, 2662 (1962).
19. Smith, R.L. and Shaw, E., J. Biol. Chem., 244, 4707 (1969).