STUDIES ON THE ACTIVE SITE OF TRYPSIN. II. THE ROLE OF THE IMIDAZOLE RING OF HISTIDINE IN THE CATALYTIC ACTION OF TRYPSIN.

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A detailed investigation of the mechanism of the esterase activity of α-chymotrypsin has shown that one serine residue and one or two residues of histidine participate in the action of this enzyme[1]. Using a specific inhibitor of α-chymotrypsin, TPCM[x], Schoellmann et al.[2,3] and Meloun and Pospíšilová[4] were able to demonstrate the substitution of one histidine residue of the α-chymotrypsin molecule and to determine that the histidine residue which was labeled by the irreversible inhibitor was bound in the peptide sequence Val.Thr.Ala.Ala.His.Cys.Gly.Val.Thr.Thr.Ser.Asp.

Trypsin contains three histidine residues and the amino acid sequences around two of these residues are very

[x] Abbreviations: TPCM: N-α-tosyl-L-phenylalanyl chloromethane; TLCM: N-α-tosyl-L-lysyl chloromethane; TLCM-trypsin: trypsin labeled with N-α-tosyl-L-lysyl chloromethane; DIP: diisopropylphosphoryl; BAEE: α-benzoyl-L-arginine ethyl ester.

similar with the neighborhood of the two histidine residues contained in the molecule of α-chymotrypsin[5,6]. Since the two proteinases, α-chymotrypsin and trypsin, are somewhat alike in their primary structure[5,6] and in the mechanism of their function, we set about investigating the inhibition of trypsin by the specific irreversible inhibitor[8], TLCM. The first part of this study[9] dealt with the investigation of the reaction conditions, the kinetics of the inhibition, and the geometry of the active site. In the present study an effort was made to determine which one of the three histidine residues of the trypsin molecule was involved in its catalytic function and was responsible for the loss of enzymatic activity after being labeled with TLCM.

DIP-trypsin (1.5 g)was converted into its S-sulfo derivative[10]. A 0.4% solution containing 1.5 g of S-sulfo--DIP-trypsin was digested with pepsin (30 mg) at 37°C and pH 1.9 for 4 hours. The peptic hydrolysate was fractionated on a Dowex 50-X2 column (130 x 2.2 cm) using a linear gradient which was developed with 5,000 ml of 0.05 M pyridine-acetic acid at pH 3.0 and 5,000 ml of 2 M pyridine-acetic acid buffer at pH 6.0. The fractionation of the hydrolysate on the column was followed by chromatography of 2 ml aliquots of each fraction on Whatman No. 3 paper in the system n-butanol-pyridine-acetic acid-water (15:3: :10:12) (cf.[11]). The peptides were detected both by ninhydrin and the Pauly reagent. Histidine-containing peptides obtained from the Dowex 50 column were purified by paper chromatography in the above system or, alternatively, by descending paper electrophoresis[12] and characterized by

quantitative amino acid analysis[13] and end-group determinations. Three histidine-containing peptides were isolated (H1, H2, H3; Table I).

TABLE I

Amino Acid Composition of Histidine Peptides of a Peptic Hydrolysate of S-Sulfo-DIP-trypsin (H1, H2, H3) and S-Sulfo-TLCM-trypsin (IH1, IH2)

Peptide	Lys	His	Ser	Glu	Pro	Gly	Ala	Cys/2[x]	Val	Ile	Leu	Tyr	Phe
H1		0.48	0.94			2.03		0.66			1.07		0.96
H2		1.00	0.90		1.11				0.89	0.90		0.68	
H3	1.06	0.93	1.05	1.05		0.92	1.92	1.03		1.07		0.90	
IH1		0.60	0.98			1.87		0.92			1.00		0.94
IH2													

[x] Determined as $CySO_3H$ after performic acid oxidation of the peptide containing S-sulfo-cysteine

In the second experiment 2.32 g of native trypsin (a 1% solution) was incubated with 530 mg of TLCM-hydrochloride (molar ratio 15:1) at 5°C and pH 7.4. The modified trypsin was dialyzed against 0.001 M HCl and lyophilized. The obtained product, TLCM-trypsin, was enzymatically inactive when tested on BAEE. The inactive TLCM--trypsin was converted into its S-sulfo derivative and S-sulfo-TLCM-trypsin was separated from the reaction mixture on a Sephadex G-25 column. Quantitative analysis of basic amino acids of the parent active trypsin and of TLCM-trypsin demonstrated that the histidine content of the inhibited enzyme had decreased by one residue (Table II). S-Sulfo-TLCM-trypsin was cleaved with pepsin under

TABLE II

Ratio of Basic Amino Acids in Trypsin and TLCM-Trypsin

Amino Acid Residues	Lys	His	Arg
Trypsin	14.00	3.21	1.88
TLCM-trypsin	13.60	2.33	2.01

conditions analogous to those shown above. The peptic hydrolysate was fractionated by the same procedure on a Dowex 50-X2 column.

Only two histidine-containing peptides were isolated from the peptic hydrolysate of S-sulfo-TLCM-trypsin (IH1 and IH2, identical with peptides H1 and H2 from trypsin). Their structures are shown in Table III.

TABLE III

Structure of Histidine Peptides of the Peptic Hydrolysate of S-Sulfo--DIP-trypsin (H1, H2, H3) and S-Sulfo-TLCM-trypsin (IH1, IH2)

Peptide	Structure	pH[x]
H1	His.Phe.Cys.Gly.Gly.Ser.Leu.	4.93
H2	Ile.Val.His.Pro.Ser.Tyr.	5.12
H3	Ala.Ala.His.Cys.Tyr.Lys.Ser.Gly.Ile.Gln.	5.12
IH1	His.Phe.Cys.Gly.Gly.Ser.Leu.	4.95
IH2	Ile.Val.His.Pro.Ser.Tyr.	5.11

[x] pH-value at which the peptide emerged from the Dowex 50 column under the conditions given above.

As may be seen TLCM substituted the imidazole ring of histidine residue 46 of peptide H3 which forms a part of the longer sequence

....Tyr.His.Phe.Cys.Gly.Gly.Ser.Leu.Ile.Asn.Ser.Gln.Trp.
28 29 30 31 32 33 34 35 36 37 38 39 40

.Val.Val.Ser.Ala.Ala.His.Cys.Tyr.Lys.Ser.Gly.Ile.Glu.Val.
41 42 43 44 45 46 47 48 49 50 51 52 53 54

.Arg....,
55

where half-cystines 31 and 47 are connected by a disulfide bond[14].

The treatment of α-chymotrypsin and trypsin with specific irreversible inhibitors results in the substitution of identical histidine residues as could be assumed on the basis of similarities in the primary structure and mechanism of function of these two proteinases.

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