STUDIES ON THE ACTIVE SITE OF TRYPSIN. I. THE REACTION OF TRYPSIN WITH THE SPECIFIC INHIBITOR N-α-TOSYL-L-LYSYL-CHLOROMETHANE AND CERTAIN PSEUDOSUBSTRATES

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The last few years have witnessed considerable success in the elucidation of the primary structures of α-chymotrypsin and trypsin[1,2]. Attention is still being focused on problems of the mechanism of action of proteinases[1,3,4]. The participation of one serine residue and of the imidazole ring of one histidine residue[5,6,7] in the catalytic action of chymotrypsin has been proved. Criteria for the characterization of the region of the active center of chymotrypsin have also been investigated and formulated in detail[8,9]. A number of papers reporting on studies confined to a comparison of primary structures and the mechanism of action of chymotrypsin and trypsin have appeared[2,10]. The investigation of the reaction of enzymes with specific inhibitors is one of the most effective methods of examining the structure of the active site of enzymes. This kind of approach has met with success in studies on proteinases[6,11], pyridoxal enzymes [12,13], and several other enzymes.

The specific inhibitor must conform to two conditions, i.e. a) it must resemble the substrate and contain the same functional groups as the natural substrate which is a prerequisite of high affinity of the pseudosubstrate to the

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active site of the enzyme, b) it must contain functional groups which can react in the active site of the enzyme, i.e. to block and thus to "label" simultaneously the area of the active site of the enzyme which is essential for its function.

At the time when this study was begun a short note appeared on the synthesis of N-α-tosyl-L-lysyl-chloromethane[+], an inhibitor of trypsin[14]. The task of the present study was to follow the reaction of trypsin with its specific inhibitor, TLCM[++].

Material and Methods

Trypsin was a three times crystallized preparation[15] which was dialyzed against 0.001 N HCl and lyophilized. The activity of trypsin was determined at 20° by the spectrophotometric assay using BAEE[16] as a substrate.

Activity measurements: To 3 ml of 0.05 M phosphate buffer at pH 8.05 in a spectrophotometric cuvette was added 25 µl of 6 x 10^{-4}M solution of BAEE. The spectrophotometer reading was zeroed and 25 µl of 4 x 10^{-4}M trypsin solution was added. The increase of optical density at 253 mµ was then measured at definite time intervals.

Determination of the Degree of Inhibition: To 0.8 ml of 0.05 M phosphate buffer at pH 8.05 was added 0.1 ml of 4 x 10^{-4}M trypsin solution and 0.1 ml of the TLCM solution of a definite concentration, and the mixture was incubated for 10 minutes at 20°. 25 µl of this solution was then added to the spectrophotometer cuvette containing 25 µl of 6 x 10^{-4}M BAEE solution in 3 ml of 0.05 M phosphate buffer at pH 8.05. The increase of absorbance at 253 mµ for a period of 15 to 45 sec. was compared and the degree of inhibition determined for each concentration.

[+]The authors are indebted to Dr.J.Rudinger and Dr.L.Jarošková for the synthesis of TLCM.

[++]Abbreviations: TLCM: N-α-tosyl-L-lysyl chloromethane; BAEE: α-benzoyl-L-arginine ethyl ester.

Results and Discussion

The interaction of TLCM with trypsin was followed in terms of the length of the pre-incubation period, the temperature, pH and also the presence of a number of substrates and pseudosubstrates which can interfere with the inhibition of trypsin by the specific inhibitor.

TLCM readily and irreversibly inactivates trypsin and the rate of inactivation decreases rapidly with decreasing temperature. At 20-fold excess of the inhibitor (concentration 4×10^{-4}M) and 20° trypsin is inactivated by 50% within 5 min. At the same temperature and 10 min. of pre-incubation the same degree of inactivation (50%) is brought about by a concentration of TLCM as low as 5×10^{-5}M. The action of TLCM is fully irreversible and reactivation of the enzyme cannot be achieved. The increase of the intensity of inhibition with increasing time of contact of trypsin with TLCM is shown in Fig. 1. The rate of inactivation was observed to be maximal at the pH-value which is cha-

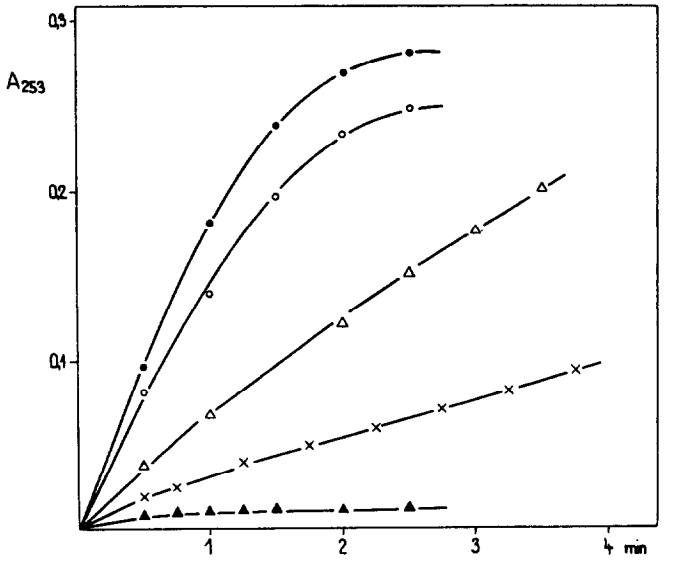

Fig. 1. Inhibition of Trypsin by TLCM as a Function of the Length of the Pre-Incubation Period.
Temperature 20°; concentration of trypsin 1.8×10^{-5}M; concentration of inhibitor 4×10^{-4}M; activity control ●●● ; activity of trypsin samples pre-incubated with TLCM for 30 sec. ooo , 5 min. ΔΔΔ , 12 min. ××× , 30 and 45 min. ▲▲▲ .

racteristic for the catalytic action of trypsin (Fig. 2). This suggests that the selective inhibition involves the functional groups of the active site which participate in the catalytic action of the enzyme.

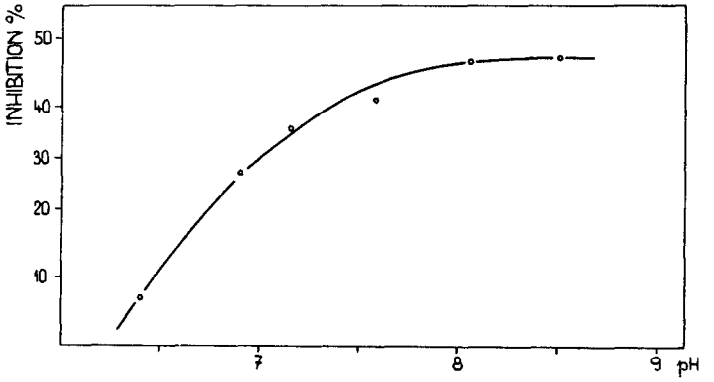

Fig. 2. Inhibition of Trypsin by TLCM as a Function of pH. Length of pre-incubation 10 min.; temperature 20°; concentration of trypsin 3.6×10^{-5}M; concentration of inhibitor 5×10^{-5}M.

The loss of activity under the above conditions could be also due to the autolysis of the enzyme. It was demonstrated in control experiments that trypsin was not inactivated due to autolysis under the conditions of the inhibition. (Fig. 3).

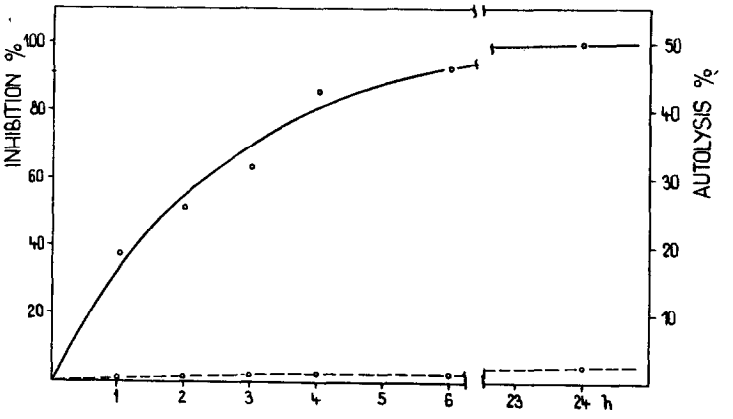

Fig. 3. Inhibition of Trypsin by TLCM and Autolysis of Trypsin. h – time in hours; —— inhibition: 96 mg of trypsin and 14.76 mg of TLCM hydrochloride in 10 ml of 0.05 M phosphate buffer at pH 7.4 (concentration of trypsin 4×10^{-4}M, concentration of TLCM 4×10^{-3}M), temperature 5°; – – – autolysis: The degree of autolysis was determined in terms of activity decrease in an experiment carried out under the same conditions as the inhibition but with the omission of TLCM.

At 15-fold excess of TLCM, a temperature of 0 to 5° and pH 7.4 the inhibition was complete within 15 to 20 min., and this was paralleled by the loss of one histidine residue as shown by amino acid analysis of the inhibited product (cf. next paper).

Additional data which show that the reaction of trypsin with TLCM takes place in the active site of the enzyme was obtained from an investigation of the inhibition in the presence of certain substrate analogs and competitive inhibitors. Some of the preliminary data are given in Table I.

TABLE I

Inhibition of Trypsin by N-αC-Tosyl-L-lysyl-chloromethane in the Presence of Certain Analogs of Substrates and Competitive Inhibitors

Competitive Inhibitor or Substrate Analog	Concentration (M)	Concentration of TLCM(M)	Inhibition %
——	——	5×10^{-5}	50
L-Lysine	1×10^{-2}	5×10^{-5}	0
	1×10^{-3}	5×10^{-5}	41
L-Diaminobutyric acid	1×10^{-1}	5×10^{-5}	42
n-Butylamine	1×10^{-2}	5×10^{-5}	0
	1×10^{-3}	5×10^{-5}	20
Benzamidine	1×10^{-3}	5×10^{-5}	0
	1×10^{-4}	5×10^{-5}	14
	1×10^{-5}	5×10^{-5}	40
Piperidine*	1×10^{-2}	5×10^{-5}	10
2-Methyl-5-ethyl piperidine	1×10^{-2}	5×10^{-5}	47

* the inhibition had a partly irreversible character

As may be seen in Table I, L-lysine at a concentration of 1×10^{-2}M completely blocks the inhibition of trypsin by TLCM. The affinity of the various compounds to the substrate-binding site of the active site of the enzyme manifests itself clearly in the case of L-lysine and L-diaminobutyric acid. A concentration of L-diaminobutyric acid 100 times greater than that of lysine is needed to achieve the same degree of protection of the enzyme against the action of the specific inhibitor. Another fact deserving interest is that even those compounds which can merely occupy the "place of the side chain" of the natural substrate are fully adequate to the protection of trypsin against the inhibitory effect of TLCM. Thus, e.g. n-butyl amine protects trypsin more than L-lysine. It may be assumed that the carboxyl group of L-lysine has a negative influence on the bond between the side chain of the amino acid and the binding site of the enzyme. The acylamino group of the substrates, in addition to its effect due to a complementary linkage, is most likely responsible for a rigid orientation of the amino acid derivative in space, thus securing the hydrolyzable group of the substrate in the proper fit at the catalytic center of the enzyme. Indeed we were able to demonstrate a complete protection of trypsin against the action of TLCM in the presence of benzamidine, an efficient competitive inhibitor of trypsin. Due to its spatial arrangement (dimensions of the binding site) the protective effect of 2-methyl-5-ethyl piperidine is not identical with the effect of piperidine itself. Our results are in agreement with the data of Rule and Lorand[17] who investigated the degree of binding of different tosylagmatines to the active site of trypsin, and also confirm the existence of a "slit" into which the side chain of the substrate must fit in the active site of trypsin[18].

The reaction of the specific inhibitor, TLCM, with trypsin occurs in the active site of trypsin. The side chain of the inhibitor containing four methylene groups and one ε-amino group occupies the place destined for the side chain of the amino acid of the substrate. Due to the rigid orientation of the molecule of the inhibitor in the active site of the enzyme the chloromethyl

group of TLCM, which replaces the carboxyl group of the amino acid, is situated in the closest vicinity of the active center. In this way there is a rapid blocking of the catalytic site of trypsin, i.e. an irreversible alkylation of the imidazole ring of histidine occurrs (cf. next paper).

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