UDC 3.4.4.5K

A STUDY OF THE REACTIONS OF THE CHEMICAL MODIFICATION OF CHYMOTRYPSIN. I

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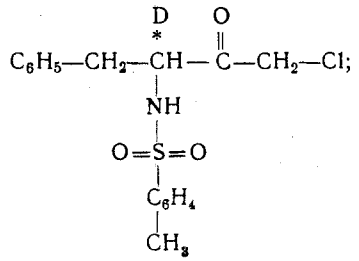
At the present time, the important role of the hydroxy group of the serine-195 [1-3] and of the imidazole ring of the histidine-57 [4, 5] in the catalytic activity of chymotrypsine is well known.

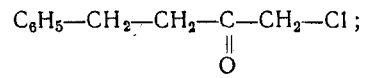
A direct proof of the participation of the histidine in position 57 [5] in the active center of chymotrypsin has been obtained by Schoellman and Shaw [4].

We have attempted to determine the significance of structural features of an inhibitor of chymotrypsin that are necessary for it to preserve its inhibiting properties. It was of interest:

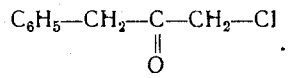
1) To synthesize the D-antipode of L-1-tosylamido-2-phenylethyl chloromethyl ketone (L-TAPK) and to determine whether the "steric requirements" for the inhibitor were fairly rigid:



2) To simplify the structure of L-TAPK by eliminating the amino group with the tosyl protection, thereby losing the asymmetry of the carbon atom, and performing the modification of chymotrypsin with phenethyl chloromethyl ketone (PECK):



3) To eliminate not only the amino group but also one CH_2 group and to carry out the modification of chymotrypsin with benzyl chloromethyl ketone (BCMK):



Experimental

The chymotrypsin was a crystalline medicinal sample (product of the Leningrad Meat Combine) with a Hammel activity of 67%.

Action of inhibitors on chymotrypsin. 1. D-TAPK—mp 101° C. The reaction with chymotrypsin was carried out in 0.1 M phosphate buffer for 3.5 hr at pH 5.0, 7.0, and 8.0 at 37° C with a 45-fold molar excess of inhibitor.

2. PECK—mp 38° C; literature data 40-41° C [8]. The reaction with chymotrypsin took place at pH 7.0 for 3.5 hr at 37° C with various molar excesses of PECK.

3. BCMK—faintly yellowish liquid, bp 124-125° C (10 mm); literature data 133-135° (19 mm) [9]. The reaction with chymotrypsin was carried out at pH 5.0 and 7.0 in 0.1 M phosphate buffer with a 45-fold molar excess of reactant. In this case, no inhibition effect was found (see Experiments 1 and 2, Table 4). Moreover, the reaction took place at pH 7.0 but with a ratio of enzyme to inhibitor of 1 : 350.

Activity of the chymotrypsin. The activity of the sample was determined from the results of the hydrolysis of the p-nitrophenyl ester of N-carbobenzoxy-DL-phenylalanine (NPE of cbz-DL-phenylalanine), obtained by the method of Bodanzky and du Vigneaud [10] with a yield of 83%, mp 102° C (literature data mp 103° C) [11]. The nitrophenyl liberated on hydrolysis was determined in a SF-4 spectrophotometer at 330 mμ. The cell of the spectrophotometer (layer thickness 1 cm) was charged with 1.5 ml of a 0.001 M solution of the NPE of cbz-DL-phenylalanine in acetone, 1.4 ml of 0.01 M phosphate buffer with pH 7.0, and 0.1 ml of the solution of chymotrypsin under investigation.

The optimum conditions for treating the chymotrypsin with the reagents mentioned were selected on the basis of the results of previous experiments with L-TAPK. Inhibition by L-TAPK generally took place at pH 7.0 with a 20-fold molar excess of inhibitor with respect to chymotrypsin [4, 7]. We have observed that the least autolysis of chymotrypsin during the reaction with inhibitors takes place in 0.1 M phosphate buffer with pH 7.0. Literature data and the results obtained permitted the selection of the following conditions for the modification of chymotrypsin: to 3 mg (0.12 μmole) of chymotrypsin in 10 ml of 0.1 M phosphate buffer with pH 7.0 was added 0.96 mg (2.7 μmole) of inhibitor in 0.15 ml of methanol—the "experimental solution". From this solution a sample was taken immediately to measure the activity of the chymotrypsin. A control experiment was set up in parallel in which the inhibitor solution was replaced by an equal volume of methanol. The experimental and control solutions were kept at 37°C for 3.5 hr. After the end of incubation, samples were taken from the solutions for the measurement of the final enzymatic activity of the chymotrypsin. As a measure of activity, we took the initial rate of hydrolysis of the substrate determined as ΔD at 330 mμ between hydrolysis of the substrate for 1 min and for 30 sec: $a = D_{60''} - D_{30''}$.

Table 1

Results of the Action of D-TAPK on Chymotrypsin

Experiment No.	Inhibitor	Conditions of modification		Inactivation of the solution, %		
		pH	ChTr : inhb, mole	control	experimental	due to inhb
1	D-TAPK	5.0	1:45	9	18	9
2		7.0	1:45	13	10	0*
3		8.0	1:45	59	48	0*
4	L-TAPK	7.0	1:20	0	64	64

*In these variants of the experiment, the autolysis in the control solutions of chymotrypsin was somewhat greater than in the experimental solutions.

Table 2

Results of the Action of PECK on Chymotrypsin

Experiment No.	Conditions of modification		Inactivation of the solution, %		
	pH	ChTr : inhb, mole	control	experimental	due to inhb
1	7.0	1:56	16	40	24
2		1:175	24	60	36
3		1:350	24	72	48
4		1:580	24	70	46

The amino acid analyses of the samples of modified chymotrypsin were carried out on an automatic amino acid analyzer of the Hitachi (Japan) type; the basic amino acids were usually analyzed on a 15-cm column. The protein was separated from the inhibitor and from the buffer salts by filtration through a column of Sephadex G-25 ("coarse") (3 × 30 cm) or by dialysis (24 hr) against distilled water at 5°C. The inactivated samples of chymotrypsin obtained after freeze-drying were hydrolyzed in a sealed evacuated tube with 6 N HCl at 105° C for 24 hr. The acid was evaporated in vacuum, the hydrolyzate was oxidized with performic acid, and traces of acid were repeatedly evaporated off with water in vacuum. The results obtained are given in Tables 1–4 below.

It follows from Table 1 that D-TAPK scarcely lowers the activity of chymotrypsin even at a 45-fold molar excess of reagent. Under the same conditions (37°C, 3.5 hr) at pH 7.0 in 0.1 M phosphate buffer with a 20-fold molar excess, L-TAPK causes 64% inactivation of chymotrypsin. The loss of the inhibiting properties in D-TAPK shows that the demands set for the stereospecificity of the inhibitor are fairly strict. These results have been confirmed by the work of Stevenson and Smillie [12], published while our experiments were being performed. These authors state that the D-antipode of TAPK does not inhibit chymotrypsin. The action of PECK on chymotrypsin can be judged from the data of

Table 3

Amino Acid Analysis of Modified Chymotrypsin

Inhibitor	Ratio of ChTr to inhb	Loss of histidine residues per mole of histidine, %
$C_6H_5CH_2CH_2COCH_2Cl$	1:350	58
$C_6H_5CH_2COCH_2Cl$ (L)	1:350	0
$C_6H_5CH_2—\overset{*}{C}H(NH—SO_2C_6H_4CH_3)—COCH_2Cl$	1:20	75

Table 4

Results of the Action of BCMK on Chymotrypsin

Experiment No.	Conditions of modification		Inactivation of the solution, %		
	pH of the buffer	ChTr : inhb, mole	control	experimental	due to inhb
1	5.0	1:45	—	—	0
2	7.0	1:45	—	—	0
3	7.0	1:350	0	23	23
4	7.0	1:350	9	22	13

Table 2. They show that PECK inactivates chymotrypsin to a considerable extent at high molar excesses of inhibitor. Thus, the loss of asymmetry of the carbon atom in L-TAPK by the elimination of the tosylamino group does not lead to the total loss of the inhibiting capacity of the reagent. Apparently the mobile "amide" hydrogen atom in L-TAPK is not so important for the reaction of the inhibitor with the enzyme as was previously considered [4]. However, PECK has a lower capacity than L-TAPK. In order to achieve appreciable inhibition by this reagent, a large molar excess of inhibitor must be added. An amino acid analysis of chymotrypsin treated with a 350-fold molar excess of PECK shows that its histidine content calculated per mole had decreased by 58% (Table 3).

Table 4 gives the results of the action of BCMK on chymotrypsin. Only at a ratio of 1 : 350 of enzyme to inhibitor was some inactivation of the enzyme observed (Experiments 3 and 4, Table 4).

An amino acid analysis of the hydrolyzate of chymotrypsin treated with BCMK (Experiments 3 and 4, Table 4) shows that the amount of histidine was unchanged (see Table 3). Consequently, the action of BCMK on chymotrypsin is apparently not connected with the alkylation of the histidine.

Thus, on the basis of the reagents studied one can trace certain characteristic features in the structure of chymotrypsin inhibitors. An inhibitor is effective if it preserves the typical characteristics of the structure of the substrate. An aromatic amino acid residue possibly favors the "anchoring" of the inhibitor molecule to the hydrophobic "center of specificity" adjacent to the active center of the enzyme.

The presence of an asymmetric carbon atom and of a tosylamino group in the inhibitor considerably increases its effectiveness, although they are not absolutely necessary for the alkylation of the histidine. When the asymmetric carbon atom is preserved, only the compound in the L-configuration possesses an inhibiting capacity. The same feature has been reported in Stevenson and Smillie's paper [12]. These authors proposed a new inhibitor—phenoxymethyl chloromethyl ketone $C_6H_5OCH_2COCH_2Cl$—which, like the phenethyl chloromethyl ketone used in our investigations reacts with the histidine of the chymotrypsin (ratio of enzyme to inhibitor 1 : 300 or 1 : 500), causing its inactivation. Stevenson and Smillie have shown that this inhibitor, just like L-TAPK, reacts with histidine-57. Apparently, for the reaction of the inhibitor with the histidine of chymotrypsin the presence of an aromatic residue and a long carbon chain is most essential. This assumption is confirmed by the negative results obtained in experiments on the inhibition of chymotrypsin with benzyl chloromethyl ketone.

Summary

The reaction of chymotrypsin with a number of modified analogs of the specific inhibitor of chymotrypsin L-1-tosylamido-2-phenylethyl chloromethyl ketone has been studied; the compounds investigated were D-1-tosylamido-2-phenethyl chloromethyl ketone, phenethyl chloromethyl ketone (PECK), and benzylethyl chloromethyl ketone (BCMK).

It has been shown that D-TAPK does not possess inhibiting activity. The inhibiting capacity of PECK is considerably reduced. A simultaneous decrease in the length of the carbon chain and the elimination of the tosylamino group (BCMK) practically destroys the inhibiting action of the reagent.

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