A NEW METHOD FOR LABELLING THE ACTIVE CENTER OF CHYMOTRYPSIN

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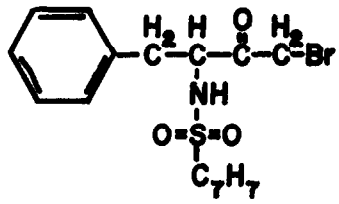
We are attempting to develop new, specific reagents for locating the active centers of enzymes by designing molecules which combine two kinds of structural features, namely, those which provide affinity to the active center and, in addition, a chemically reactive grouping which may anchor irreversibly to the enzyme permitting subsequent identification of key amino acid residue(s) involved. As an example of this type of approach we wish to describe a reagent devised for labelling the active center of chymotrypsin and give evidence that it achieves this goal.

N-Tosyl-L-phenylalanine methyl ester is a substrate for chymotrypsin (Hummel, 1959). It was decided that a bromoketone incorporating N-tosyl-L-phenylalanine, that is I, would have the desired affinity for the active center and ability to alkylate the enzyme at this site.



I

TPBK

The bromoketone I, TPBK, was synthesized from tosyl-L-phenylalanine by a standard method (Bachmann and Struve, 1942) through an intermediate diazoketone which was treated with HBr to yield crystals, m.p. 94-95° [1]. (Anal.

[1] It is now realized that in successive preparations of the bromoketone, at the diazoketone step N-methylation at the sulfonamide nitrogen took place as a side reaction to a variable degree not clearly discernable in the melting point but leading to less active preparations. This difficulty has been avoided in recent syntheses of chloroketone to be described.

calcd. for $C_{17}H_{18}BrNO_3S$; C, 51.52; H, 4.58; N, 3.53; Br, 20.17. Found: C, 52.40; H, 5.39; N, 3.30; Br, 20.31.)

When the bromoketone was incubated with alpha-chymotrypsin (Worthington) at pH 6.0, 37°, a loss of enzymic activity was observed on aliquots removed for spectrophotometric assay with N-acetyl-L-tyrosine ethyl ester (Schwert and Takenaka, 1955) as substrate (Fig. 1). The irreversible loss of activity was apparently due to reaction at the active center since a known reversible inhibitor of chymotrypsin, that is, hydrocinnamic acid (Neurath and Schwert, 1950) protected the enzyme from the bromoketone (Table 1).

The foregoing evidence based on activity studies has been confirmed by observations with radioactive TPBK synthesized from uniformly labelled L-phenylalanine-C^{14}. It was found that on mixing radioactive TPBK with chymotrypsin in a 20/1 molar ratio (conditions as in Fig. 1) followed by extraction with ether (7 x 15 ml., previously saturated with water) and dialysis against distilled water at 4° for 48 hours, no radioactivity remained bound

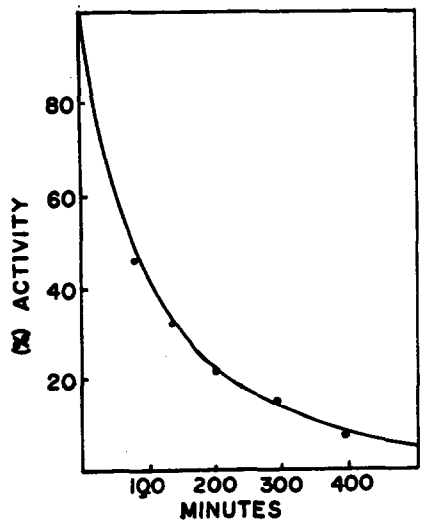


Fig. 1 - Progressive inactivation of chymotrypsin by TPBK. Chymotrypsin, 0.1 micromole and TPBK 1.9 micromole, incubated in 0.1 M phosphate buffer, pH 6.0, final volume 25 ml. containing 3% methanol.

TABLE 1

Protection of Chymotrypsin by Hydrocinnamic Acid
Against Inactivating Action of TPBK

	% ENZYMATIC ACTIVITY				
	0 MINS	100 MINS	200 MINS	300 MINS	400 MINS
A - CHYMOTRYPSIN	100	100	100	100	100
B - CHYMOTRYPSIN + TPBK	100	35	17	11	9
C - CHYMOTRYPSIN + TPBK + HYDROCINNAMIC ACID	100	82	75	62	60

Chymotrypsin, 0.1 micromole, TPBK, 1.9 micromole, hydrocinnamic acid, 430 micromoles; incubated at 37° in 0.1 M phosphate buffer, pH 6.0, final volume 25 ml., containing 3% methanol.

(Because of the low solubility of the bromoketone in water, a methanolic solution of it was added to the buffer solution used for the inactivation studies. The final concentration of methanol (3%) had no effect on chymotrypsin stability, that is, full activity was retained throughout the experiment as indicated in experiment A.)

to the protein unless time had been allowed for inactivation of the enzyme to proceed. For quantitative study of the binding, in order to avoid variable quenching effects due to protein, a standard was prepared containing 0.0129 micromoles of chymotrypsin and 0.328 micromoles of TPBK-C^{14} in 2 ml. of water; this was added to 18 ml. of phosphor solution for scintillation counting (Bray, 1960) and 1278 c.p.m. were observed in a Packard liquid scintillation counter. In inactivation studies, care was taken to analyze essentially the same amount of protein that had been used in the standard in determining the bound radioactivity. The results of two experiments shown in Table 2 indicate that the radioactivity bound by chymotrypsin when totally inactivated is of the order of one mole of inhibitor per mole of enzyme.

TABLE 2

Binding of TPBK to Chymotrypsin (CT) During Inactivation

	CT Micromoles Determined*	TPBK-C^{14} Micromoles Calculated	c.p.m. Observed	Binding Ratio Molar CT : TPBK
Exp. 1	0.0137	0.0131	50.9	1 : 0.95
Exp. 2	0.0126	0.0121	47.1	1 : 0.96

For CT, MW = 25000

* Protein determined by the Lowry method (Lowry, et al., 1951)

The specificity of TPBK for the functioning enzyme is shown by its failure to inactivate chymotrypsinogen. After prolonged exposure to TPBK and following removal of the inhibitor, the zymogen could be fully activated by trypsin, as shown by the following experiment. Chymotrypsinogen (0.1 micromole) was incubated in 25 ml. of 0.1 M phosphate buffer, pH 6.0, with TPBK (2.0 micromoles) for eight hours, then extracted with ether and dialyzed as described above. When the zymogen was then activated by trypsin (0.09 mg.) for 255 minutes at room temperature, the resultant chymotrptic activity was identical to that of a control solution of chymotrypsinogen similarly treated in the absence of TPBK. Consistent with this finding was the observation that no radioactivity was bound to chymotrypsinogen when it was incubated with radioactive TPBK under the above conditions.

Chemical studies on the nature of chymotrypsin - TPBK compound are under way to relate these findings to results obtained by other methods (cf. Summary by Neurath and Hartley, 1959).

The application of this type of approach to other enzymes is being investigated.

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