A PHOTOAFFINITY LABELING OF THE ACTIVE SITE OF α-CHYMOTRYPSIN WITH TPDK*

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Received 7 September 1973

1. Introduction

In chemical modification of enzymes, affinity-labeling techniques which use site-specific reagents for the object enzymes have great utility and availability [1]. For example, the reaction of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) with α-chymotrypsin, demonstrated by Shaw et al., originally established the presence of His-57 in the active site of the enzyme [1–3]. Westheimer et al., pioneered in the use of photolyzable reagents in site-labeling reactions for enzymes [4]. In 'photoaffinity labeling,' a reagent R–P is used, where R is the portion that directs to the active site under study, and P is a group that is ordinarily unreactive, but can be converted by photolysis to a reactive intermediate P* [5]. The P* species used by Westheimer et al. were carbenes, while Knowles et al., to improve the process, employed nitrenes for labeling antibodies [6]. In the course of our studies on the photoaffinity labeling, we have explored the photoirradiation of chymotrypsin in the presence of L-1-tosylamido-2-phenylethyl diazomethyl ketone (TPDK), a precursor of TPCK [2], in an attempt to see the relation between the reactive species and the reaction site with this well-known site-specific example.

2. Materials and methods

α-Chymotrypsin from Worthington, three times crystalized, lot CDi-2DA, was used. TPDK was prepared by the method of Schoellmann and Shaw [2].

* Chemical modification of proteins. VII.

Enzymatic activity was observed with *N*-benzoyl-L-tyrosine *p*-nitroanilide as substrate [7]. The amino acid analyses were performed on a Hitachi Amino Acid Analyzer type KLA-3B, according to the procedure of Spackman et al. [8]. Protein samples were hydrolyzed for 24 hr in 6 N HCl at 110°C before analysis.

The photolysis was carried out as follows: A solution (1×10^{-5} M) of chymotrypsin in 0.05 M phosphate buffer (pH 7.0) was mixed with 25–50-fold molar excess of TPDK in acetonitrile solution (final concetration was 5%) in a Pyrex tube. The solution was degassed in high vacuum initially at the cold in a liquid-nitrogen jar and then at room temperature, and this process was repeated three times. The solution was irradiated with a 500 W high-pressure mercury lamp (Eikosha Halos, PIH-500) for 7 min at 5°C. The mixture was dialyzed against several changes of 10^{-3} M HCl at 4°C and lyophilized. The same treatments with the solution in the absence of TPDK were performed as control experiments.

3. Results

Under photoirradiation, TPDK very rapidly inactivated chymotrypsin. No loss of activity was observed without irradiation. α-Chymotrypsin showed a 65% loss of activity when the molar ratio of inhibitor to enzyme was 25, and a 75% loss in a 50:1 molar ratio (table 1). In the case of the control enzyme sample irradiated in the absence of TPDK, the loss of activity found was only 30%, although prolonged irradiation (20 min) brought about more than 50% loss of activity. The amino acid compositions of the

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Table 1
Relative activity of the irradiated chymotrypsin with and without TPDK.

	Activity (%)
Chymotrypsin	70
Chymotrypsin + TPDK (2.5×10^{-4} M)	35
Chymotrypsin + TPDK (5.0×10^{-4} M)	25

irradiated chymotrypsin with and without TPDK were nearly identical except for a significant change in the His residues as shown in table 2. 0.6 His residue less is found in the hydrolysate of the irradiated chymotrypsin with TPDK, while no loss was observed in the one without TPDK. Since only two residues of His are present in the enzyme molecule this analytical change is distinct. No new peaks were obrained on acid hydrolysis of TPDK-irradiated chymotrypsin that could be unambiguously assigned to a new His derivative, nor in the case of TPCK-chymotrypsin [2].

Table 2
Amino acid compositions of irradiated and non-irradiated chymotrypsin.

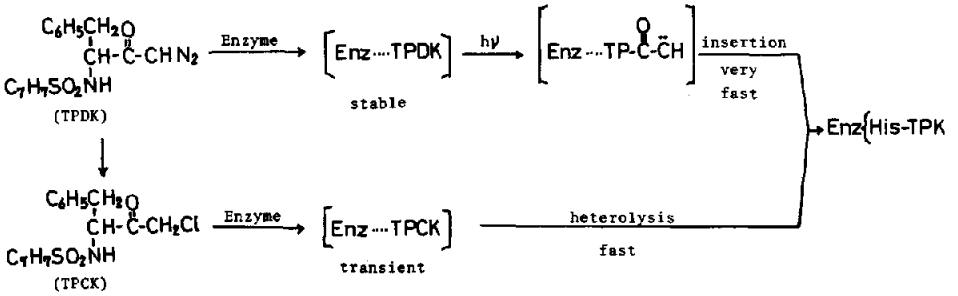
Amino acid	Non-irradiated chymotrypsin	Irradiated chymotrypsin	TPDK-irradiated chymotrypsin
Asp	21.4	21.7	20.9
Ser	19.4	19.2	18.9
Thr	22.7	22.3	21.8
Glu	17.6	17.4	17.3
Pro	9.2	9.3	9.2
Gly	22.5	23.0	21.8
Ala	22.0	21.3	20.7
Cys/2	9.7	9.7	9.2
Val	21.2	21.2	21.8
Met	1.7	1.7	1.7
Ile	9.6	8.9	9.0
Leu[a]	18.0	18.0	18.0
Tyr	4.2	3.7	4.0
Phe	5.8	5.8	6.0
Lys	14.0	13.5	13.0
His	2.0	2.0	1.4
Arg	2.8	2.6	2.7

[a] For calculation of molar ratios of amino acid residues the micromoles of leucine were assumed to be 18 residues.

4. Discussion

α-Ketodiazo compounds are known to produce carbenes on loss of nitrogen by photolysis [4,9]. When such a reagent as TPDK is applied, it initially forms a complex with the enzyme and on irradiation may allow the formation of a carbene, an extremely reactive intermediate (scheme 1). Usually heterolytic reactants used for conventional labeling direct only to nucleophiles in protein molecules. However, the carbene will react with the surrounding molecules and is even capable of insertion into a C–H bond [4].

Thus the foregoing loss of the activities of chymotrypsin on photolysis in the presence of TPDK is most likely attributed to modification of the amino acid residues in the active site. Loss of some of His was evident. Although two His residues are present in the enzyme, one may well assume that the active site His-57 was involved particularly in view of the established affinity of TPCK miety with the active site [2, 3]. A fairly small K_i value (5.4×10^{-3} M) of TPDK for chymotrypsin also supports this reasoning. Reactions of carbenes with imidazole rings have not yet been described in literature, and the finding, that the insertion of the carbene from TPDK and the hetero-



Scheme 1

lysine of the halide (TPCK) both seem to lead to the reaction with the His. is of interest (scheme 1). Generally the α-ketocarbene so produced readily undergoes both the intramolecular Wolff rearrangement to a ketene and reactions with solvents [4]. The above result, that the loss of His is not stoichiometric, is probably due to these diverse and high reactivities of a carbene. Although our experiments have shown neither the structures of the products nor involvement of other amino acid residues, the photoaffinity labeling approach illustrated here is expected to offer a versatile addition of modification studies reasonably supplementing the conventional heterolytic methods

Acknowledgements

This work was supported in part by grants from the Ministry of Education and the Naito Foundation.

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