Direct Evidence for the Presence of Histidine in the Active Center of Chymotrypsin*

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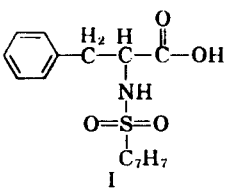
Received September 26, 1962

The synthesis of the chloromethyl ketone derived from *N*-tosyl-L-phenylalanine, *i.e.*, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK, III), is described. On incubation of this ketone with chymotrypsin, a progressive irreversible inactivation of chymotrypsin was observed. The *p*H optimum for this reaction was 7.2. In view of the stoichiometric nature of the reaction and the fact that amino acid analysis of the inhibited enzyme on acid hydrolysis revealed loss of one histidine residue, it was concluded that direct evidence had been obtained for the presence of histidine at the active center of chymotrypsin. No loss of histidine was observed after incubation of TPCK with chymotrypsin previously inactivated with diisopropylfluorophosphate. Trypsin was not affected by the inhibitor. The view is presented that the selectivity of TPCK in attaching to the active center of chymotrypsin is the result of an enzymatic process.

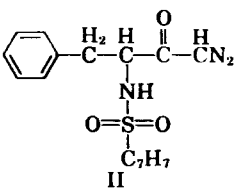
In attempts to locate the active centers of enzymes, protein group reagents or known drugs have occasionally found use if they caused an irreversible inhibition by combination with the enzyme at a single site. This has permitted the identification of an important amino acid residue involved in enzyme action by allowing degradation of the inactivated enzyme and isolation of a particular altered amino acid residue. The role of diisopropylfluorophosphate in labeling a single serine residue while inactivating chymotrypsin, trypsin, and a number of other hydrolytic enzymes (Balls and Jansen, 1952; Schaffer *et al.*, 1953; Cohen *et al.*, 1955) is a classic example of this approach which gives the most direct evidence of the participation of a given amino acid residue in the function of an enzyme. In these enzymes, the importance of amino acid residues other than serine has been appreciated from a variety of indirect evidence, but direct evidence comparable to that obtained for serine has not been forthcoming.

In the hope of obtaining additional direct information about the active centers of chymotrypsin and trypsin and of demonstrating the fruitfulness of a general approach to active center studies based on the rational design of a specific reagent for any given enzyme we have approached the problem in a new way.[1] For alkylating the active center of chymotrypsin, we combined in a single molecule *N*-tosyl-L-phenylalanine (I) to confer affinity to the active center and a halomethyl ketone grouping to achieve irreversible attachment. Initial work with the bromomethyl ketone (III, X = Br) demonstrated the possibility of labeling the active center of chymotrypsin with an agent of this type (Schoellmann and Shaw, 1962a). More recently the chloromethyl ketone has been preferred because of its convenient synthesis, which is described in this paper together with information on the specificity of the reaction and on the probable point of attachment in the enzyme structure.

The preparation of halomethyl ketones derived from *N*-tosyl-L-phenylalanine (I) was adapted from the general procedure (Bachmann and Struve, 1942); the acid chloride was treated with diazomethane to yield a diazomethyl ketone[2] (II) which, on treatment with HBr or HCl, yielded the corresponding haloketone. The use of the tosyl group was dictated by the difficulty in forming acid chlorides from acylamino acids owing to azolactonization. However, the sulfonamide group with its acidic hydrogen may undergo *N*-methylation during the formation of the diazoketone and produce an inactive product (IV) unless precautions are taken. This difficulty was evident in our earlier syntheses of the bromoketone (Schoellmann and Shaw, 1962a) and was eventually best resolved by a synthesis of the chloroketone as described.



I



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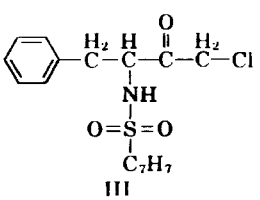
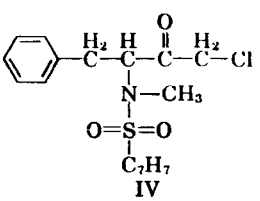


III
TPCK



IV

EXPERIMENTAL

α-Chymotrypsin (Worthington, three-times crystallized, lot 6018-19) and trypsin (Nutritional Biochemical Corporation, twice crystallized) were used in these experiments. Enzymatic activity of chymotrypsin and trypsin was determined by the procedure of Schwert and Takanaka (1955) with the use of *N*-acetyl-L-tyrosine ethyl ester and *N*-benzoyl-L-arginine ethyl ester, respectively. Standard assays for chymotrypsin were performed in 0.1 M phosphate buffer, *p*H 7.5; for trypsin, 0.01 M phosphate buffer, *p*H 7.5, was used. Freshly prepared, fully active enzyme solutions were used throughout. The enzymatic reactions were followed in a Perkin-Elmer recording spectrophotometer, and initial velocities were calculated from the slope of the curves. Fractions of these values were expressed as per cent activity.

Diisopropylphosphoryl-chymotrypsin was prepared by the method of Naughton *et al.* (1960).

* The support of the National Science Foundation is gratefully acknowledged. A preliminary report has appeared (Schoellmann and Shaw, 1962b).

[1] In other laboratories, similar, independent approaches are being made to study the active centers of enzymes (Baker *et al.*, 1961) and of antibodies (Wofsy *et al.*, 1962), among others.

[2] The diazomethyl ketone itself is of interest as a potential active center reagent for chymotrypsin and is being investigated.

Protein concentrations were determined with the Folin reagent by the method of Lowry *et al.* (1951).

Radioactivity was measured in a windowless gas-flow Geiger counter.

Synthesis of TPCK, the Chloromethyl Ketone Derived from N-Tosyl-L-phenylalanine (III).—To a stirred, chilled suspension of *N*-tosyl-L-phenylalanine acid chloride (0.95 g, 3 mmoles) prepared by the method of Popenoe and du Vigneaud (1954) in anhydrous ether (30 ml) was added a solution of diazomethane (6 mmoles standardized by titration) in anhydrous ether. The reaction was allowed to stand overnight and finally completed by refluxing for 15 minutes. From this solution the diazoketone (II) could be isolated. (Recrystallization from ether gave slightly yellow crystals, m.p. 94–96°. The infrared spectrum showed the characteristic peak at 4.68 μ for the diazoketone.) The ethereal solution of the diazoketone was treated with dry hydrogen chloride for 2 hours and the chloroketone isolated on removal of the solvent. Recrystallization from ethanol (95%) gave colorless crystals, 0.74 g, m.p. 102–103°, 75% yield.

Anal. Calcd. for $C_{17}H_{18}O_3NSCl$: C, 58.03; H, 5.16; N, 3.98; Cl, 10.08. Found: C, 57.80; H, 5.17; N, 3.76; Cl, 9.80.

For the synthesis of radioactive TPCK, uniformly labeled L-phenylalanine-C^{14} (Schwarz) was carried through the above procedure.

Synthesis of the Chloromethyl Ketone Derived from N-methyl-N-tosyl-L-phenylalanine (IV).—This compound was prepared by the same procedure as described for the *N*-tosyl-L-phenylalanine haloketone except that a 7-fold molar excess of diazomethane was used in the reaction with the acid chloride. The compound, which was obtained in a 65% yield as crystals from ethanol (95%), m.p. 104–106°, considerably depressed the melting point of the *N*-tosyl-L-phenylalanine chloroketone (III).

Anal. Calcd. for $C_{18}H_{20}O_3NSCl$: C, 59.09; H, 5.51; N, 3.83. Found: C, 58.91; H, 5.67; N, 3.27.

Reaction of TPCK with α-Chymotrypsin.—A 3×10^{-4} M solution of chymotrypsin (mw 25,000) in 0.1 M phosphate buffer, *p*H 6.0, was incubated with a 10–20 fold molar excess of the chloroketone dissolved in methanol. The final concentration of methanol was not higher than 3%. The reaction was stopped by adjusting the *p*H to 3.0. The mixture was then dialyzed against several changes of distilled water at 4° and lyophilized. Such preparations were used for amino acid analysis.

For binding studies of TPCK-C^{14} and chymotrypsin in the presence and absence of urea, 5 mg of chymotrypsin was dissolved in 25 ml of 0.05 M phosphate buffer, *p*H 7.0. To this and to a similar solution containing 8 M urea was added TPCK-C^{14} (1.6 mg, 34.3×10^4 cpm/μmole) corresponding to a 20:1 ratio of inhibitor to enzyme. After 2 hours the chymotryptic activity of the solution without urea as measured in aliquots removed for assay was shown to have completely disappeared. Both solutions were dialyzed and lyophilized. Aliquots were analyzed for protein and for radioactivity.

Amino Acid Analyses.—The amino acid analyses were performed on a Beckman-Spinco Amino Acid Analyzer according to the procedure of Spackman *et al.* (1958). For hydrolysis, 1.0 ml of concentrated HCl was added to 1.0 ml of an aqueous solution containing 3–5 mg protein and the mixture was heated at 110° for 22 hours in an evacuated, sealed tube. To calculate the molar ratio of amino acid residues the micromoles of aspartic acid plus arginine were assumed to be 24 residues (21 and 3 respectively).

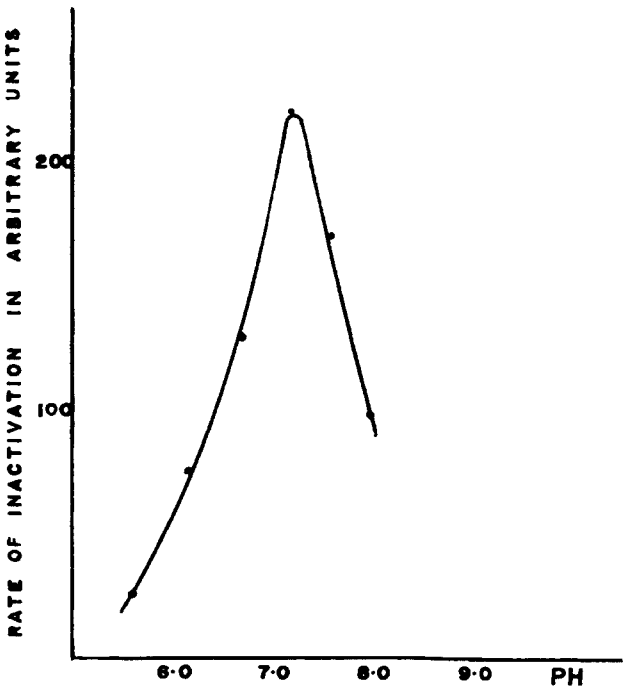


FIG. 1.—The *p*H dependence of the inactivation of chymotrypsin by the chloromethylketone derived from *N*-tosyl-L-phenylalanine (TPCK, III).

RESULTS

Inhibition of Chymotrypsin by TPCK; Stoichiometry and Effect of Urea and of pH.—The chloromethyl ketone, TPCK, rapidly inactivated chymotrypsin. When the molar ratio of inhibitor to enzyme was 10, a 50% inactivation was observed at *p*H 6 in about 50 minutes. This *p*H was generally used because it permitted long observation of chymotrypsin solutions without loss of activity. However, the reaction was considerably more rapid at *p*H 7. The rate of inactivation as a function of *p*H is shown in Figure 1.

The molar stoichiometry observed during inactivation by the bromomethylketone (Scheollmann and Shaw, 1962a) was confirmed with a more highly radioactive preparation of the chloromethylketone as shown by the data in Table I, which were obtained at *p*H 7.0. In the presence of 8 M urea, however, very little TPCK remained bound to chymotrypsin, that is, less than 3% of that observed in the absence of urea. This low binding may have resulted during dialysis by exposure of the reactivated enzyme to incompletely removed TPCK.

Action of N-Methyl-TPCK (IV) on Chymotrypsin.—When *N*-methyl TPCK (IV) was examined as a possible inhibitor of chymotrypsin, it was found that under standard conditions for 8 hours no loss of activity could

TABLE I
BINDING OF TPCK-C^{14} TO CHYMOTRYPSIN IN PRESENCE AND ABSENCE OF UREA

	Observed cpm	Chymotrypsin (μM $\times 10^{-3}$)	TPCK (μM $\times 10^{-3}$)	Binding Chymotrypsin/TPCK
Chymotrypsin + TPCK-C^{14}	3523	10.8	10.2	1:0.96
Chymotrypsin + TPCK-C^{14} in 8 M urea	119	12.7	0.34	1:0.027

be observed. Under the same conditions the enzyme was completely inactivated by TPCK.

Contrast Between the Susceptibility of Trypsin and Chymotrypsin to TPCK.—When trypsin and chymotrypsin were incubated with the chloromethylketone (III) under comparable conditions, the activity of the chymotrypsin dropped to 8% in 55 minutes, whereas the activity of the trypsin solution was not altered. At 2.5 hours, the tryptic activity was still essentially that of the control, both having dropped slightly (to 93–94% of the initial value). The chymotrypsin solution was totally inactive at this time. These experiments were carried out in 0.01 M Tris-maleate buffer, *p*H 6.0, containing 0.005 M calcium chloride for increased trypsin stability.

Amino Acid Composition of Chymotrypsin Before and After Inactivation by TPCK.—The amino acid composition of unmodified chymotrypsin was compared with that of chymotrypsin completely inactivated by the chloromethylketone. The results shown in Table II represent the average of five independently inactivated and hydrolyzed samples and the same number of chymotrypsin samples hydrolyzed without prior inactivation. The first two columns of the table include analyses taken from the literature as cited. The results show clearly that only one significant change was found on analysis of the inactivated enzyme, a change seen in every individual analysis. One histidine residue less is found in the hydrolysate of the inactivated enzyme. Since only two residues of histidine are present in chymotrypsin, this analytical change is quite distinct. No new peaks were obtained on acid hydrolysis on TPCK-inactivated chymotrypsin that could be unambiguously assigned to a new histidine derivative.

When diisopropylfluorophosphate-inactivated chymotrypsin was incubated with TPCK for 8 hours under standard conditions followed by isolation and acid hydrolysis, no loss of histidine was found (Table II).

TABLE II

AMINO ACID ANALYSIS OF CHYMOTRYPSIN BEFORE AND AFTER REACTION WITH TPCK

	No. of Amino Acid Residues per Molecule				
	Chymotrypsin			Modified Chymotrypsin	
	Wilcox *et al.*[a] (1957)	Keil *et al.*[a] (1962)	Our Results	TPCK	CT-DIP[b] + TPCK
Aspartic acid	20.8	21.3	21.0	21.0	21.0
Threonine	22.0	21.0	22.3	21.7	22.3
Serine	29.1	28.1	27.9	28.4	28.5
Glutamic acid	14.2	14.9	15.2	14.9	14.9
Proline	8.7	9.1	9.1	9.4	9.1
Glycine	23.3	22.2	22.3	22.1	22.6
Alanine	21.7	22.0	21.4	21.3	21.5
Valine	22.4	22.0	20.5[c]	20.9[c]	21.2[c]
Methionine	1.9	1.8	2.0	1.9	1.9
Isoleucine	9.9	9.8	9.7	9.9	9.9
Leucine	18.8	19.2	18.6	18.7	18.5
Tyrosine	4.1	4.2	4.2	4.3	4.2
Phenylalanine	6.5	6.2	6.0	6.1	6.1
Lysine	13.2	13.5	13.4	13.4	13.3
Histidine	2.0	1.9	2.0	1.1	2.0
Arginine	3.0	2.9	3.0	3.0	3.0
Half-cystine	10.0	9.8	10.1	10.2	9.9

[a] Values obtained with chymotrypsinogen and corrected for 4 amino acids released during activation to chymotrypsin (asp.arg, ser, thr). [b] Diisopropylfluorophosphate-inactivated chymotrypsin. [c] Uncorrected. For calculation of molar ratios of amino acid residues the micromoles of aspartic acid + arginine were assumed to be 21 + 3 = 24 residues.

DISCUSSION

The evidence implicating histidine in the active center of chymotrypsin has been summarized by Koshland (1959). Particularly pertinent to the present work are the results obtained earlier with fluorodinitrobenzene. Whitaker and Jandorf (1956) found that during reaction with this reagent under conditions most favoring specificity, *i.e.*, at *p*H 10.7, the loss of activity of chymotrypsin paralleled the disappearance of histidine. However, it was acknowledged that almost an equivalent amount of labeling was taking place at unknown sites. Consequently, the loss of activity could not be attributed unequivocally to substitution at a histidine residue. At *p*H values nearer neutrality (*i.e.*, in bicarbonate solution) fluorodinitrobenzene will react with one of the two histidines not only in chymotrypsin but also in chymotrypsinogen and in diisopropylphosphoryl-chymotrypsin (Massey and Hartley, 1956). Under these conditions the reaction with chymotrypsin diminished substrate binding but not maximum velocity.

Our results with TPCK (III) contrast with the foregoing results in several respects. A single mole of this reagent becomes bound to the chymotrypsin molecule with complete loss of enzymic activity, and analysis of acid-hydrolyzed product indicates loss of one histidine residue. These findings strongly suggest that the inhibitor alkylates a histidine residue in chymotrypsin. Indeed, any other interpretation would be difficult to explain. However, since such a histidine derivative has not yet been found, such a conclusion must be confirmed by further work.

Some evidence that TPCK reacts at the active center of chymotrypsin was deduced from our earlier observation that the inhibition was delayed by a reversible, competitive inhibitor of chymotrypsin, β-phenylpropionic acid, and that no combination of the inhibitor with chymotrypsinogen occurred (Schoellmann and Shaw, 1962a). Additional observations suggest, in fact, that in the reaction of TPCK with chymotrypsin one is dealing with a reagent that reacts with an enzymatic site. Thus, TPCK reacts with histidine only in enzymatically active chymotrypsin; it cannot in this reaction be considered merely a reagent for histidine since at least one histidine is also equally accessible to chemical reaction in chymotrypsinogen and diisopropylfluorophosphate-inactivated chymotrypsin as judged by the results with dinitrofluorobenzene in aqueous ethanolic bicarbonate (Massey and Hartley, 1956), yet TPCK does not lead to a deletion of histidine from diisopropylfluorophosphate-inactivated chymotrypsin or combine in any way with chymotrypsinogen or urea-disoriented chymotrypsin. The *p*H dependence of the inactivation of chymotrypsin by TPCK (Fig. 1) also indicates that the reaction is enzymatically promoted, since the curve is similar to that for the *p*H dependence of enzymatic activity.[3] If the alkylation by TPCK were the result of a displacement of chloride by an uncharged imidazole ring, as would be expected in the case of a simple imidazole reagent, the sharp drop in alkylation of the enzyme observed above *p*H 7.2 would not be expected.

The failure of the *N*-methyl derivative of TPCK (IV) to inactivate chymotrypsin is consistent with this

[3] A possible contribution to the dropping off of activity with rising *p*H may be increasing ionization of the sulfonamide group in TPCK (pK = 6.9, measured in 35% aqueous dimethylformamide).

interpretation of an essential substrate-like behavior of TPCK in the inactivation process, since methylation of the α-amino nitrogen in typical chymotrypsin substrates is known to diminish greatly the ability of such modified derivatives to serve as substrates (Hein and Niemann, 1961). In the chloromethyl ketones, it is unlikely that such a change alters the chemical reactivity of the halogens greatly.

A common criticism of active center studies based on the loss of enzymatic activity due to chemical modification is that the reagent possibly deforms the secondary and tertiary structure of the enzyme in such a way as to disrupt the interaction of groups essential for activity but that it may do so by reaction at a point in the structure not necessarily near the essential groups themselves. From the foregoing discussion it follows that such a criticism cannot be leveled at the conclusion that TPCK reacts at the active center of chymotrypsin, since a functioning enzyme is absolutely required for the reaction to take place. TPCK is thus in a class with organophosphorus compounds such as diisopropylfluorophosphate which have reactivity for an enzymatically active site (Dixon and Webb, 1958) and appears to be even more specific a reagent.

Our initial goal in combining in a single molecule a phenylalanine residue and a chloromethylketone function was to increase the probability of an alkylation at the active center of chymotrypsin. It was realized that a direct displacement of the halogen atom from the carbon to which it was attached by some group on the enzyme was a reaction that ostensibly required attack one bond distance away from the usual site for chymotrypsin; this did not appear prohibitive. In view of the striking results obtained with TPCK, however, an alternative mechanism seems more likely. A transition state for the nucleophilic displacement of halogen from α-haloketones has been proposed in which the carbonyl carbon participates (Bartlett, 1953). If this situation obtains in the interaction of TPCK and chymotrypsin, we may consider that the geometry corresponds closely to that required by the specificity of the enzyme and that the first step of the inactivation process may be the same as that of the normal enzymatic process. Tentatively, this view and our observation of a loss of histidine would favor that mechanism in which the initial nucleophilic agent is the imidazole ring of histidine rather than the ionized serine hydroxyl group (Westheimer, 1959). However, precise information about the nature of the binding of the TPCK residue in inactivated chymotrypsin is clearly desirable, together with a unified picture which includes a role for serine in the inactivation process, before any final conclusion can be reached.

Structural studies of the alkylated enzyme are in progress.

Acknowledgment

Messrs. J. Edwards, G. Moore, and P. Petra provided able technical assistance in these experiments.

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