Evidence for an Active-Center Histidine in Trypsin through Use of a Specific Reagent, 1-Chloro-3-tosylamido-7-amino-2-heptanone, the Chloromethyl Ketone Derived from N^α-Tosyl-L-lysine*

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ABSTRACT: The chloromethyl ketone derived from N^α-tosyl-L-lysine, TLCK (1-chloro-3-tosylamido-2-heptanone), has been synthesized as an active-center labeling reagent for trypsin since it combines specificity features and a reactive grouping. It inactivated trypsin with a second-order rate constant of 5.6 l. mole^{-1} sec^{-1}, at pH 6, but did not do so in the presence of a competitive inhibitor of trypsin, benzamidine, or in 8 M urea.

These observations indicate that TLCK reacted at the active center of trypsin. With radioactive inhibitor the reaction was shown to be equimolar, and analysis revealed loss of one histidine residue. Chymotrypsin was unaffected.

T he identification of the amino acid residues comprising the active centers of proteolytic enzymes, in order to arrive at an understanding of enzyme specificity and mechanism of action, has been a subject of intensive study in recent years. The essential role of serine was made clear from investigations of organophosphorus compounds which, in the case of chymotrypsin and trypsin, began with the observation of a stoichiometric inactivation with formation of a diisopropylphosphoryl-enzyme (Jansen et al., 1949a,b). This chemical modification permitted the identification of the functional serine residue in a characteristic peptide sequence (Schaffer et al., 1957; Oosterbaan et al., 1955; Dixon et al., 1956) and, ultimately, in the primary structure of chymotrypsin and trypsin.

These important observations emerged from an analysis of the action of a broadly reactive class of enzyme inhibitors. However, the possibility of designing specific inhibitors to permit chemical modification of other amino acid residues in the active centers has been relatively neglected until recently. TPCK[1] which stoichiometrically inactivates chymotrypsin by alkylation of a histidine residue is one of the few studied (Schoellmann and Shaw, 1963). This reagent is a chloroketone derivative of tosyl-L-phenylalanine which has the virtue of being similar to a substrate in structure and containing a reactive functional group. Analysis of the alkylated derivative has permitted identification of this essential histidine in the primary sequence of the enzyme (Ong et al., 1964, 1965; Meloun and Pospisilova, 1964; Smillie and Hartley, 1964).

The failure of TPCK to act on trypsin was in accord with the specificity differences between chymotrypsin and trypsin. It was of interest, therefore, to determine whether an analogous approach taking into account the specificity of trypsin would have similar success both as a demonstration of the general applicability of the approach and to provide information on the functional groups of trypsin. An additional stimulus came from the knowledge that structural studies on the primary sequences of chymotrypsin (Hartley, 1964; Keil et al., 1963) and trypsin (Walsh et al., 1964) were nearing completion and would facilitate studies on the chemical modification of these enzymes.

In the case of trypsin a substrate-derived chloromethyl ketone should be either an arginine or lysine compound; synthesis of the lysine derivative seemed more promising and work centered on it. When N^α-tosyl-N^ϵ-carbobenzyloxy-L-lysine was carried through the usual chloromethyl ketone synthesis, the penultimate product, N^ϵ-carbobenzyloxy-TLCK (II), was readily obtained, but removal of the carbobenzyloxy group caused difficulty. As anticipated, the usual palladium-catalyzed hydrogenolysis was not applicable because of simultaneous dehalogenation (Baltzly and Phillips, 1946) of the chloromethyl ketone to the methyl ketone (IV). A hydrolytic procedure was developed which consistently provided crystalline TLCK (III) in a 14% yield from compound II and made possible an investi-

[1] Abbreviations used in this work: TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; TPCK, tosylphenylalanine chloromethyl ketone; DL-BAPA, benzoylarginine p-nitroanilide.

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gation of its properties as described in part in a preliminary way (Mares-Guia and Shaw, 1963).

Experimental[2]

Trypsin (two-times-crystallized, salt free) and trypsinogen (once-crystallized) were purchased from Worthington Biochemical Corp. Samples prepared for amino acid analysis were purified on carboxymethylcellulose columns (Maroux *et al.*, 1962).

Enzymic activity of trypsin was determined with DL-benzoylarginine *p*-nitroanilide (DL-BAPA) hydrochloride as substrate (Erlanger *et al.*, 1961). The enzymic reactions were followed in a Beckman DB recording spectrophotometer. Protein concentrations were determined from ultraviolet absorption at 280 mμ, where $A_{1cm}^{1\%} = 12.9$. Radioactivity was measured in a Nuclear-Chicago Model 722 liquid scintillation spectrometer. Amino acid analyses were performed on a Beckman-Spinco amino acid analyzer (Spackman *et al.*, 1958). For hydrolysis, 1.0 ml of concentrated hydrochloric acid was added to 1.0 ml of an aqueous solution containing 3–5 mg of protein and the mixture was heated at 110° for 22 hr in an evacuated, sealed tube. To calculate the molar ratio of amino acid residues the micromoles of glycine were assumed to be 25 residues.

Conversion of N^α-*Tosyl-*N^ϵ-*carbobenzyloxy-L-lysine to the Diazomethyl Ketone (I).* Powdered N^α-tosyl-N^ϵ-carbobenzyloxy-L-lysine (Swallow *et al.*, 1958) (6.54 g) was suspended in dry benzene (100 ml) and stirred at room temperature with phosphorus pentachloride (2.6 g) for 1.5 hr protected from moisture. The mixture was quickly filtered and the solvent removed in a rotary evaporator at 40°. The residue was redissolved in anhydrous ether (100 ml) and treated in an ice bath with dry ethereal diazomethane (2 equiv) which was added with stirring. The product crystallized out and, after standing overnight at 4°, the suspension was filtered, yielding 4.2 g, 72%, mp 134–135°. The melting point did not change on recrystallization from ethyl acetate and hexane.

Anal. Calcd for $C_{22}H_{26}N_4O_5S$: C, 57.62; H, 5.72; N, 12.22; S, 6.99. Found: C, 57.47; H, 5.93; N, 11.09; S, 7.14.

The nitrogen analyses by the Dumas procedure were inconsistent and generally low, perhaps a property of the diazo ketone group.

1-Chloro-3-tosylamido-7-carbobenzyloxyamido-2-heptanone (N^ϵ-*Carbobenzyloxy-TLCK, II*). The diazomethyl ketone (3.6 g) was dissolved in ethyl acetate (50 ml) and anhydrous ether (200 ml) and treated with dry hydrogen chloride at room temperature for 2 hr. The solvent was removed and the residue was crystallized from ethyl acetate and hexane, yielding 3.27 g, mp 156–157°, 89%. A sample recrystallized for analysis from the same solvents gave mp 158–160°.

Anal. Calcd for $C_{22}H_{27}N_2O_5ClS$: C, 56.51; H, 5.83;

N, 6.00; Cl, 7.59. Found: C, 56.73; H, 5.88; N, 6.31; Cl, 7.55.

The Chloromethyl Ketone Derived from N^α-*Tosyl-L-lysine (TLCK, III).* N^ϵ-Carbobenzyloxy-TLCK (1.5 g) was dissolved in ethanol (100 ml) by warming; concentrated hydrochloric acid was added (40 ml), and the solution was kept at 75° for 2 hr. Water (50 ml) was added, and the mixture was reduced to a small volume in a rotary evaporator at 45°. The residual syrup containing some solid was taken up in water (50 ml) and filtered free of starting material. Usually two identical runs were combined at this point, and the recovered starting material from them was 1.15 g. The aqueous filtrate was taken to a syrup again under reduced pressure. The residue was desiccated *in vacuo* with alkali to absorb final traces of hydrogen chloride and taken up in about 5 ml of water. Any insoluble material was removed by centrifugation, and the supernatant solution was again concentrated under reduced pressure with additions of absolute ethanol to ensure dehydration. The hydrochloride crystallized slowly from a concentrated solution in absolute ethanol; it was thinned with an ethanol–anhydrous ether mixture for collection and washing. After recrystallization in the same way there was obtained 0.32 g, mp 150–153° dec, a conversion of 14% or a yield of 22% based on unrecovered starting material. (Some preparations reached mp 156–158°.)

Anal. Calcd for $C_{14}H_{22}N_2O_3Cl_2S$: C, 45.53; H, 6.00; S, 8.68. Found: C, 44.94; H, 6.06; S, 8.25.

Attempted removal of the carbobenzyloxy group with hydrogen bromide in acetic acid caused extensive formation of *p*-toluenesulfonamide. The use of different blocking groups such as *t*-butoxycarbonyl gave some success but has not yet led to a reliable procedure.

The duration of the hydrolytic treatment was somewhat arbitrarily chosen. The use of a colorimetric ninhydrin procedure to follow the liberation of the ϵ-amino group gave variable low results as has been found by others in lysine derivatives (Park and Kim, 1964).

Methyl Ketone Derived from Tosyl-L-lysine (IV). N^ϵ-Carbobenzyloxy-TLCK (1.0 g) in acetic acid (65 ml) and water (5 ml) was stirred with 5% palladized charcoal (200 mg) in a stream of hydrogen for 1.5 hr. The solvent was removed from the filtrate in a rotary evaporator at 40° with added water to facilitate the distillation of the acid. The residue crystallized and was thinned with absolute alcohol and ether for filtration, yielding 0.57 g, mp 133–135°. A sample recrystallized from absolute alcohol gave mp 135–137°.

Anal. Calcd for $C_{14}H_{23}N_2O_3ClS$: C, 50.22; H, 6.93; N, 8.37; S, 9.56. Found: C, 50.56; H, 7.16; N, 8.04; S, 9.42.

TLCK-^3H*.* TLCK was labeled with tritium by the Wilzbach technique by New England Nuclear Corp. by treating cold material with 3 curies of tritium gas at a pressure of 250 mm for 4 days at 0°. The product was exchanged with water and recrystallized to constant specific activity from absolute ethanol and ether.

N^α-*Tosyl-L-lysylmethyltrypsin (TLCK-Inhibited Trypsin).* Trypsin (500 mg, 20.8 μmoles) was added in small

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[2] Melting points were taken on a Fisher-Johns block. Microanalyses were performed by Alfred Bernhardt, Mulheim, Germany.
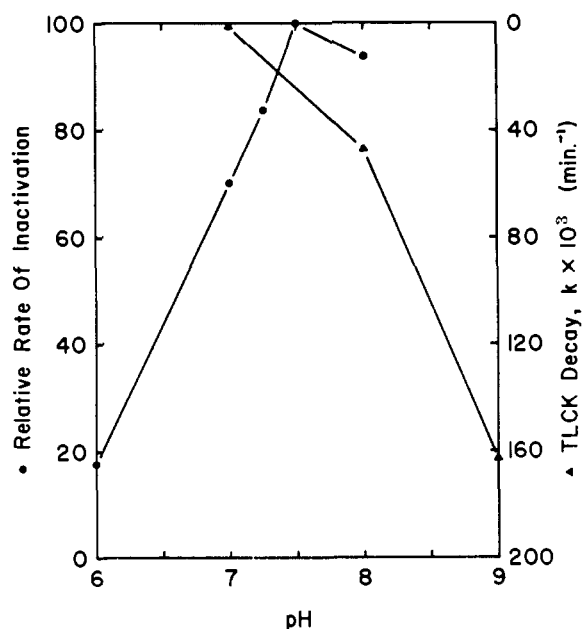
FIGURE 1: Effect of pH on the reactivity of TLCK with trypsin and on TLCK stability. The values for the rate of inactivation are not corrected for decomposition of TLCK.

FIGURE 2: Protection of trypsin against inactivation by TLCK with benzamidine as a reversible inhibitor. Trypsin, 4.56×10^{-5} M; TLCK, 6.17×10^{-4} M. Curve 1: benzamidine, 7.83×10^{-4} M; curve 2: benzamidine, 1.88×10^{-4} M; curve 3: benzamidine, 7.83×10^{-5} M; curve 4, no benzamidine. All solutions are in 0.02 M Veronal buffer, final pH 8.0.

aliquots during 1 hr to a stirred solution of TLCK (20.8 mg, 56.2 μM) freshly dissolved in 100 ml of 0.05 M Tris–maleate–calcium chloride buffer, pH 7.0, at 25°. Stirring was continued for an additional 3 hr; assay of enzymic activity showed the reaction to be completed. The solution was adjusted to pH 4, dialyzed at 4° against several changes of distilled water, readjusted to pH 4.0, and lyophilized. Purification was achieved by the procedure used for trypsin. Trypsinogen was exposed to tritiated TLCK under these conditions to determine the extent of binding.

Results

Inhibition of Trypsin by TLCK. The chloromethyl ketone derived from N^{α}-tosyl-L-lysine, TLCK, caused a rapid inactivation of trypsin as hoped. The rate of inhibition of trypsin by TLCK at 25° was determined by assay of aliquots of an incubating solution which was prepared by mixing 1 volume of 13×10^{-6} M trypsin dissolved in 0.2 M maleate buffer, pH 6.0, with 1 volume of 32.49×10^{-6} M TLCK dissolved in water. About 25% inhibition occurred after 1 hr. The second-order rate constant equals 5.6 M^{-1} sec^{-1}. This can be compared favorably with the rate of reaction of trypsin with DFP at pH 7.2 (Fahrney and Gold, 1963), 5.0 M^{-1} sec^{-1}, with the rate of acylation at pH 7.0 by diphenylcarbamyl chloride (Erlanger and Cohen, 1963), 8.2 M^{-1} sec^{-1}, and with the rate of alkylation of chymotrypsin by TPCK (Schoellmann and Shaw, 1962) at pH 6.0, 0.79 M^{-1} sec^{-1}. The relative observed rate of inactivation as a function of pH was determined in a similar manner, dissolving

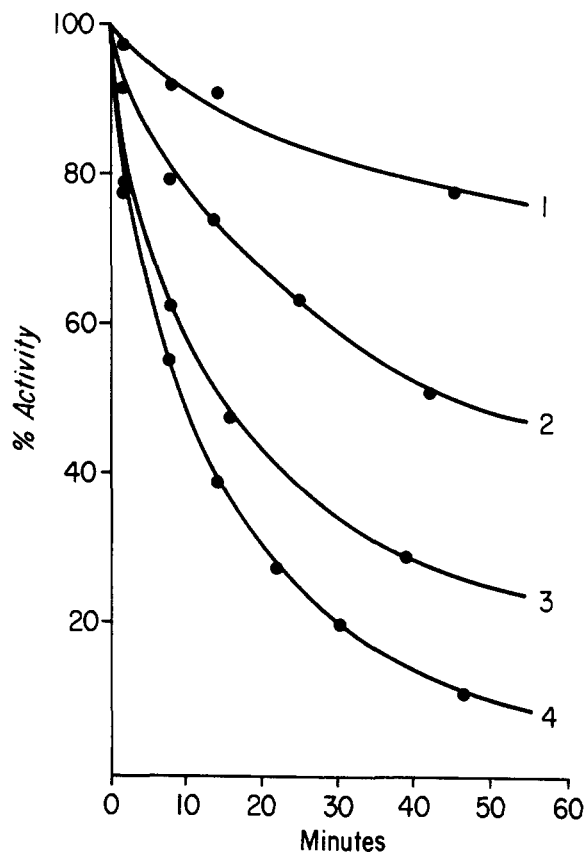trypsin in 0.2 M Tris–maleate buffers. The values shown in Figure 1 were estimated from the initial 25% inhibition. Trypsin solutions were always tested concurrently as controls and maintained full activity. The rate of the reaction increased rapidly as the pH was raised from pH 6 to 7.5, which was the maximum, and then dropped. Observations under alkaline conditions were complicated by the instability of the reagent, which prevented more extended measurements. The data plotted in Figure 1 are the experimentally observed values not corrected for the instability of TLCK.

Complete inactivation of trypsin (*i.e.*, more than 99.5% inhibition) was readily achieved as judged by kinetic (DL-BAPA) or "all or none" assay (Ray and Koshland, 1963) using p-nitrophenyl acetate.

Stability of TLCK as a Function of pH. The stability of TLCK at 25° as a function of pH was studied by observing the decreased ability to inhibit trypsin at pH 6.0 after incubation over a range of pH values. Aliquots of 32.49×10^{-6} M TLCK solutions dissolved in 0.02 M Tris–maleate buffers were mixed with equal volumes of 13×10^{-6} M solutions of trypsin dissolved

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in 0.2 M maleate, pH 6.0, after suitable time intervals, and the rates of inhibition were measured. TLCK is stable at pH 6.0; thus the concentration of TLCK present in any sample can be calculated from the rate of inhibition and the second-order rate constant of this reaction, which has been established. The first-order rate constants for decomposition are plotted in Figure 1.

The instability in alkaline media will markedly effect the apparent rate of inhibition of trypsin and even the extent of inhibition if a large excess of TLCK is not employed when the reaction is carried out under such conditions. For example, 48% of TLCK will decompose in 5 min at pH 9.0. On the other hand, considering the conditions of its synthesis, TLCK must be judged relatively stable to acid.

Evidence for Covalent Binding of TLCK at the Active Center of Trypsin. To determine whether or not the action of TLCK was at the active center of trypsin, the protective effect of a reversible inhibitor was investigated. Benzamidine, which has been shown to be an effective competitive inhibitor of trypsin (Mares-Guia and Shaw, 1965), did retard considerably the inactivation process even at very low concentrations. The data plotted in Figure 2 indicate that, under conditions in which TLCK produced 90% inactivation, the presence of 7.83×10^{-4} M benzamidine limited inactivation to 20%. (This was not due to a chemical interaction between the amidine and the chloro ketone since the protective effect was seen at once and a condensation reaction is generally very slow (Woolley *et al.*, 1962).)

When tritiated TLCK was used and the inhibited enzyme measured for nondialyzable radioactivity, it was shown that a combination had taken place in essentially molar proportions (Table I). The slightly low

TABLE I: TLCK Binding Studies.

	dpm/μM	Moles TLCK Bound/ Mole Protein
Trypsin binding study		
TLCK preparation	12.6×10^6	
TLCK bound to trypsin	9.62×10^6	0.8
Trypsinogen binding study		
TLCK preparation	9.78×10^5	
TLCK bound to trypsinogen	8.28×10^4	0.085

value may in part be due to lability of the tosylamido group of the inhibitor residue even after binding to trypsin.[3] A small amount of binding, which could be attributed to the trypsin content of the sample used

[3] P. Petra, E. Shaw, and W. Cohen, unpublished observations.

(specified as below 10%), was found in the case of trypsinogen (Table I).

Interference of Reaction of TLCK with Trypsin by 8 M Urea. Trypsin dissolved in 0.05 M Tris–maleate, pH 7.0 buffer, 8 M in urea, was mixed with TLCK dissolved in the same buffer. The concentrations were 1.51×10^{-5} M and 4.54×10^{-5} M, respectively. Aliquots were removed and diluted fivefold with acid to lower the pH to 5.0 at several time intervals from 1 to 150 min. The assays (final urea concentration 0.53 M), employing DL-BAPA as substrate, indicated activity equal to a control, with no TLCK, run in parallel for the entire experiment. Under these conditions but in the absence of 8 M urea, 50% inhibition occurred in less than 3 min.

Histidine as the Site of Alkylation of Trypsin by TLCK. When the amino acid composition of trypsin was compared with TLCK-inhibited trypsin, a drop in the histidine content from three to two residues was noted. The actual data indicated 2.9 residues before inhibition and 2.0 residues afterward (average of three determinations). The same change was observed on material not purified by column chromatography. When only a small number of residues of an amino acid is present in a protein as in the case of histidine in trypsin, the possibility of error in assessing the number of residues is minimal. The presence of three histidines in trypsin corresponds to the observations of others (Viswanatha *et al.*, 1960; Zmrhal, 1963; Walsh *et al.*, 1964).

Since only 1 mole of TLCK reacted with 1 mole of trypsin (Table I), the change in histidine content fully accounts for the chemical modification accompanying inactivation.

Failure of TLCK to Inhibit Chymotrypsin. Chymotrypsin (1 ml) dissolved in 0.2 M Tris–maleate buffer, pH 6.78, at a concentration of 6.66×10^{-5} M was incubated with 1 ml of 2.55×10^{-3} M TLCK in water for 90 min at 25°. Chymotrypsin activity was measured using p-nitrophenyl acetate as substrate. For this purpose 1 ml of the reaction mixture was mixed with 2 ml of the above buffer and 0.2 ml of p-nitrophenyl acetate (2.85×10^{-2} M in methanol), and the rate of production of p-nitrophenol was followed by the increase in optical density at 400 mμ. Chymotrypsin was not inhibited at all, whereas under the same conditions trypsin was inhibited 50% in about 30 sec.
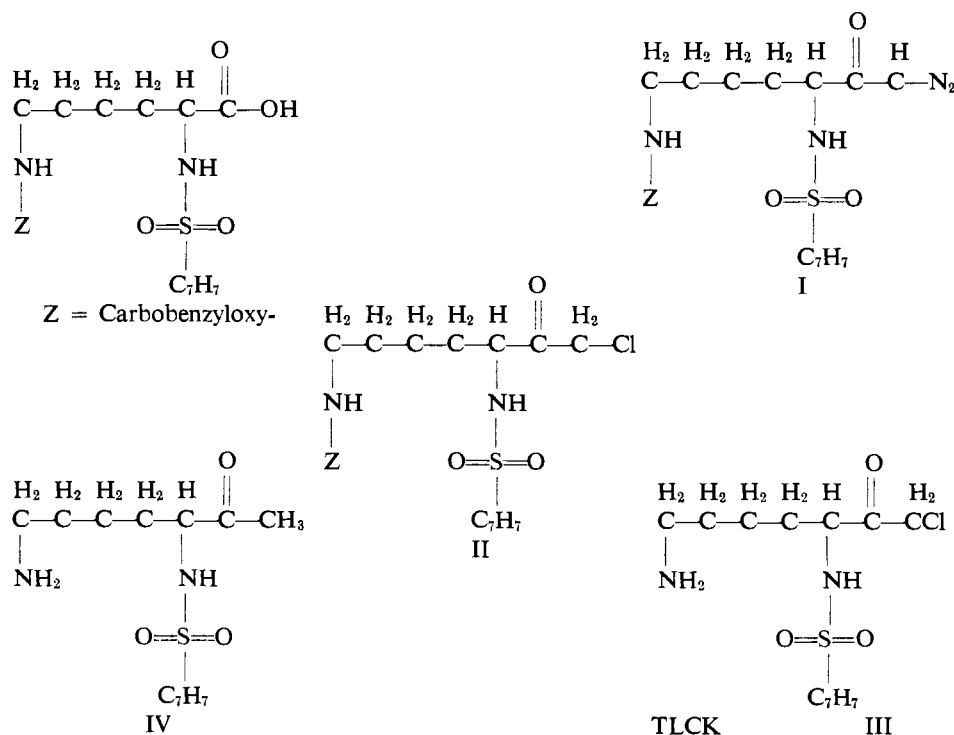
Discussion

The results obtained with TLCK demonstrate the effectiveness of using substratelike features in designing specific enzyme inhibitors. Although TLCK and TPCK, as described earlier (Schoellmann and Shaw, 1963), are both chloromethyl ketones, each will inactivate only that enzyme whose specificity requirements have been satisfied in the inhibitor structure. Thus a specific inactivator has now been produced for either chymotrypsin or for trypsin. This is in contrast to diisopropyl fluorophosphate which inactivates both enzymes (and others of unrelated specificity) by phosphorylation

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                    O
H₂ H₂ H₂ H₂ H    ‖
C—C—C—C—C—C—OH
|              |
NH            NH
|              |
Z            O=S=O
              |
             C₇H₇
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Z = Carbobenzyloxy-

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                       O
H₂ H₂ H₂ H₂ H    ‖    H
C—C—C—C—C—C—C—N₂
|              |
NH            NH
|              |
Z            O=S=O
              |
             C₇H₇
              I
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H₂ H₂ H₂ H₂ H    O   H₂
C—C—C—C—C—C—C—Cl
|              |
NH            NH
|              |
Z            O=S=O
              |
             C₇H₇
              II
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                    O
H₂ H₂ H₂ H₂ H    ‖
C—C—C—C—C—C—CH₃
|              |
NH₂           NH
              |
             O=S=O
              |
             C₇H₇
              IV
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H₂ H₂ H₂ H₂ H    O    H₂
C—C—C—C—C—C—CCl
|              |
NH₂           NH
              |
             O=S=O
              |
             C₇H₇
  TLCK        III
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of a serine residue of their active centers (Jansen et al., 1949b) and indicates that a new level of selective action has been reached.

As in the case of chymotrypsin, a role for histidine in catalysis by trypsin has been inferred for some time, for example, from studies of the pH dependence of the trypsin-catalyzed hydrolysis of benzoylarginine ethyl ester (Gutfreund, 1955) which indicated dependence on a group with pK' of 6.25. The pH dependence of K_m and V_m for tosylarginine methyl ester hydrolysis (Ronwin, 1959) as well as for the acylation and deacylation steps of various nonspecific substrates (Bender and Kaiser, 1962) led to a similar conclusion along with acknowledgment of the reservation that an α-amino group might fulfill the pK requirement as well as an imidazole group (Ronwin, 1959).

The present work gives direct evidence for the participation of a histidine residue in the catalytic mechanism of trypsin by providing a reagent that rapidly inactivates trypsin in a stoichiometric manner and does so only with functioning enzyme. The loss of a histidine residue found on analysis of the acid-hydrolyzed, inhibited enzyme indicates that one of the three histidines present in trypsin has become covalently bound to the inhibitor and can no longer carry out its catalytic function. The reagent not only indicates the essential role of histidine but, through its attachment, permits identification of the active-center histidine residue in the trypsin primary structure, as was made possible in the case of chymotrypsin by the chloromethyl ketone derived from L-phenylalanine (Ong et al., 1964).

The finding that two histidines in trypsin (as in chymotrypsin) are closely linked by a disulfide bridge has led to the supposition that both residues function in the catalytic process (Walsh et al., 1964). The present

results with TLCK do not shed any light on this question but indicate that alkylation of one of them is sufficient to cause complete loss of activity.

The altered histidine residue formed by reaction of trypsin with TLCK does not appear as a new peak under the standard conditions of amino acid analysis due to high affinity for the chromatographic resin but can be isolated under special circumstances.[3]

The fact that TPCK reacts with a histidine in chymotrypsin (Schoellmann and Shaw, 1963) and that TLCK reacts with a histidine in trypsin as shown in the present paper does not mean that they are general reagents for determining the essentiality of histidine in any enzyme. As was stressed in a discussion of the properties of TPCK (Schoellmann and Shaw, 1963), that reagent reacted only with histidine in functioning chymotrypsin and not in trypsin. The present work shows that trypsin does have a histidine at its active center, as revealed by a chloromethyl ketone with substrate features. Misleading conclusions about the nonessentiality of histidine in any given enzyme may be drawn from negative evidence, that is, the failure of enzymic activity to be influenced by TPCK or TLCK. For example, the inertness to thrombin of TPCK has led to the idea that histidine is not important in thrombin action (Seegers et al., 1965). Such a conclusion would not be warranted without actual demonstration of histidine alteration. Since thrombin is similar to trypsin in its specificity and not to chymotrypsin, it was expected that TLCK might provide an active-center reagent for thrombin. This has now been confirmed,[4] although the site of attachment in thrombin is not known. In the

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[4] E. B. Ong and E. Shaw, unpublished observations.

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widest sense, even when the substrate specificity of an enzyme is satisfied in the chloromethyl ketone structure, it should not be assumed that the reagent provides a test for the essentiality of histidine since, in the inhibitor–enzyme complex, alkylation with some other important functional group may be favored.

Chymotrypsin is said to have some inherent trypsin-like activity and, similarly, trypsin, to have a definite chymotryptic activity (Inagami and Sturtevant, 1960). From this point of view the high degree of specificity of TPCK and TLCK for their respective enzymes was not expected to reach the essentially absolute level demonstrated. However, the nonspecific hydrolyses are generally of an esterase type and depend considerably on structural features. For example, while benzoylarginine ethyl ester is split by chymotrypsin (Inagami and Sturtevant, 1960), tosylarginine ethyl ester apparently is not (Schwert et al., 1948; Hummel, 1959). It might be that substrate-derived chloromethyl ketones with side chains other than the tosyl of TPCK and TLCK would have cross reactivity toward both trypsin and chymotrypsin.

Enzymes with substrate specificity similar to trypsin are widely distributed in nature. They include thrombin, plasmin, and kallikrein, as well as papain and ficin. Each might be expected to be inhibited by TLCK.[5]

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[5] Papain has been found to be rapidly inhibited by TLCK, but, interestingly, also by TPCK (W. Cohen, unpublished observations).