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ACTIVE SITE OF TRYPSIN-LIKE ENZYME FROM *STREPTOMYCES ERYTHREUS*

SPECIFIC INACTIVATION BY NEW CHLOROMETHYL KETONES DERIVED FROM N^{α}-DINITROPHENYL-L-LYSINE AND N^{α}-TOSYL-L-ARGININE

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SUMMARY

In order to elucidate the active site of the trypsin-like enzyme from *Streptomyces erythreus* by the use of site-specific reagents without isotope labeling, L-1-chloro-3-(2,4-dinitroanilino)-7-aminoheptan-2-one (DLCK), *i.e.* N^{α}-2,4-dinitrophenyllysine chloromethyl ketone has been examined in comparison with L-1-chloro-3-tosylamido-7-aminoheptan-2-one (TLCK) as an active site-directed irreversible inhibitor. Although the apparent rate of inactivation of the enzyme by DLCK was about half of that by TLCK, it was shown that complete inactivation by DLCK involves a stoichiometric reaction which can be determined by spectral analysis of the 2,4-dinitrophenyl group, and amino acid analysis of the inhibited enzyme reveals loss of one histidine residue.

It was also shown that, like TLCK, DLCK inactivates bovine trypsin irreversibly, though with a rate of inactivation about five times less than that by TLCK.

In addition, a detailed study of the inactivation reaction of the chloromethyl ketone from N^{α}-tosyl-L-arginine, L-1-chloro-3-tosylamido-6-guanidinohexan-2-one (TACK) with trypsin and the trypsin-like enzyme from *S. erythreus* showed that there is little difference between the reactivities of TACK and TLCK to the trypsin-like enzyme, though TACK inactivates trypsin more rapidly than TLCK does.

INTRODUCTION

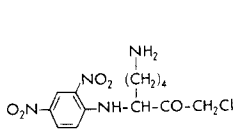
Three proteases possessing substrate specificity similar to that of bovine

Abbreviations: DLCK, L-1-chloro-3-(2,4-dinitroanilino)-7-aminoheptan-2-one; TLCK, L-1-chloro-3-tosylamido-7-aminoheptan-2-one; TACK, L-1-chloro-3-tosylamido-6-guanidinohexan-2-one; DLME, N^{α}-2,4-dinitrophenyl-L-lysine methyl ester; TLME, N^{α}-tosyl-L-lysine methyl ester; TAME, N^{α}-tosyl-L-arginine methyl ester; *p*-NO_2-ZACK, N^{α}-(*p*-nitrobenzyloxycarboxyl)-arginylchloromethane.

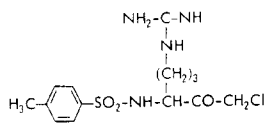
pancreatic trypsin have recently been isolated from *Streptomyces fradiae*[1], *Streptomyces griseus*[2] and *Streptomyces erythreus*[3]. Chemical structure in the region near the active site has been confirmed for the *S. griseus* enzyme, the amino acid sequence in this region showing remarkable similarity to that in bovine trypsin[4]. In order to obtain an insight of the enzymatic reaction of trypsin-like enzymes, it is worth-while determining whether or not there is any further homology between the bacterial proteases from *Streptomyces* and mammalian trypsins.

It has been shown that a chloromethyl ketone derivative of N^{α}-tosyl-L-lysine, L-1-chloro-3-tosylamido-7-aminoheptan-2-one (TLCK) inhibits trypsin by a reaction involving alkylation of N-3 of His-57 (chymotrypsinogen numbering scheme)[5]. In the present work we have studied the specific inhibition of the *S. erythreus* trypsin-like enzyme and of bovine trypsin by L-1-chloro-3-(2,4-dinitroanilino)-7-aminoheptan-2-one (DLCK) as an active site-directed irreversible inhibitor. Whereas investigation of an enzyme active site with TLCK requires the use of an isotope tracer technique, this is not necessary when DLCK is used. This is because, unlike the chloromethyl ketone derivatives employed so far, DLCK is colored as a result of containing the yellow 2,4-dinitrophenyl chromophore. Thus it should be possible to simply follow the stoichiometry of chemical modification resulting from incorporation of DLCK by the enzyme by spectrophotometric analysis.

For the purpose of comparison with the results obtained previously with TLCK[3], we also examined in the present study the inactivation of *S. erythreus* trypsin-like enzyme and trypsin by a chloromethyl ketone derivative of N^{α}-tosyl-L-arginine, L-1-chloro-3-tosylamido-6-guanidinohexan-2-one (TACK).



(DLCK)



(TACK)

MATERIALS AND METHODS

Materials

Trypsin-like enzymes from *S. erythreus* and *S. fradiae* were prepared by the method described earlier[3]; bovine pancreatic trypsin (twice crystallized, salt free) was obtained from Worthington and used without further purification. Sephadex G-25 (coarse) was purchased from Pharmacia Fine Chemicals and TLCK from Cyclo Chemical Corp. Benzoyl-L-arginine *p*-nitroanilide and N^{α}-tosyl-L-arginine methyl ester (TAME) were purchased from the Protein Research Foundation, Osaka, Japan. N^{α}-Tosyl-L-lysine methyl ester (TLME) and N^{α}-2,4-dinitrophenyl-L-lysine methyl ester (DLME) were prepared by methylesterification of N^{α}-tosyl-N^{ε}-benzyloxycarbonyl-L-lysine and N^{α}-2,4-dinitrophenyl-N^{ε}-benzyloxycarbonyl-L-lysine, respectively, with diazomethane, followed by treatment with trifluoroacetic acid[6].

Analysis of TLME: Calculated for $C_{14}H_{22}O_4N_2S \cdot HCl$ (mol. wt 360.87): C, 47.93; H, 6.61; N, 7.98; S, 9.14; Cl, 10.10. Found: C, 47.70; H, 6.73; N, 7.96; S, 9.79; Cl, 10.14.

Analysis of DLME: Calculated for $C_{13}H_{18}O_6N_4 \cdot HCl$ (mol. wt 362.78): C, 43.04; H, 5.28; N, 15.44; Cl, 9.77. Found: C, 42.98; H, 5.32; N, 15.40; Cl, 9.93.

DLCK and TACK were synthesized from diazoketones of N^{α}-2,4-dinitrophenyl-N^{ε}-benzyloxycarbonyl-L-lysine and N^{α}-tosyl-N^{G}-nitro-L-arginine, respectively (Inouye, K., Sasaki, A. and Yoshida, N., unpublished). Both the purified chloromethyl ketones were homogeneous in thin-layer chromatography and gave the following analytical result: DLCK: Calculated for $C_{13}H_{17}N_4O_5Cl \cdot HCl \cdot 2H_2O$: N, 13.43; Cl, 17.00. Found: N, 13.43; Cl, 17.39. TACK: Calculated for $C_{14}H_{22}N_4O_3SCl \cdot HCl \cdot H_2O$: N, 13.49; Cl, 17.07. Found: N, 13.13; Cl, 17.95.

Assay of enzymatic activity

The determination of enzymatic activity in modification was carried out using benzoyl-L-arginine *p*-nitroanilide[7]. For the kinetics of various synthetic ester substrates, the initial rate was determined using a Radiometer pH-stat coupled to a Titrigraph. The titration method used followed a method given in the literature[8]. All measurements were made at pH 8.0 and 30 °C. In the experiment with the N^{α}-tosyl amino acid esters, the reaction volume of TAME was 200 ml and that of TLME 50 ml, because of their extremely low K_m values.

Inactivation of enzyme by chloromethyl ketone

Solution of trypsin and trypsin-like enzymes from *S. erythreus* in 0.05 M Tris–HCl buffer, pH 7.0, containing 0.02 M $CaCl_2$, were treated with the buffer solutions of TLCK, DLCK and TACK at 25 °C. The final enzyme concentration was $5 \cdot 10^{-6}$ M. The residual activity of the inhibited enzyme was examined at periodic intervals. With the trypsin-like enzyme, a 20-μl aliquot of the reaction mixture was removed and added to 20 μl of 0.1 M HCl to stop the inactivation reaction; with trypsin, 50 μl of 0.1 M HCl was added to 100 μl of reaction mixture. Incubation of a solution containing only enzyme ($5 \cdot 10^{-6}$ M) was run in parallel with the inactivation experiment to determine the uninhibited enzymatic activity level.

Preparation for study of the stoichiometry of binding of DLCK

The pH of a solution of the trypsin-like enzyme (20.5 mg, 1 μmole) in 2 ml of 0.05 M Tris–HCl buffer (pH 7.0, 0.02 M $CaCl_2$) was adjusted to pH 7.0 by the addition of 0.27 ml of 0.05 M Tris–HCl, pH 8.0, since the enzyme is acidic protein. Then 0.5 ml of a solution of DLCK in the pH 7.0 buffer (2.0 mg, 5.2 μmoles) was added to the stirred enzyme solution in the dark. Thus, the final concentrations of enzyme and DLCK in the reaction mixture were $3.6 \cdot 10^{-4}$ and $1.9 \cdot 10^{-3}$ M, respectively. After 5 min, a 5-μl aliquot of the reaction mixture was diluted with 2 ml of 1 mM HCl and assayed. The remaining activity of the preparation was less than 5%. After a further 15 min the reaction mixture was passed through a Sephadex G-25 column in 1 mM HCl (2 cm × 50 cm), dialyzed against 1 mM HCl and lyophilized. The molar concentration of this modified enzyme was determined by the method of Lowry *et al.*[9]. In this calculation, native protein concentration of trypsin-like enzyme from *S. erythreus* was determined from the absorbance at 280 nm using a molar extinction coefficient of $2.5 \cdot 10^4$. Since the ultraviolet spectra of trypsin and the trypsin-like enzyme show little absorption over 320 nm, the stoichiometry of incorporation was taken as $A/1.2 \cdot 10^4 \cdot M$, where A and M are the absorbance value at 355 nm in 1 mM HCl of

the enzyme inactivated by DLCK and the molar concentration of enzyme, respectively, and $1.2 \cdot 10^4$ is the molar extinction coefficient of DLCK. Amino acid analyses were performed by the method described in the previous paper[3].

RESULTS AND DISCUSSION

Kinetic study of inactivation by DLCK and TLCK

The inactivation of bovine trypsin and trypsin-like enzyme from *S. erythreus* at pH 7.0, 25 °C, by DLCK and TLCK was carried out at a number of inhibitor concentrations, and reciprocals of the apparent first-order inactivation rate constants were plotted against inhibitor concentration as described by Kitz and Wilson[10]. The progressive irreversible inhibition of trypsin-like enzyme from *S. erythreus* by DLCK is shown in Fig. 1. These results gave the limiting rate of inactivation (k_3), which is

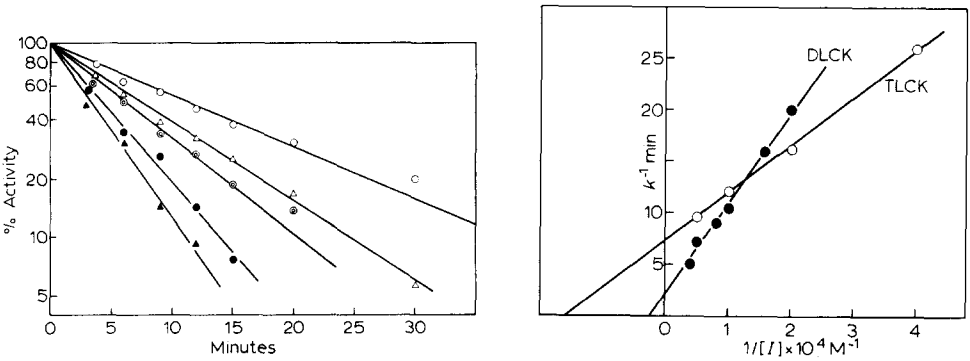


Fig. 1. The inactivation of trypsin-like enzyme from *S. erythreus* ($5 \cdot 10^{-6}$ M) by L-1-chloro-3-(2,4-dinitroanilino)-7-aminoheptan-2-one (DLCK) at pH 7.0, 25 °C: ○, $0.625 \cdot 10^{-4}$ M; △, $1.0 \cdot 10^{-4}$ M; ◎, $1.25 \cdot 10^{-4}$ M; ●, $2.0 \cdot 10^{-4}$ M; ▲, $2.5 \cdot 10^{-4}$ M DLCK.

Fig. 2. Saturation kinetics in the inactivation of trypsin-like enzyme from *S. erythreus* by L-1-chloro-3-(2,4-dinitroanilino)-7-aminoheptan-2-one (DLCK) and L-1-chloro-3-tosylamido-7-aminoheptan-2-one (TLCK) at pH 7.0, 25 °C.

the first-order rate constant for the conversion of the reversible complex to the irreversibly inhibited enzyme, and the dissociation constant for the initial reversible complex (K_i). Fig. 2 shows the reciprocal plots of inactivation of the trypsin-like enzyme by DLCK and TLCK. The kinetic data for the four inactivation systems are given in Table I*.

Both of the enzymes are more efficiently inhibited by TLCK than by DLCK. It is inferred from the saturation kinetics of the inactivation that the more potent inactivation by TLCK is attributable to its smaller K_i value, since no distinguishable difference could be found in the rates of alkylation, k_3, in these four systems. In trypsin inactivation the K_i (DLCK)/K_i (TLCK) is 5.6, and in trypsin-like enzyme inactivation 5.7. This is consistent with the kinetic data obtained from the enzyme-catalyzed hydrolysis of ester substrates having chemical structures corresponding to

* Although the previous paper[3] indicates that the inactivation rate constant (k_3) of trypsin by TLCK is one-fifteenth of that for trypsin-like enzyme from *S. erythreus*, reexamination shows the nearly equal k_3 value for the enzymes as shown in this table.

TABLE I

KINETIC DATA FOR THE IRREVERSIBLE INHIBITION OF TRYPSIN AND TRYPSIN-LIKE ENZYME FROM *S. erythreus* BY TLCK AND DLCK AT pH 7.0, 25 °C

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E \cdot I \xrightarrow{k_3} \text{inactive modified enzyme.}$$

Enzyme	*Inhibitor*	K_i (10^{-4} *M*)	k_3 (min^{-1})	k_3/K_i ($M^{-1} \cdot min^{-1}$)	*Relative** k_3/K_i
Trypsin	TLCK	2.5	0.16	0.064	32
	DLCK	14.0	0.20	0.014	7
Trypsin-like enzyme from *S. erythreus*	TLCK	0.7	0.14	0.20	100
	DLCK	4.0	0.42	0.11	55

* Relative value when k_3/K_i for the trypsin-like enzyme from *S. erythreus*–TLCK system was assumed to be 100.

the respective inhibitors. The kinetic constants for the enzyme-catalyzed hydrolyses of N^{α}-tosyl-L-lysine methylester and N^{α}-dinitrophenyl-L-lysine methyl ester were also determined and compared as shown in Table II. Although the k_{cat} values for the substrates differ slightly with both enzymes, the K_m values for TLME are smaller than those for DLME by about two orders of magnitude. It may be that steric hindrance by the more bulky dinitrophenyl group decreases the affinity towards the active sites of the enzymes.

TABLE II

KINETIC DATA FOR THE ENZYMATIC HYDROLYSIS BY TRYPSIN AND TRYPSIN-LIKE ENZYME FROM *S. erythreus* AT pH 8.0 (0.1 M KCl, 0.05 M $CaCl_2$), 30 °C

Enzyme	*Substrate*	K_m (10^{-3} *M*)	k_{cat} (s^{-1})
Trypsin	TLME	0.1	127
	DLME	4.4	23.2
Trypsin-like enzyme from *S. erythreus*	TLME	0.06	56.5
	DLME	4.2	30.7

However, the speed of inactivation by chloromethyl ketones is not necessarily a function of their K_I value, but dependent upon their molar concentration in the reaction system. When it is necessary to inactivate the trypsin-like enzyme by DLCK very rapidly, the use of inhibitor at 10^{-3} M, a concentration higher than the K_i value by one order of magnitude, gives complete inactivation within several minutes; whereas at 10^{-4} and 10^{-5} M concentration about 20 min and more than 1 h, respectively, are needed for complete inactivation of the enzyme. These results suggest that, like TLCK, DLCK acts in a substrate-like manner towards trypsin and trypsin-like enzyme from *S. erythreus*.

Inactivation by TACK

Inactivation of the enzymes by TACK was examined and compared to inactivation by TLCK. From the reciprocal plots, a k_3 of 0.22 min^{-1} and $K_i = 0.9 \cdot 10^{-4}$ M for the trypsin–TACK system (Fig. 3), and a k_3 of 0.11 min^{-1} and $K_i = 0.5 \cdot 10^{-4}$ M

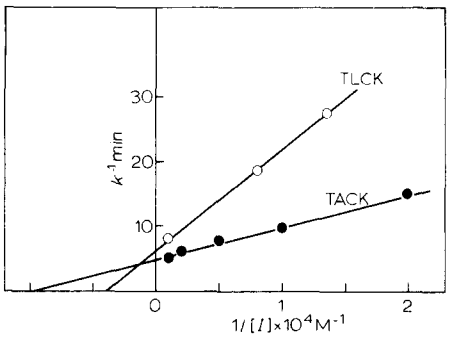


Fig. 3. Saturation kinetics in the inactivation of bovine trypsin by L-1-chloro-3-tosylamido-6-guanidinohexan-2-one (TACK) and L-1-chloro-3-tosylamido-7-aminoheptan-2-one (TLCK) at pH 7.0, 25 °C.

for the trypsin-like enzyme–TACK system were determined. Table III summarizes the comparable data from inactivation of the enzymes by TACK and TLCK With trypsin, a comparison of k_3/K_i values for the two chloromethyl ketones reveals that TACK is 3.4 times more rapid than TLCK, while with the other enzyme no difference is distinguishable between their apparent rates and their efficiencies of inhibition of the trypsin-like enzyme are the same as that of inhibition of trypsin by TACK. These results are seemingly related to the degree of affinity between the enzymes and the reagents as was discussed in the case of DLCK. The K_i values for inactivation by TACK and TLCK and K_m values for the hydrolysis of ester substrates corresponding to the inhibitors, *N*-tosyl-L-arginine methyl ester and *N*-tosyl-L-lysine methyl ester, are given in Table IV. The K_m value of TLME with trypsin is higher

TABLE III

KINETIC DATA FOR THE IRREVERSIBLE INHIBITION OF TRYPSIN AND TRYPSIN-LIKE ENZYME FROM *S. erythreus* BY TACK AND TLCK AT pH 7.0, 25 °C

Enzyme	*Inhibitor*	K_i (10^{-4} *M*)	k_3 (min^{-1})	*Relative** k_3/K_i
Trypsin	TACK	0.9	0.22	110
	TLCK	2.5	0.16	32
Trypsin-like enzyme	TACK	0.5	0.11	75
from *S. erythreus*	TLCK	0.7	0.14	100

* Relative values were determined as in Table I.

TABLE IV

K_m FOR THE ENZYMATIC HYDROLYSES AND K_i FOR IRREVERSIBLE INHIBITION OF TRYPSIN AND TRYPSIN-LIKE ENZYME FROM *S. erythreus*

Enzyme	*Substrate*	K_m (10^{-4} *M*)	*Inhibitor*	K_i (10^{-4} *M*)
Trypsin	TLME	1.0	TLCK	2.5
	TAME	0.1	TACK	0.9
Trypsin-like enzyme	TLME	0.6	TLCK	0.7
S. erythreus	TAME	0.2	TACK	0.5

than that of TAME by an order of magnitude, but a not so marked difference is observed between the K_m values of the substrates with trypsin-like enzyme from *S. erythreus*. The synthesis of N^{α}-(*p*-nitrobenzyloxycarbonyl)-arginylchloromethane (*p*-NO_2-ZACK) has been reported, though unfortunately its chemical purity is low because of self condensation during preparation[11]. However, it has been estimated by Shaw *et al.*[13] that *p*-NO_2-ZACK is at least two orders of magnitude more rapid than TLCK in the inactivation of trypsin at pH 7, the same conditions as used in our experiment with TACK. As TACK is only several times more potent than TLCK for the inactivation of trypsin (Table III), it may be that *p*-nitrobenzyloxycarbonyl or benzyloxycarbonyl group lead to remarkable enhancement on inhibition of trypsin. The effect on the potency of trypsin inactivation of a substituent on the α-nitrogen, as possessed by the chloromethyl ketones derived from arginine and lysine, would be similar to those in the inactivation of α-chymotrypsin and subtilisin[12]. It appears that TACK and TLCK alkylate a similar amino acid residue on the enzymes, *i.e.* His-57, though we lack direct evidence at present.

Stoichiometry of DLCK inhibition of trypsin-like enzyme from S. erythreus

The stoichiometry of the DLCK incorporation which causes loss of enzymatic activity of trypsin-like enzyme from *S. erythreus* was determined by two methods; spectrophotometric titration of the dinitrophenyl group incorporated, and an examination of the amino acid residues modified by the reagent. The reaction between DLCK and an amino acid side chain in the enzyme results in the introduction of a yellow chromophore with a molar extinction coefficient of $1.2 \cdot 10^4$ at 355 nm. Fig. 4 shows the spectra of DLCK and the inactive trypsin-like enzyme modified with DLCK at pH 3. In both spectra λ_{max} was observed at 355 nm, attributable to the 2,4-dinitrophenyl group. On the basis of this, we estimated the incorporated DLCK to be 0.93 mole/mole of completely-inhibited enzyme isolated chromatographically from the reaction mixture (see experiment). This method for determining the stoichiometry is more convenient than that using an isotopically-labeled compound such as [^{3}H]- or [^{14}C]TLCK.

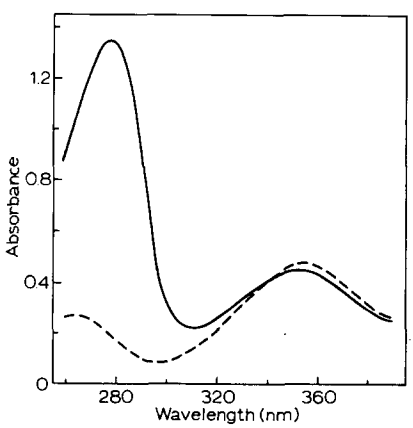


Fig. 4. Absorbance spectra of inactive trypsin-like enzyme from *S. erythreus* modified with L-1-chloro-3-(2,4-dinitroanilino)-7-aminoheptan-2-one (DLCK) (——) and DLCK (----) at pH 3.0.

The molar stoichiometry was also studied by examining the amino acid composition of unmodified native enzyme and that of the enzyme inactivated by DLCK. Amino acid analyses were identical within experimental error except for the disappearance of one histidine residue in the hydrolysate of the inactivated enzyme. However, no modified histidine derivative could be obtained on amino acid analysis of the hydrolysate of the DLCK-inactivated trypsin-like enzyme.

The data in Table V show that the number of DLCK mole bound per mole of protein is estimated by two different methods to be 0.9 and 0.8, respectively. These data support the conclusion that a specific histidine residue of the trypsin-like enzyme from *S. erythreus* is modified in a stoichiometric manner by DLCK, just as in the inactivation of trypsin by TLCK[13]. This alkylated histidine residue presumably participates in the catalytic mechanism of the trypsin-like enzyme, and it is anticipated that its position on the amino acid sequence corresponds to His-46 in bovine trypsin[5].

TABLE V

STOICHIOMETRY OF CRUCIAL ALKYLATION EVENTS DURING INACTIVATION OF TRYPSIN-LIKE ENZYME FROM *S. erythreus* BY DLCK

Spectroscopic analysis	$\varepsilon_{355\ nm}$	*Moles DLCK bound/mole protein*
DLCK	$1.20 \cdot 10^4$	
Trypsin-like enzyme from *S. erythreus* modified with DLCK	$1.12 \cdot 10^4$	0.93
Amino acid analysis	*His residues/ mole protein*	*Modified His residues/mole protein*
Trypsin-like enzyme from *S. erythreus*	2.9	
Trypsin-like enzyme from *S. erythreus* modified with DLCK	2.1	0.8

When DLCK reacted with trypsin-like enzyme from *S. fradiae*, which contains a single histidine residue[3], a complete loss of the histidine residue was found on analysis of acid hydrolysate of the inhibited enzyme. On the other hand, spectroscopic analysis of DLCK-modified bovine trypsin shows that 0.6 of the three histidine residues of the trypsin molecule have become bound to the inhibitor and resulted in total loss of enzymatic activity. This estimated number of DLCK-modified histidine residues is consistent with the result for TLCK-inhibited trypsin[5,14]. Shaw and Springhorn[5] suggested that in this case the content of unmodified trypsin is about 33%, since the bovine trypsin preparation consists of inert protein. This suggestion, together with our result, might be accounted for by assuming that commercial trypsin is approx. 60% active (refs 15 and 16).

Finally, it should be taken into consideration whether or not the reagents deform the conformation of the enzyme. We have, at present, no means of understanding this problem. Our preliminary observations of the circular dichroism spectra

of the modified- and native trypsin-like enzyme showed no remarkable difference between the CD bands, and thus gave no information which could throw light upon the secondary structure of the protein. A slight distortion of the disulfide bond is anticipated by some change of CD spectrum near the 255-nm region. This same effect had been also observed more clearly in the CD spectrum of α-chymotrypsin modified with L-1-chloro-3-tosylamido-4-phenylbutan-2-one and DFP (Yoshida, N., Twata, T. and Kuriyama, K.) unpublished). Nevertheless, it seems unlikely that such a twist of the disulfide bridge should cause inactivation of the enzymes. The relation between conformational change and inactivation as a consequence of chemical modification is an important problem to be solved. Therefore, circular dichroism studies on α-chymotrypsin and trypsin modified by various active site-directed inhibitors are now in progress, the results of which will be reported elsewhere.

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