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INTERACTION OF METHYLCHYMOTRYPSIN WITH *STREPTOMYCES* SUBTILISIN INHIBITOR

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Summary

The effect of methylation of histidine-57 of α-chymotrypsin with *Streptomyces* subtilisin inhibitor was examined. Methylchymotrypsin was isolated by affinity chromatography on inhibitor-Sepharose, and the interaction of this inactive enzyme with inhibitor was quantitatively analyzed by two different methods: the spectrophotometric titration of difference spectrum resulted in the complex formation and the application of competitive enzyme assay by using substrates of large K_m values. The former method gave values of $8.6 \cdot 10^{-6}$ M as dissociation constant (K_d) of methylchymotrypsin · inhibitor complex and 0.91 as the number of binding sites (n) per inhibitor monomer, both of which were almost equivalent to those for native enzyme · inhibitor complex. By the latter novel method, values of $7.9 \cdot 10^{-6}$ M and 1.08 were obtained for K_d and n, respectively, for interaction of inhibitor with α-chymotrypsin, and $8 \cdot 10^{-6}$ M as K_d for methylchymotrypsin · inhibitor complex. These results indicate that methylation of histidine-57 of active site in α-chymotrypsin molecule does not affect essentially the binding ability to inhibitor and the modified enzyme binds stoichiometrically to inhibitor, as the native enzyme does, with a molar ratio of 1 : 1 per inhibitor monomer.

Introduction

In 1972, a proteinase inhibitor designated *Streptomyces* subtilisin inhibitor was isolated by Murao et al. [1] from *S. albogriseolus* and shown to be a specific inhibitor for microbial alkaline proteinases such as subtilisins and *Streptomyces* proteinase. Recently, however, Inouye et al. [2] reported that the inhibitor also inhibits competitively α-chymotrypsin, though the K_d value ($3 \cdot 10^{-6}$ M) of the enzyme · inhibitor complex is larger than that ($<10^{-9}$ M) for subtilisin · inhibitor complex. This suggests that the inhibitor can strictly discriminate the structural difference in the active site of enzymes, though the

active site structure of both the enzyme is shown to be somewhat resemble each other.

Quite recently we [3] examined the interaction of the inhibitor with thiol-subtilisin, in which serine-221 of the native enzyme was converted to cysteine residue [4,5] and found that this modified enzyme could bind to the inhibitor with a K_d value of $4 \cdot 10^{-5}$ M which was 10^5-fold greater than that of the native enzyme-inhibitor interaction. This finding prompted us to investigate systematically the interaction of chemically-modified and catalytically inactive enzymes with the inhibitor. The interaction of the inhibitor with methylchymotrypsin, which is characteristic of having a small substituent, a methyl group, at the 3-N position of histidine-57 in α-chymotrypsin, was examined first, since the enzyme is catalytically inactive and the investigation of protein-protein interaction can be performed without risk of autolysis.

The present report describes the quantitative analyses of interaction of methylchymotrypsin with *Streptomyces* subtilisin inhibitor by a method applied the competitive enzyme assay procedure as well as the spectrophotometric titration method. The results obtained have indicated that the inactive enzyme can also bind stoichiometrically to the inhibitor at almost the same strength as that of native enzyme-inhibitor interaction at neutral pH region.

Materials and Methods

α-Chymotrypsin from bovine pancreas (crystallized 3 times) was purchased from Sigma Chemical Co., U.S.A. and further purified by using a *Streptomyces* subtilisin inhibitor-Sepharose column immediately prior to its use. The enzyme concentration was estimated assuming that the molar absorbance at 280 nm (ϵ_{280}) was $5 \cdot 10^4$ at pH 7. Crystalline *Streptomyces* subtilisin inhibitor was a generous gift from Professor S. Murao, University of Osaka Prefecture. The concentration of the inhibitor is expressed in terms of molar concentration of monomer throughout this paper, as a molecule of the inhibitor consists of two identical subunit monomers (mol. wt. 11 500) under the common condition [6]. The molar concentration was determined by using the absorbance $A^{1\%}_{1cm,276nm} = 8.29$ at pH 7.0 [6]. *Streptomyces* subtilisin inhibitor-Sepharose was prepared according to the method of Sato et al. [7]. Methyl *p*-nitrobenzenesulfonate was obtained from Eastman Kodak Co., U.S.A. Sepharose 4B was a product of Pharmacia Fine Chemicals Co., Sweden, and cyanogen bromide was purchased from Nakarai Chemical Co., Kyoto, Japan.

Preparation and purification of methylchymotrypsin

Methylchymotrypsin was prepared by the method described by Ryan and Feeney [8] with a slight modification and purified by affinity chromatography on a *Streptomyces* subtilisin inhibitor-Sepharose as follows: α-Chymotrypsin, 250 mg, was dissolved in 20 ml of 0.1 M Tris . HCl buffer containing 20 mM $CaCl_2$, pH 8.0. A 2-fold molar excess of methyl *p*-nitrobenzenesulfonate in 0.5 ml of methanol was added to the enzyme solution. When the reaction mixture was kept at room temperature for 30 min, the activity toward *p*-nitrophenyl acetate was reduced by approximately 60%. Phenylmethanesulfonyl fluoride, 5 mg, in 0.5 ml of acetonitrile was added to the reaction mixture to

inactivate the residual activity. When the activity toward *p*-nitrophenyl acetate was no longer detectable, reaction mixture was applied directly to a column (2.7 × 9.2 cm) of *Streptomyces* subtilisin inhibitor-Sepharose equilibrated with 30 mM Tris · HCl buffer containing 0.1 M NaCl and 10 mM $CaCl_2$, pH 7.5, and the column was washed with several times the void volume of buffer. Phenylmethanesulfonylated chymotrypsin (PMS-chymotrypsin) and other impurities were found in non-adsorbed fractions, and the methylchymotrypsin adsorbed was recovered from the column by elution with 30 mM acetate buffer, pH 3.5 (See Fig. 2). The fractions were combined, dialyzed against 1 mM HCl and then lyophilized. The yield was 103 mg. The methylchymotrypsin purified in this way was found to be almost completely inert toward casein and *p*-nitrophenyl acetate (Ac-OPhNO_2) substrates. The preparation was demonstrated to contain one less histidine residue than native chymotrypsin by amino acid analysis using a Hitachi automatic amino acid analyzer type KLA-3B and to have the ability to bind proflavin as shown in Fig. 1. The dissociation constant, K_d, for the modified enzyme · dye complex was estimated to be $2.7 \cdot 10^{-5}$ M according to the method of Brandt et al. [9]. This value is approximately equivalent to that of the native enzyme · proflavin complex [9,10]. In the calculation of K_d, a value of $1.89 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ was used for $\Delta\epsilon_{465}$ at $8.44 \cdot 10^{-6}$ M dye concentration. Tos-PheCH_2Cl-chymotrypsin was prepared by the method of Schoellman and Show [11].

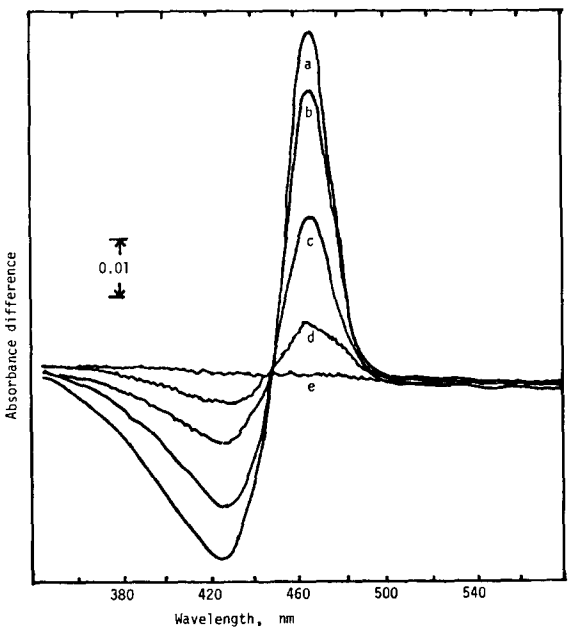

Fig. 1. Visible difference spectra observed by mixing methylchymotrypsin with proflavin. Varying concentrations of methylchymotrypsin were incubated with a constant concentration ($8.44 \cdot 10^{-6}$ M) of proflavin in 50 mM phosphate buffer, pH 7.44, with an ionic strength of 0.1 (NaCl) at 23°C. The concentrations of methylchymotrypsin were $1.94 \cdot 10^{-5}$ M (curve a), $1.49 \cdot 10^{-5}$ M (curve b), $0.78 \cdot 10^{-5}$ M (curve c) and $0.2 \cdot 10^{-5}$ M (curve d). Curve e is base line. The tandem double cuvette system was employed. Sample side: proflavin-methylchymotrypsin mixture dissolved in the buffer solution in a front cuvette and only the buffer solution in the other; Reference side: methylchymotrypsin in a front cuvette and proflavin in the other.

Ultraviolet and visible difference spectra

Ultraviolet difference spectra observed upon inhibitor · enzyme complex formation and visible difference spectra caused by the proflavin binding were measured at 23°C using a Hitachi 323 recording spectrophotometer equipped with the tandem cuvette system.

Synthesis of substrates

N-Acetyl-L-tyrosine-*m*-nitroanilide (*N*-Ac-L-Tyr · *m*-nitroanilide): *N*-Ac-L-Tyr was converted to the anilide by the method of Anderson et al. [12]. A solution containing 100 mg (0.448 mmols) of *N*-Ac-L-Tyr and 5.35 μl of *N*-methylmorphine in 10 ml of tetrahydrofuran (freshly distilled off calcium hydride) was cooled at 0°C and mixed with 58 μl of isobutyl chloroformate under vigorous stirring. 1 min later, 0.492 mmol of *m*-nitroaniline dissolved in 5 ml of tetrahydrofuran were further added and the reaction mixture was stirred for 2 h at room temperature. After the solvent was removed under vacuum, 8 ml of ethylacetate and 6 ml of 1/3 M citric acid were added to the residue, the mixture being shaken vigorously. The water layer was discarded and the ethylacetate layer was washed with water, subsequently with saturated sodium bicarbonate and finally with saturated sodium chloride. The ethylacetate solution was dried over anhydrous sodium sulfate and was concentrated in vacuo. The resulted precipitate was washed with cold water and recrystallized from ethylacetate: yield, 70 mg; mp, 192—193°C; Calculated, H 4.95, C 59.47, N 12.24; Found, H 5.03, C 58.91, N 11.68.

N-Acetyl-L-tyrosine *p*-methylanilide (*N*-Ac-L-Tyr · *p*-methylanilide): This was synthesized from *N*-Ac-L-Tyr (100 mg) and *p*-toluidine (53 mg) by a method similar to that described above: yield, 100 mg; mp, 205—206, Calculated, H 6.41, C 69.23, N 8.97; Found, H 6.76, C 69.12, N 8.64.

Hydrolysis of *N*-Ac-L-Tyr · *m*-nitroanilide and *N*-Ac-L-Tyr · *p*-methylanilide was followed by measuring the release of *m*-nitroaniline and *p*-toluidine at 376 and 305 nm, respectively, using a Hitachi 323 recording spectrophotometer with a cell compartment maintained at 25°C by means of a thermostat.

Results and Discussion

I. Binding of methylchymotrypsin to the Streptomyces subtilisin inhibitor-Sepharose

If methylchymotrypsin can bind to the inhibitor, it is expected that the modified enzyme should be adsorbed and purified on *Streptomyces* subtilisin inhibitor-Sepharose column. Thus, the crude methylchymotrypsin preparation was directly applied on a column of *Streptomyces* subtilisin inhibitor-Sepharose. Fig. 2 shows the chromatographic profile of the crude enzyme. Methylchymotrypsin was adsorbed on the column at pH 7.5 and eluted at pH 3.5 (peak B). By this procedure, methylchymotrypsin was easily separated from PMS-chymotrypsin and other impurities, both of which were found in break-through fractions (peak A). Native α-chymotrypsin could also be purified by the same chromatographic procedure. These results indicate that methylchymotrypsin can interact with the inhibitor as α-chymotrypsin does.

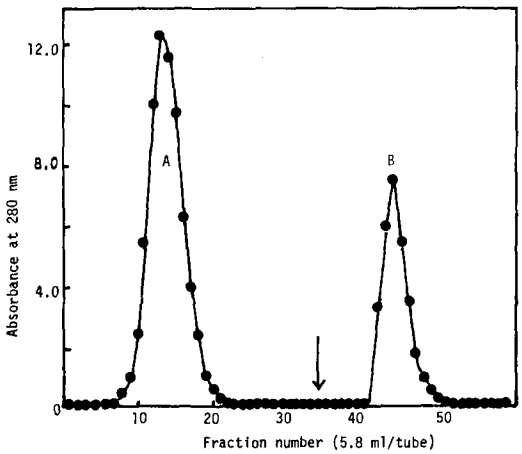

Fig. 2. Affinity chromatographic purification of methylchymotrypsin on inhibitor-Sepharose. A crude preparation of methylchymotrypsin, 250 mg, in 20 ml of 30 mM Tris · HCl buffer containing 0.1 M NaCl and 10 mM $CaCl_2$, pH 7.5, was loaded on a column (2.7 X 9.2 cm) of inhibitor-Sepharose equilibrated with the above buffer. Arrow indicates change from the starting buffer to 30 mM acetate buffer, pH 3.5. The other experimental details were described in the text.

II. Spectrophotometric analysis of interaction between methylchymotrypsin and the inhibitor

Inouye et al. [2] have reported that difference spectrum attributable to aromatic amino acid residues is observed by α-chymotrypsin-*Streptomyces* subtilisin inhibitor interaction. The same type of ultraviolet difference spectrum was also observed by mixing the solution of methylchymotrypsin and the inhibitor (Fig. 3). The difference absorptivities at 287 nm and 293 nm at a

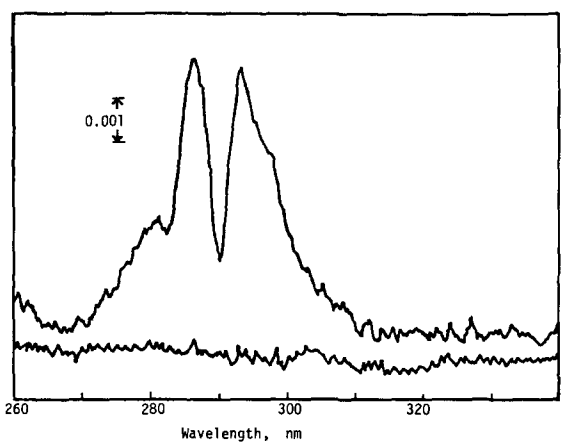

Fig. 3. Ultraviolet difference spectrum observed on binding of inhibitor with methylchymotrypsin. The concentrations of methylchymotrypsin and the inhibitor in 50 mM phosphate buffer, pH 7.44, with ionic strengths of 0.1 (NaCl) were $1.7 \cdot 10^{-5}$ M and $2.9 \cdot 10^{-5}$ M, respectively. The tandem double cuvette system was employed. Sample side: methylchymotrypsin and inhibitor in the buffer were in a front cuvette and only the buffer solution was in the other; Reference side: methylchymotrypsin in a front cuvette and inhibitor in the other.

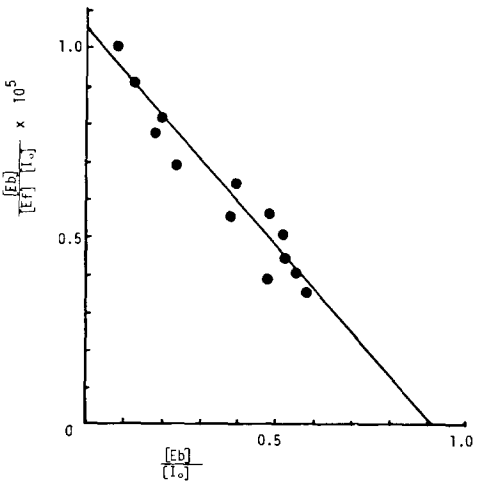

Fig. 4. Estimation of K_d and n values of methylchymotrypsin-inhibitor interaction by Scatchard plot. The K_d and n values were graphically estimated by following the equation of Scatchard:

$$\frac{[E_b]}{[E_f][I_0]} = \frac{n}{K_d} - \frac{1}{K_d} \cdot \frac{[E_b]}{[I_0]}$$

where $[I_0]$ = initial concentration of inhibitor, $[E_f]$ and $[E_b]$ = molar concentrations of free and bound enzymes. Both enzyme concentrations were calculated from the saturation curve of difference absorbances, ΔA_{287}, observed by mixing varying concentrations of the inhibitor (0.53 to approx. $18.55 \cdot 10^{-5}$ M) with a constant concentration ($1.74 \cdot 10^{-5}$) of methylchymotrypsin in 50 mM phosphate buffer, pH 7.44, with an ionic strength of 0.1 (NaCl) at 23° C.

constant concentration of methylchymotrypsin were dependent on the concentrations of the inhibitor, giving a saturation curve. Using these data, the dissociation constant, K_d, of the complex and the number of binding sites, n, per mole of the inhibitor monomer can be graphically estimated from the analysis by Scatchard plot [13] as shown in Fig. 4. Using $1.44 \cdot 10^3$ $cm^{-1} \cdot M^{-1}$ as the molar difference absorbance at 287 nm ($\Delta\epsilon_{287}$), a K_d value of $8.6 \cdot 10^{-6}$ M and n value of 0.91 were obtained at pH 7.44 and 23° C. This K_d value is comparable to that of native α-chymotrypsin-*Streptomyces* subtilisin inhibitor complex obtained from competitive type inhibition of the inhibitor for α-chymotrypsin-catalyzed hydrolysis of Ac-OPhNO$_2$ (K_i = $3.8 \cdot 10^{-6}$ M) and to those calculated from the spectrophotometric titration of native enzyme-inhibitor interaction by Inouye et al. [2] at pH 7.0 ($2.2 \cdot 10^{-6}$ M) and by us at pH 7.44 ($8 \cdot 10^{-6}$ M) in the present experiment.

III. Application of competitive enzyme assay procedure for the estimation of dissociation constant in the interactions of native and modified chymotrypsin with the inhibitor

The most general method for the estimation of K_d is an analysis according to Michaelis-Menten equation assuming one-to-one interaction. There is, however, a possibility that the inhibitor combines more than one mole of enzyme or conversely the enzyme has two or more binding sites for interaction with the inhibitor, as has been discussed in the case of α_2-macroglobulin proteinase interaction [14—16]. To estimate the number of binding sites, n, per mole of inhibitor

TABLE I

KINETIC PARAMETERS FOR α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ANILIDE SUBSTRATES AND K_S OF *m*-NITROANILIDE · METHYLCHYMOTRYPSIN COMPLEX

K_m and k_{cat} values of α-chymotrypsin were determined from a Lineweaver-Burk plot. The enzyme reaction mixtures were composed of $6.7 \cdot 10^{-6}$ M α-chymotrypsin, 0.12 to approx. $5.0 \cdot 10^{-3}$ M *m*-nitroanilide or 0.6 to approx. $4.0 \cdot 10^{-3}$ M *p*-methylanilide in 0.1 M Tris · HCl buffer, pH 7.8, containing 7% dimethylformamide. The reaction temperature was 25°C. The K_S value of *m*-nitroanilide · methylchymotrypsin complex was determined by proflavin displacement method described by Brandt et al. [9]. The concentrations of proflavin and methylchymotrypsin were $6.5 \cdot 10^{-6}$ M and $1.5 \cdot 10^{-4}$ M, respectively, and the concentrations of *m*-nitroanilide were 0.1 to approx. $4.0 \cdot 10^{-3}$ M. The other experimental conditions were the same as those in A.

	α-chymotrypsin		Methylchymotrypsin K_S (mM)
	K_m (mM)	k_{cat} ($s^{-1} \cdot 10^2$)	
Ac-L-Tyr · *m*-nitroanilide	2.7 ± 0.4	4.9 ± 0.4	4.0 ± 0.5
Ac-L-Tyr · *p*-methylanilide	13 ± 4	8.5 ± 0.3	—

monomer, we analyzed the data of spectrophotometric titration of the difference absorbance spectrum by the Scatchard plot as shown in Fig. 4.

We devised another analytical method, which is based on the competitive enzyme assay procedure, for the estimation of K_i and n values. In this method, it is preferable to use substrates giving K_m greater than K_i ($2-8 \cdot 10^{-6}$ M) of α-chymotrypsin-inhibitor interaction. Thus, we synthesized two substrates, Ac-L-Tyr · *m*-nitroanilide and Ac-L-Tyr · *p*-methylanilide. Table I summarizes kinetic parameters of α-chymotrypsin-catalyzed hydrolysis of both substrates and K_s value of methylchymotrypsin-Ac-L-Tyr · *m*-nitroanilide interaction. If α-chymotrypsin is bound to the number (n) of equivalent sites on the inhibitor monomer molecule and inhibitor inhibits the enzyme reaction competitively, K_i can be represented by Eqn. 1:

$$K_i = \frac{([E_0] - [E_i])(n[I_0] - [E_i])}{[E_i] \cdot \left(1 + \frac{[S]}{K_s}\right)} \quad (1)$$

Under the condition of $[E_0] < K_s$, $[S] = [S_0]$ and using:

$$f = \frac{[E_i]}{[E_0]} \quad (2)$$

Eqn. 1 is arranged to Eqn. 3

$$f \cdot \frac{[E_0]}{[I_0]} = n - \frac{f}{1-f} \cdot \frac{1}{[I_0]} \cdot \left(1 + \frac{[S_0]}{K_s}\right) \cdot K_i \quad (3)$$

$[E_i]$ is obtained experimentally from Eqn. 4

$$[E_i] = \frac{V_0 - V_i}{V_0} \cdot [E_0] \quad (4)$$

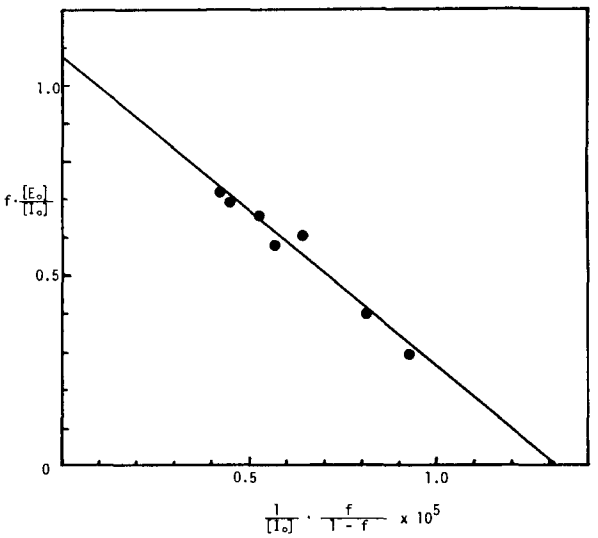

Fig. 5. Estimations of K_i and n values of α-chymotrypsin inhibitor interaction by titration of amidase activity toward Ac-L-Tyr · *m*-nitroanilide. The reaction mixtures were composed of $2.3 \cdot 10^{-5}$ M α-chymotrypsin, $1.0 \cdot 10^{-4}$ M Ac-L-Tyr · *m*-nitroanilide and varying concentrations (0.79 to approx. $6.66 \cdot 10^{-5}$ M) of the inhibitor in 0.1 M Tris · HCl buffer, pH 7.8. Reaction temperature was 25°C. The final concentration of dimethylformamide was 7%.

where $[E_i]$ is the molar concentration of enzyme bound to inhibitor and $[E_0]$, $[S_0]$ and $[I_0]$ are initial molar concentrations of enzyme, substrate and inhibitor, respectively, and Ks is the dissociation constant of ES complex (substrate constant) and is assumed to be identical to K_m. The V_0 and V_i mean the initial enzyme reaction velocities in the absence and presence of the inhibitor, respectively. A plot of $f \cdot [E_0]/[I_0]$ against $f/(1-f) \cdot 1/[I_0]$ gives a straight line, from which K_d and n value of α-chymotrypsin-*Streptomyces* subtilisin inhibitor interaction were estimated to be $7.9 \cdot 10^{-6}$ M and 1.08 respectively, (Fig. 5). When Ac-L-Tyr · *p*-methylanilide was used as the substrate, values of $8.5 \cdot 10^{-6}$ M and 1.05 were obtained for K_d and n.

The K_d value of the inhibitor-methylchymotrypsin complex was attempted to estimate by the competitive enzyme assay system composed of native α-chymotrypsin, methylchymotrypsin, the inhibitor and the substrate, Ac-L-Tyr · *m*-nitroanilide, according to the following consideration.

$$E + I \overset{K_i}{\rightleftharpoons} EI \tag{5}$$

$$E + S \overset{K_s}{\rightleftharpoons} ES \rightarrow E + P \tag{6}$$

$$E' + I \overset{K_d}{\rightleftharpoons} E'I \tag{7}$$

$$E' + S \overset{K_{s'}}{\rightleftharpoons} E'S \tag{8}$$

where E = α-chymotrypsin, E′ = methylchymotrypsin, I = *Streptomyces* subtilisin inhibitor, S = substrate, and K_i, K_s, K_d and $K_{s'}$ are dissociation constants of EI, ES, E′I and E′S, respectively.

In case of $[E] < K_s$ and $[E'] < K_d$, $[S] = [S_0]$ and K_d can be expressed by Eqn. 9

$$K_d = \frac{[E'][I]}{[E'I]} = \frac{\left(1 + \frac{[S_0]}{K_s}\right)[EI]K_i\left\{([E'_0] - [I_0] + [EI])([E_0] - [EI]) + K_i[EI]\left(1 + \frac{[S_0]}{K_s}\right)\right\}}{\left(1 + \frac{[S_0]}{K'_s}\right)([E_0] - [EI])\left\{([E_0] - [EI])([I_0] - [EI]) - K_i[EI]\left(1 + \frac{[S_0]}{K_s}\right)\right\}} \quad (9)$$

and [EI] is obtained experimentally from Eqn. 10

$$[EI] = \frac{v_0 - v_i}{v_0} \cdot [E_0] \quad (10)$$

where v_0 = initial velocity of α-chymotrypsin-catalyzed hydrolysis of Ac-L-Tyr · *m*-nitroanilide in the absence of methylchymotrypsin and *Streptomyces* subtilisin inhibitor, and v_i = the initial velocity of the enzyme reaction in the presence of varying concentrations of methylchymotrypsin and a constant concentration of the inhibitor.

By introducing the experimental values into Eqn. 9, the K_d value of methylchymotrypsin-inhibitor complex can be calculated. Thus, the experiments were carried out with the reaction system composed of $1.7 \cdot 10^{-5}$ M α-chymotrypsin, $5.0 \cdot 10^{-5}$ M inhibitor, $1.0 \cdot 10^{-4}$ M Ac-L-Tyr · *m*-nitroanilide and varying concentrations (1.43 to approx. $4.0 \cdot 10^{-5}$ M) of methylchymotrypsin under the condition shown in Table I. An average value of K_d was $8 \cdot 10^{-6}$ M at pH 7.8 from six series of the experiments.

The interaction of the inhibitor with Tos-Phe CH_2Cl-chymotrypsin, in which tosyl-L-phenylalanine was introduced into 3-N position of histidine-57 in α-chymotrypsin, was also investigated. In contrast to the results obtained with methylchymotrypsin, no difference spectrum was observed even when considerably high concentrations of both proteins (2.2 to approx. $11.1 \cdot 10^{-5}$ M inhibitor and $1.63 \cdot 10^{-5}$ M Tos-Phe CH_2Cl-chymotrypsin) were mixed, suggesting that Tos-Phe CH_2Cl-chymotrypsin has no binding affinity to the inhibitor. This suggestion was supported from the fact that Tos-Phe CH_2Cl-chymotrypsin was not adsorbed on the inhibitor-Sepharose column (Fujiwara and Tsuru, unpublished observation). Thus, it should be concluded that introduction of methyl group into histidine-57 does not affect essentially the interaction of the enzyme with the inhibitor and that Tos-Phe CH_2Cl-chymotrypsin which contained larger substituent than methyl group can not interact with the inhibitor presumably because of steric hindrance or conformational change by the introduction of tosyl-L-phenylalanine.

The present results indicate that α-chymotrypsin retains unaltered its binding ability to the inhibitor after methylation of the active site histidine. This suggests that the native enzyme does not fit compactly with the inhibitor around the region of 3-N position of histidine-57, though there are several pieces of evidence that it binds to some proteinases at or near the active site of the enzyme [1—3,6,7,17,18]. The present experiments also proved that there is

one binding site per inhibitor monomer for native and the modified chymotrypsins. Since the inhibitor molecule consists of two identical subunits, the complex of the enzyme inhibitor should exist as E_2I_2 form, in which E is α-chymotrypsin or methylchymotrypsin and I is inhibitor monomer. The formation of similar type of complex has previously been reported in inhibitor-subtilisin interaction by Sato and Murao [17].

In conclusion, it was found that methylation of histidine-57 in α-chymotrypsin did not affect the binding ability to *Streptomyces* subtilisin inhibitor as judged by spectrophotometric titration method and competitive enzyme assay procedure.

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