Intermediate Formation Process in Thermolysin Catalysis Observed Using a Fluorescent Displacement Probe in the Stopped-Flow Method

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The thermolysin-catalyzed hydrolysis of *N*-Cbz-tripeptides was studied by means of a fluorescence stopped-flow method using *N*-dansyl-L-phenylalanine (Dns–Phe) as a displacement probe at lower pH.

To determine the probe features of Dns–Phe, the equilibrium and kinetics of the binary interaction between Dns–Phe and the enzyme were studied by means of static differential fluorescence and by fluorescence temperature-jump methods in the pH range 4.5—5.6.

The dissociation constant (K_d) increased along with an increase in pH. Both the decreasing (apparent) binding rate and the increasing (apparent) dissociation rate with increasing pH were attributed to this decline of complex formation at higher pH.

The pre-steady state process in the substrate hydrolytic reaction was studied with this displacement probe, and the observed rate constant for the four tripeptide substrates had saturation kinetics that indicated the existence of a new intermediate during the sequence of the enzyme reaction. Four tripeptide substrates had comparable k_2 values (rate constant of intermediate formation), whereas the K_S value of Cbz–Phe–Leu–Ala was about twice that of the others.

Thermolysin, a thermostable microbial neutral protease containing zinc as a cofactor,[1] has been a target of many studies of catalytic properties[2—6] and structures.[7,8] We also studied some of the kinetic aspects of this enzyme in hydrolytic[9—12] and condensation[13—16] reactions. This enzyme has attracted attention in relation to membrane-bound metal endopeptidase,[17—19] and the structural and mechanistic aspects of inhibitor interactions have been studied.[20—24]

Concerning the pre-steady state kinetics of this enzyme, however, there have been fewer reports than for serine proteases, such as chymotrypsin.[25,26] This is primarily because thermolysin does not seem to form a metastable intermediate, such as an acyl-enzyme. Although some pioneering observations of pre-steady state processes for fluorescent depsipeptide and peptide substrates have been reported,[6,27] they were considered to be binding processes of the substrate.[6] However, we reported that *N*-acyl-tripeptide substrates, such as Fua–Gly–Phe–Ala, showed a burst process at a pH value below 6;[11] the analysis of a limited number of profiles implied the presence of an intermediate. Multi intermediates were also supported by a study on the temperature dependence and the isotope effects on the steady state rate by Izquierdo and Stein.[28]

Another important reason for the lack of pre-steady state kinetic studies of thermolysin is its substrate specificity. Thermolysin cannot hydrolyze normal esters of amino acids. The lack of a suitable reporting probe which can reversibly bind to the active site of the enzyme, such as proflavin for chymotrypsin,[25] is also significant.

Since some *N*-acyl amino acids competitively inhibit thermolysin,[29] we showed that fluorescence displacement could be used to study the rapid kinetics of thermolysin[30] for *N*-dansylated phenylalanine (Dns–Phe). This method is based on an energy transfer between the intrinsic tryptophan of the enzyme and the dansyl group of the probe, providing a distinct fluorescence signal upon close proximity of the two groups.[27,31,32]

Here, we confirmed the fundamental background of this displacement method and extended it to substrate specificity in the pre-steady state process among four *N*-acyl-tripeptides, in order to understand the mechanism of thermolysin catalysis.

Experimental

Materials: Thermolysin was obtained from Daiwa Kasei (Osaka, Japan; lot T4DB191). Bovine pancreatic carboxypeptidase A^{Cox}) (E.C.3.4.17.1) was purchased from Sigma (St. Louis, Mo, USA) as an aqueous suspension with a toluene preservative (Lot. 12F8186, 122F-8055). Purification and determination of the active concentrations of these enzymes proceeded as described.[9,33] Dns–Phe, Dns–Leu, Cbz–Phe, Cbz–Gly–Phe–Ala, Cbz–Gly–Leu– Ala, Cbz–Gly–Leu–Gly, Cbz–Phe–Leu–Ala, and Leu–Leu were purchased from Tokyo Kasei (Tokyo, Japan), Sigma (St. Louis, USA) or the Peptide Institute (Minoh, Japan). Dimethyl sulfoxide (DMSO) and acetonitrile (MeCN) were of spectroscopic

grade, obtained from Nacalai Tesque (Kyoto, Japan) or Cica (Tokyo, Japan). Other chemicals, such as Mes buffer, were commercially available. Distilled and deionized water was used throughout this study.

Methods: Interactions of the fluorescent inhibitor with the enzyme were monitored using a Hitachi-850 (Tokyo, Japan) or Shimadzu-RF5000 fluorophotometer (Kyoto, Japan). A temperature-jump apparatus of the Joule-heat type was constructed by Ohtsuka Electronics Co. (Type RA-410; Hirakata, Japan). The cell volume was around 2 cm^3 and the capacitance of the coaxial cable was 0.05 μF. The uprise time and the temperature increase under the conditions frequently used in this study (0.1 M KBr and 25 °C, M = mol dm^{-3}) were determined to be 5 μs and 5 °C, respectively. The stopped-flow apparatus, also made by Ohtsuka Electronics (Type Ra-401), had a gas (N_2)-driven flow cell of a front-stop type; the dead-time was determined to be around 3 ms under the usual conditions. In both of these two rapid reaction measurements, a 200 W D_2 lamp was use as the light source. The excitation beam was monochromated and the emitted light was filtered by a cut-off filter (L-39; 50% cut at 390 nm) before reaching the photomultiplier. Both pieces of apparatus were connected with a microcomputer through an A/D converter; if necessary, several successive runs were integrated and analyzed. Steady state kinetics and other spectrophotometer measurements were performed using an ultraviolet/visible spectrophotometer SM401 (Union Giken; Hirakata, Japan) or UV-2200 (Shimadzu). Some steady state kinetics were performed by HPLC using a Shimadzu LC10A-Cosmosil 5C18P, as described.[16]

Results

Complexation Equilibrium of Dns–Phe/Thermolysin: When thermolysin was mixed with a Dns–Phe solution (in 5% DMSO/buffer), the fluorescence emission of the Dns-group at around 550 nm, with excitation at 330 nm, increased (Fig. 1a). With excitation of the tryptophan residues in thermolysin, the presence of Dns–Phe decreased the emission at around 340 nm and increased that at 550 nm (Fig. 1b). Although all of these fluorescence changes caused by thermolysin can be monitored for the Dns–Phe/thermolysin interaction, λ_{ex} 280 nm/λ_{em} 550 nm gave the highest S/N and λ_{ex} 330 nm/λ_{em} 550 nm showed the best reproducibility in the equilibrium measurements. When the fluorescence change is described by a simple one-step binary interaction of thermolysin (E) and Dns–Phe (D),

$$E + D \underset{K_d}{\rightleftharpoons} E \cdot D \quad (1)$$

$$K_d = [E][D]/[E \cdot D], \quad (2)$$

then the fluorescence change (ΔF) is described with initial concentrations of E and D ($[E]_0$ and $[D]_0$) as

$$\Delta F = 2\Delta f[E]_0[D]_0/\{A + SQR(A^2 - 4[E]_0[D]_0)\}, \quad (3)$$

where Δf is the difference between the fluorescent coefficients and $A = K_d + [E]_0 + [D]_0$. The K_d was evaluated by a non-linear regression of the fluorescence increment at various concentrations of $[E]_0$ and $[D]_0$. The quality of the curve-fitting is shown in Fig. 2 as a modified Scatchard plot, where the reproduced ΔF with the calculated K_d are drawn in a continuous curve. The three combinations of excitation/emission wavelengths in Fig. 1 gave essentially consistent K_d values; their pH dependence is shown in Fig. 3a. In the same figure, the K_d for Dns–Leu/thermolysin is also given. They gave very similar K_d values and pH dependence. At pH values above 5.5, K_d increased steeply, which is in accordance with the pH dependence of the kinetic inhibition constant (K_i) of Cbz–Phe measured for the hydrolysis of Fua–Gly–Leu–NH_2.[9]

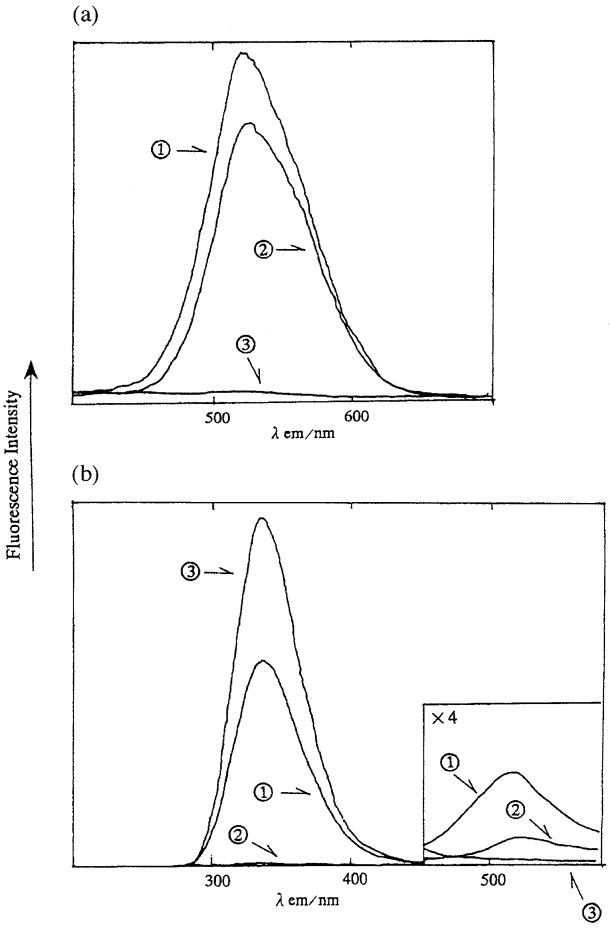


Fig. 1. Fluorescence spectra of thermolysin and Dns–Phe solutions. (a) Excitation at 330 nm. (b) Excitation at 280 nm. pH = 4.50, 25 °C. 0.01 M $CaCl_2$, 2.5%(v/v) DMSO. $[E]_0 = 5$ μM, [Dns–Phe] = 50 μM. For both; curves 1, 2, and 3 correspond to thermolysin + Dns–Phe, Dns–Phe, and thermolysin, respectively.

Cbz–Phe can also inhibit carboxypeptidase A, and K_i of this enzyme is less dependent upon the pH.[33] In accordance with this, the K_d values evaluated for Dns–Phe + Carboxypeptidase A by a similar experiment were less pH dependent, as shown in Fig. 3b.

The values of K_d for Dns–Phe + thermolysin were 4- to 10-times larger than those of K_i of Cbz–Phe towards thermolysin, and, therefore, the fluorescence change was completely diminished by adding 0.1 mM Cbz–Phe. Through such competitive ejection, the K_d for other non-fluorescent inhibitors (K_{dI}) can be evaluated (data not shown). When the condition $[E]_0 \ll [D]_0, [I]_0$ is satisfied, K_{dI} is obtained from the $[I]_0$ dependence of the apparent K_d value of the fluo-

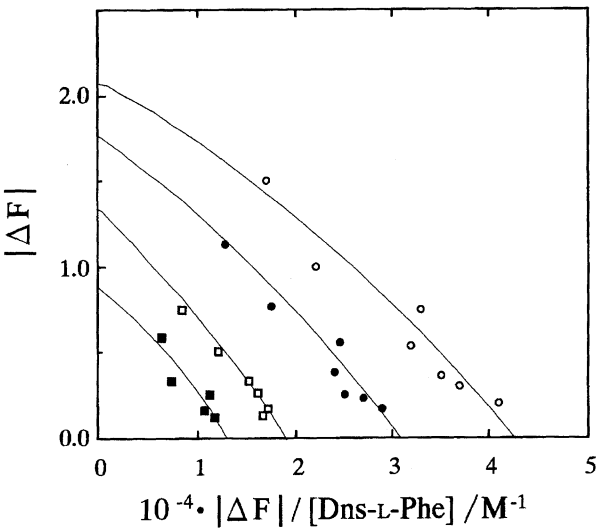


Fig. 2. Changes in the fluorescence intensity difference (ΔF) with various initial concentrations of the probe at 25 °C, shown as a modified Scatchard plot. The continuous fine curves are drawn using K_d values obtained by regression procedure. pH=4.53 (○), 4.75 (●), 4.96 (□), and 5.25 (■). 0.1 M KBr, 0.01 M $CaCl_2$, $[E]_0$=10 μM, [Dns–Phe]=5—100 μM, and 2.5%(v/v) DMSO. λ_{ex}=330 nm and λ_{ex}=540 nm.

rescence probe. Thus, the K_{dI} for Cbz–Phe+thermolysin and Leu–Leu+thermolysin, both of which are competitive inhibitors of this enzyme, were 6.75 μM (pH 4.85, 25 °C) and 1.05 mM (pH 4.80, 25 °C), respectively. These were roughly comparable to the K_i values obtained from kinetic measurements: 5.05 μM (pH 4.60, 25 °C)[9] and 1.1 mM (pH 4.95, 25 °C),[11] respectively.

Kinetics of the Complexation: Figure 4 shows typical temperature-jump observations for a mixture of Dns–Phe and thermolysin. In this kinetic measurement, excitation at 280 nm and emission at >390 nm was applied to gain S/N. Several trajectories were integrated and the averaged curve was fitted to a single exponential decay in order to evaluate a single relaxation time (τ;—). With various concentrations of thermolysin and Dns–Phe, the τ values were obtained and analyzed using an independently determined K_d value under identical conditions, assuming that the observed process is the single-step opposing reaction,

$$E + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot D, \tag{4}$$

and follows

$$\tau^{-1} = k_1([E]_{eq} + [D]_{eq}) + k_{-1}. \tag{5}$$

The dependence of the τ^{-1} value on the sum of the equilibrium concentrations of thermolysin and Dns–Phe is shown in Fig. 5. The k_1 and k_{-1} values were obtained from the slope and the y-intercept, respectively, at some pH values, as listed in Table 1.

The crucial issue for extending this study to the ternary system, comprising the enzyme, the fluorescent inhibitor, and a substrate, was that the reaction time necessary to reorder

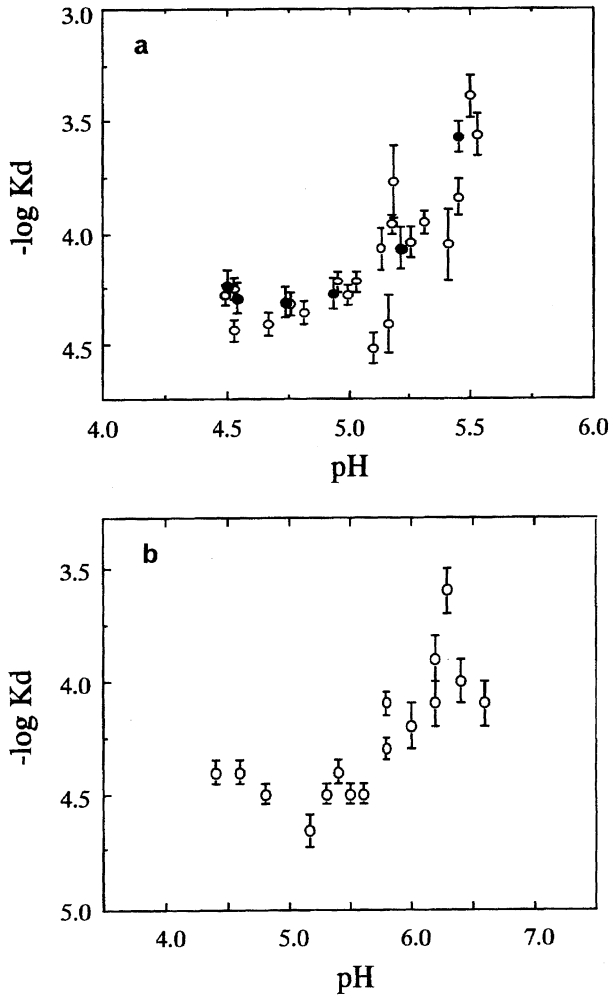


Fig. 3. The pH dependence of the K_d upon the complex of Dns–Phe of Dns–Leu with thermolysin (a) and carboxypeptidase A (b) at 25 °C. 0.1 M KBr, 0.01 M $CaCl_2$, $[E]_0$=10 μM, [Dns–Phe] or [Dns–Leu]=5—100 μM, and 2.5%(v/v) DMSO. ○, Dns–Phe; ●, Dns–Leu.

for a new equilibrium state be less than one ms under the present experimental conditions. This value is well below the time scale of the flow method kinetics. Therefore, the complexation of Dns–Phe and thermolysin can be soundly used in a displacement stopped-flow study to monitor the fast (pre-steady state) reaction in the substrate hydrolysis.

Stopped-Flow Study in the Ternary System: Figure 6 represents the fluorescence change observed after an enzyme solution containing Dns–Phe was mixed with a solution of Cbz–Phe–Phe–Ala containing Dns–Phe at the same concentration in a stopped-flow apparatus of λ_{ex}=280 nm and λ_{em}>390 nm. The whole trace comprises three portions (a very rapid decrease in the fluorescence, a flat portion, and a slow increase), corresponding to the pre-steady state process, the steady state, and a breakdown of the steady state (leading to the complete hydrolysis of the substrate). This correspondence was ascertained by steady and post-steady state HPLC measurements.[31]

A single relaxation time constant ($\tau=k_{obs}^{-1}$) was obtained

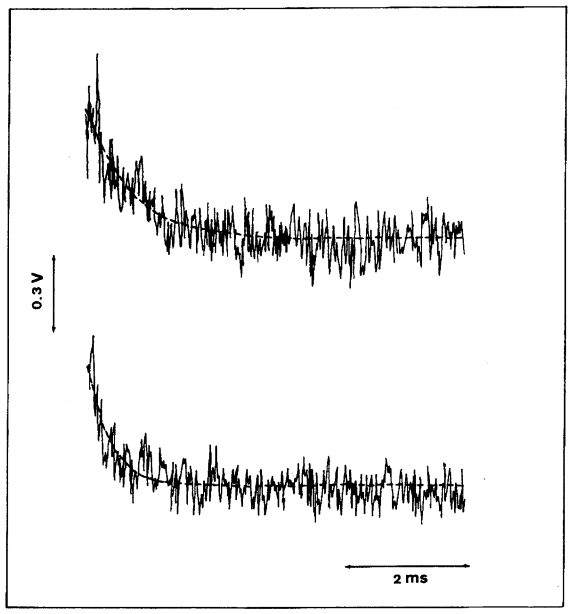


Fig. 4. Typical temperature-jump traces for the system thermolysin+Dns–Phe. pH=4.54 (upper) and 4.75 (lower). 25 °C (after temperature jump), 0.1 M KBr, 0.01 M $CaCl_2$, and 2.5%(v/v) DMSO. $[E]_0$=10 or 20 μM, [Dns–Phe]=50 μM. λ_{ex}=330 nm and λ_{em}>390 nm. The fine curves are drawn based on the non-linear regression of the data assuming a single relaxation time.

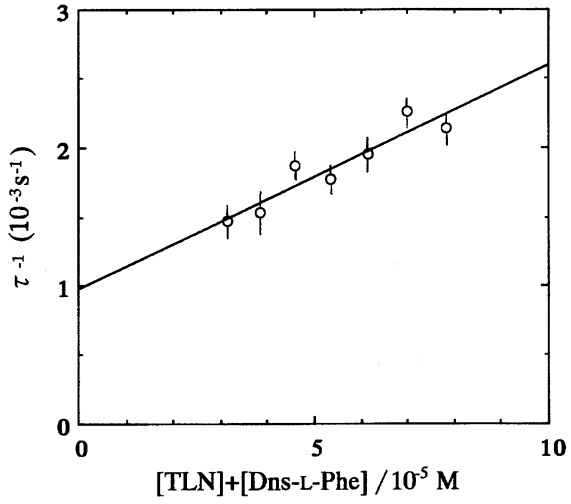


Fig. 5. Concentration dependence of the reciprocal relaxation time of the system thermolysin+Dns–Phe in a temperature-jump at pH 4.75, 25 °C. 0.1 M KBr, 0.01 M $CaCl_2$, and 2.5%(v/v) DMSO. $[E_0]$=20] μM.

from each pre-steady state portion; the concentration dependence of k_{obs} showed nonlinear features (Fig. 7) for all four substrates measured. This is quite different from the findings of Morgan and Fruton,[6] who measured the reaction of thermolysin with mansylated tripeptides. It is difficult to correlate the process to the isomerization of free enzyme, since the concentration dependence of k_{obs} for this process must give rise to a decrease in the substrate concentration.[34] Thus, the intermediate most probably exists in the sequence

Table 1. Kinetic Parameters of the Interactions of Dns–Phe with Thermolysin[a]

pH	$k_1/10^7 M^{-1} s^{-1}$	k_{-1}/s^{-1}	$k_{-1}/k_1/10^{-5}$M	K_d[b]$/10^{-5}$M
4.54	2.94 +/−0.41	770 +/−180	2.6	2.8
4.75	1.60 +/−0.17	997 +/−96	6.2	4.8
4.94	1.35 +/−0.52	1420 +/−260	10.5	6.2

a) $[E]_0$=10 or 20 μM, [Dns–Phe]=5—100 μM. b) Determined by equilibrium measurements.

of the enzyme reaction,

$$E+S \underset{K_S}{\rightleftharpoons} E\cdot S \xrightarrow{k_2} E\cdot S' \xrightarrow{k_3} E+P. \quad (6)$$

If so, the pre-steady state relaxation time can be described by Eq. 7.

$$k_{obs} = \tau^{-1} = k_2[S]_0/\{[S]_0 + K_S(1+[D]_0/K_d)\} + k_3. \quad (7)$$

The k_{cat} value ($=k_2k_3/(k_2+k_3)$) was determined independently by colorimetric or chromatographic means (0.52, 0.73, 7.4, and 11 s^{-1} for Cbz–Gly–Leu–Gly, Cbz–Gly–Leu–Ala, Cbz–Gly–Phe–Ala, and Cbz–Phe– Leu–Ala, respectively); the obtained k_{obs} data were analyzed by non-linear least-squares method on Eq. 7 with three variables (k_2, k_3, and K_S), using $k_{cat}=k_2k_3/(k_2+k_3)$ as a restrict condition. The quality of the evaluated parameters is shown in the fine continuous curve in the figure. The numerical values of K_S, k_2, and k_3 for the four substrates are listed in Table 2.

Discussion

Enzyme-Inhibitor Complex Formation: The values of k_1 evaluated here fore the binding rate of Dns–Phe to thermolysin (Table 1) are much smaller than those usually considered for a simple diffusion-limited collisional process.[34] It is difficult to consider other mechanisms, because measuring the τ values at lower $[E]_{eq}+[D]_{eq}$ was difficult due to very small fluorescence changes. However, the occurrence of an intramolecular isomerization process after the formation of the first complex between the enzyme and the inhibitor is likely (Eq. 8).

$$E+D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot D \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\cdot D' \quad (8)$$

Here, the τ value for the observed process would involve the equilibrium constant of the first step ($K_d^\circ=k_{-1}/k_1$) and the rate constants of the second step, as described by

$$\tau^{-1} = k_2([E]_{eq}+[D]_{eq})/(K_d^\circ + [E]_{eq}+[D]_{eq}) + k_{-2}. \quad (9)$$

The $[E]_{eq}+[D]_{eq}$ dependence of $\tau^{-1}-k_{-2}$ then gave a hyperbolic curve approaching k_2 at an extremely high value of $[E]_{eq}+[D]_{eq}$. When $K_d^\circ \gg [E]_{eq}+[D]_{eq}$ can be assumed, Eq. 9 becomes the same form as Eq. 5, and the apparent k_1 ($k_{1(app)}$) obtained above is equal to k_2/K_d° and $k_{-1(app)}$ to k_{-2}. Since the value at the saturated level should be well below 0.5 ms, k_2 would be above 2000 s^{-1}; thus, K_d° would be less than 10^{-4} M.

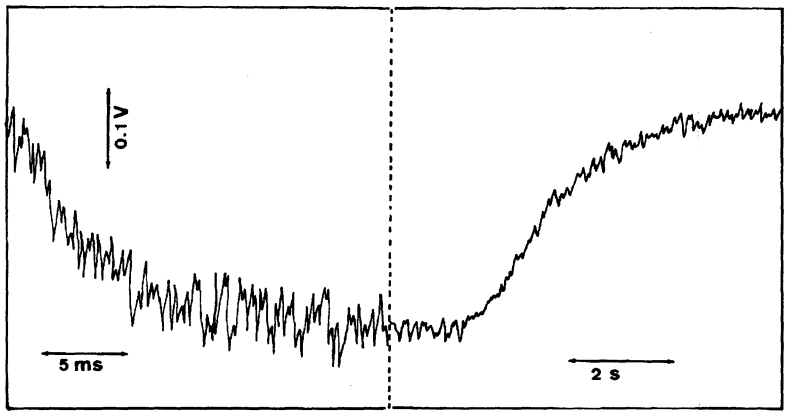


Fig. 6. Stopped-flow reaction trace after mixing thermolysin with Cbz–Gly–Leu–Ala in the presence of Dns–Phe as a fluorescent displacement probe. pH 4.5, 25 °C, 0.1 M KBr, 0.01 M $CaCl_2$, and 2.5%(v/v) DMSO. $[E]_0=10$ μM, $[S]_0=0.25$ mM, and [Dns–Phe]=50 μM. $\lambda_{ex}=280$ nm and $\lambda_{em}>390$ nm. The left and right halves were recorded with a rapid and a slower sweep, respectively. The whole trace is composed of three parts: a rapid pre-steady state, steady-state, and the breakdown of this state (see text).

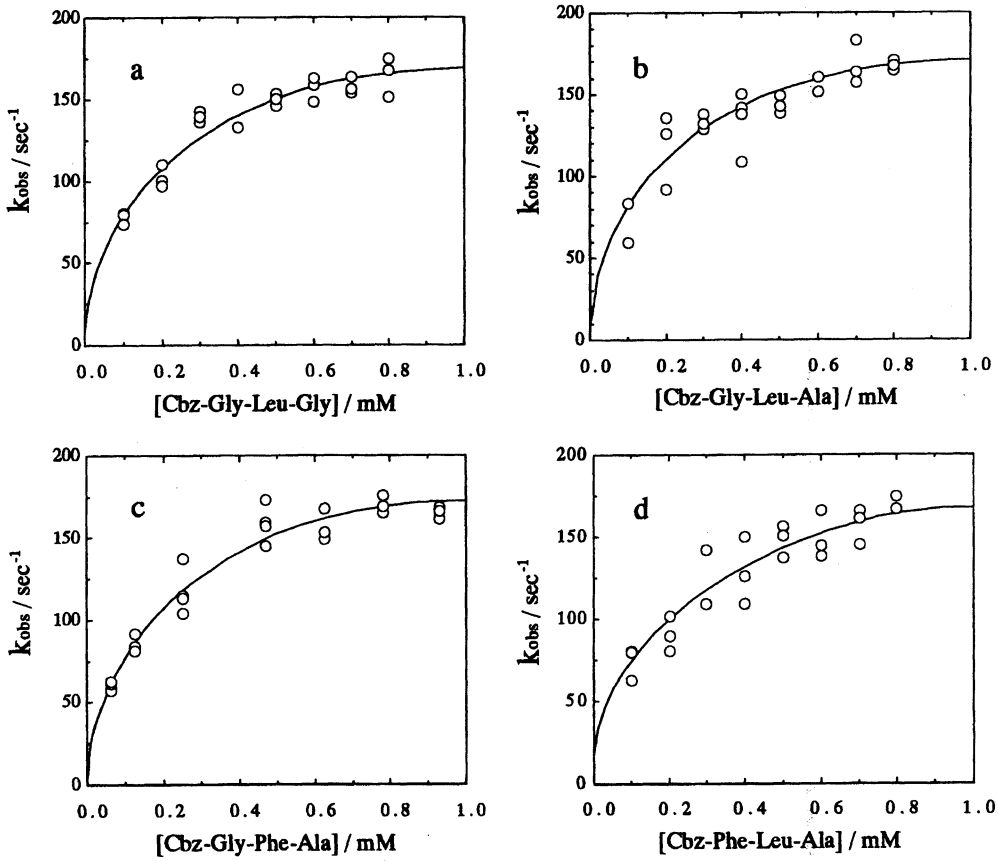


Fig. 7. Dependence of k_{obs} on [thermolysin + Cbz–tripeptide]. (a) Cbz–Gly–Leu–Gly, (b) Cbz–Gly–Leu–Ala, (c) Cbz–Gly–Phe–Ala, and (d) Cbz–Phe–Leu–Ala. pH 4.54, 25 °C, 0.1 M KBr, 0.01 M $CaCl_2$, and 2.5%(v/v) DMSO. $[E]_0=10$ μM, [Dns–Phe]=50 μM. $\lambda_{ex}=280$ nm and $\lambda_{em}>390$ nm. The fine curves were drawn based on the k_2, k_3, and K_S values (Eq. 7) obtained by non-linear regression of the plots with experimental K_d and k_{cat} values.

With an increase in pH, $k_{1(app)}$ became smaller while $k_{-1(app)}$ became larger. The steep increase in the K_d value with increasing pH was then attributed to the binding and the dissociation processes. The primary binding forces of acyl–amino acid towards thermolysin are the Coulombic attraction, hydrophobic interaction, and coordination on the active site zinc.[8,29] Among these, the Coulombic attraction would be most sensitively influenced from any change in the pH, or weakening of the electrostatic attraction results in both the deceleration of the binding rate (or increase in K_d°) and the acceleration of the dissociation rates (k_{-1} and perhaps even k_{-2}). A change in the coordination environment of the

Table 2. Parameters for the Pre-steady State Process of Cbz-tripeptide Hydrolysis by Thermolysin Observed by Fluorescence Displacement[a)]

Substrate	K_s/μM	k_2/s^{-1}	k_3/s^{-1}
Cbz–Gly–Leu–Gly	56 +/−4	196 +/−4	0.52 +/−0.02
Cbz–Gly–Leu–Ala	59 +/−7	198 +/−7	0.73 +/−0.03
Cbz–Gly–Phe–Ala	64 +/−4	195 +/−4	7.7 +/−0.2
Cbz–Phe–Leu–Ala	103 +/−13	208 +/−10	11.5 +/−0.6

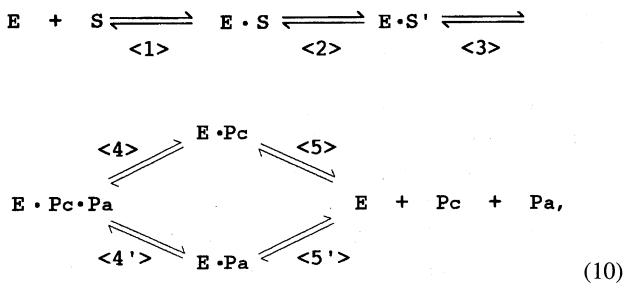
a) pH=4.54, 25 °C, 0.1 M KBr, 0.01 M $CaCl_2$, and 2.5%(v/v) DMSO. $[E]_0$=10 μM, [Dns–Phe]=50 μM, [S]=100—900 μM.

active site Zn might also influence the stability of the binary complex.

As described in detail by Brandt et al.,[25] the displacement method is premised on the realization of the rapid pre-equilibrium of a reporting competitive inhibitor interacting with an enzyme. Irrespective of the binding (kinetic) mechanism of the present probe of thermolysin (Eq. 4 or Eq. 8), this condition was proven in this study. An excess in the concentrations of the species, $[S]_0 \gg [E]_0 < [D]_0$, was realized. Compared with the proflavin–chymotrypsin system, the dissociation rate of the probe-thermolysin complex is slower. However, a simulation study indicated that our system can monitor the processes in the stopped-flow method without a significant delay due to the chemical reaction.

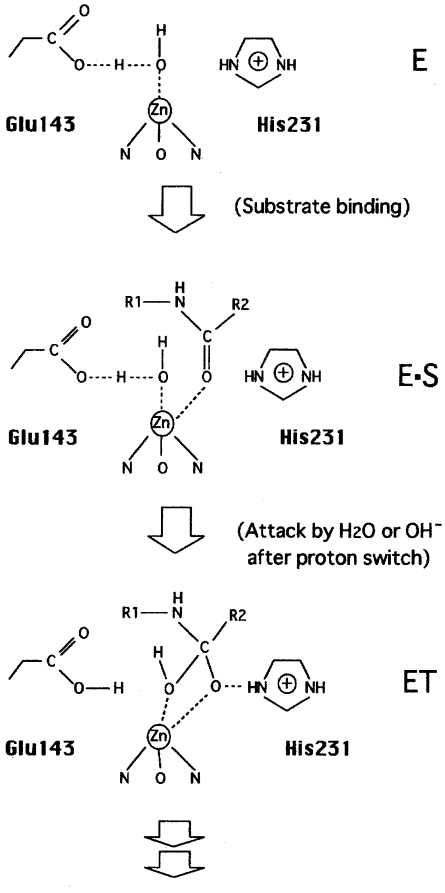
Intermediate in the Hydrolytic Reaction: The nonlinear concentration dependence of k_{obs} for all four substrates indicated that thermolysin catalysis involves an interconversion process. Our finding[11] of such a process in a thermolysin catalysis was limited to a specific chromophoric substrate (Fua–Gly–Leu–Ala), and the amount of data was insufficient. Thus, the discrepancy between our results and those of Morgan and Fruton[6] could be attributed to either the substrate[31] or the pH. Furthermore, photochemical cis–trans isomerization was claimed for furylacryloylpeptides,[35] and the possible unknown contribution of such a phenomenon to the observed isomerization process in the pre-steady state kinetics was not easily eliminated. With the present methodology we can measure these processes involved in more general substrates and analyze them thoroughly.

Since our kinetic study on the peptide condensation reaction[16] indicated that the binding of the carboxyl and amine components onto thermolysin can be described by a random Bi–Bi mechanism, the entire sequence of the peptide hydrolysis/condensation reaction catalyzed by this enzyme can be formally described as follows:



where P_a and P_c denote the amine and carboxyl component products, respectively. The inhibitor complexation of the present instance, itself, is part of the reaction of Eq. 10, starting from the right end (step 5′). Also the above consideration implies that this step involves an isomerization process.

The most likely reaction mechanism of the thermolysin-catalyzed hydrolysis of peptides[9,23,28,36] involves a general base catalysis or a proton switch by Glu_{143} to assist the attack on the carbonyl carbon of the scissile bond by water (or OH^-) coordinated on the active site zinc and the formation of a tetrahedral intermediate (TI) (ET in Scheme 1). This TI species decomposes upon general acid catalysis or a zwitterion decomposition. The process observed here can be deduced as TI formation or a successive process after TI formation, before its complete decomposition, such as proton movements. It might even be a non-chemical step of protein isomerization or a conformational change. Four substrates showed a k_2 of comparable values, whereas the K_S value of Cbz–Phe–Leu–Ala was about twice that of the others. An increase in the K_S value with a Gly/Phe change at the S_1 position was similar to the result in K_m,[4] and indicates the limited size of the S_1 pocket of this enzyme. The change in Gly/Ala at the P_2' and that of Leu/Phe at the P_1' positions resulted in a different k_{cat} at neutral pH;[4] also, at this acidic pH, they were not reflected much in the rate of the new intermediate formation process (k_2). This insensitivity might preferably



Scheme 1.

relate to the observed process with a non-chemical event(s).

The limitation of the present method is within the applicable range of pH. In two senses an increase in the medium pH narrows the observation of the pre-steady state. The complex formation of the present probe is much weakened at higher pH, and the pre-steady state process, itself, becomes much faster at higher pH. The k_2 value at around 200 s^{-1} is comparable to the k_2 values of the specific ester substrates of the α-chymotrypsin reaction at optimum pH (k_2 for Fua–Phe–OMe is 203 s^{-1} at pH 8.9).[26] Thus, we can only state that the thermolysin catalysis contains some interconversion step at least at a slightly acidic pH. However, if the efforts to observe the pre-steady state process(es) at neutral or alkaline pH were unsuccessful, not simply because of the physical limitations imposed by the apparatus, and if the new intermediate is only formed under acidic conditions, some process related to proton movements, such as from the activated water to Glu 143 or Glu 143 to the scissile amide, could be involved.

Abbreviations. Fua-, 3-(2-furyl)-acryloyl-; Dns- or dansyl-, 5-(dimetylamino)-1-naphthylsulfonyl; mansyl, 6-(*N*-methylanilino)-2-naphthylsulfonyl; -OMe, methyl ester; Mes; 2-Morpholinoethanesulfonic acid; DMSO, dimethyl sulfoxide; E, enzyme; D, displacement probe; I, inhibitor; S, substrate; P, product; E D and E D′, enzyme-probe complexes, E S and E S′, enzyme-substrate complexes; P_a and P_c, amine and carboxyl component product, respectively; $[\ ]_{eq}$, equilibrium concentration.

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