Mechanism of Dipeptidyl Carboxypeptidase Activity of Thermolysin

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(Received May 18, 1984)

Steady state and presteady state kinetic analyses were performed for thermolysin-catalyzed hydrolysis of chromophoric tripeptide substrates with free carboxyl terminal. The pH dependence of k_{cat} showed fairly higher pK_a (≃7) than that of the second-order rate parameter(k_{cat}/K_m)(≃5) and the pH-dependence of K_m resembled that of K_i for *N*-blocked dipeptidyl inhibitor observed before (S. Kunugi *et al.*, *Eur. J. Biochem.* **124**, 157 (1982)). These findings indicated that the reaction with this type of substrate involves a nonproductive binding mode. In a presteady state kinetic study by stopped-flow method, a burst process of 10—20 ms order was observed before the linear steady state at a relatively low pH and temperature. This process showed moderate pH dependence. Considering these experimental results coupled with those accumulated so far on this enzyme, a unified mechanism including a nonproductive binding and an isomerization process in prior to the cleavage of the peptide bond was proposed for the dipeptidyl carboxypeptidase activity of this enzyme.

Thermolysin is a microbial thermostable zinc-containing protease.[1] This enzyme shows no exo-activity. Dipeptides or *N*-blocked amino acids act as inhibitors.[2] It shows, however, dipeptidyl carboxypeptidase activity towards certain *N*-blocked peptides with free carboxyl terminal, such as *N*-(benzyloxycarbonyl)glycyl-L-leucyl-L-leucine (ZGlyLeuLeu), *N*-benzyloxycarbonyl-L-phenylalanylglycyl-L-leucyl-L-alanine (ZPheGlyLeuAla)[3] and 3-(2-furyl)acrylolglycyl-L-leucyl-L-alanine (FuaGlyLeuAla).[4] The K_m values of thermolysin against several synthetic peptides are relatively high: that for 3-(2-furyl)acryloylglycyl-L-leucinamide (FuaGlyLeuNH_2) or 3-(2-furyl)acryloylglycyl-L-phenylalaninamide (FuaGlyPheNH_2), widely used for the assay of the activity of this enzyme, is around 0.5 mM (1M=1 mol dm^{-3}) at acidic pH and 5 mM at neutral pH.[5] These low affinities limited detailed kinetic analysis of the catalytic reactions. Analysis of the presteady state in this enzyme reaction was performed only for special tripeptide or pentapeptide substrates having fluorescent acyl group[6,7] and thorough discussions of the reaction processes have not been given as yet.

In the research on the interaction of various substrate analogues with several zinc-containing proteases[5,8,9] we noticed that the C-free amino acid or dipeptide inhibited these enzymes very strongly at acidic pH and showed very steep pH dependences. An X-ray crystallographic study by Kester *et al.*[10] showed that an anionic inhibitors of this type binds on the active site of thermolysin in an inversed manner with the carboxylate directly coordinating on the active site zinc, which is to have strong relation with the peculiar pH dependence of the inhibition. Such a binding mode could be common in the case of the substrates having free carboxyl end, such as *N*-acylated tripeptides. Thus we report here results of detailed steady-state kinetic analysis of the hydrolysis of 3-(2-furyl)acryloyl tripeptide substrates catalyzed by thermolysin, as well as the observation of the presteady state process of the reaction and discuss the mechanisms of the dipeptidyl carboxypeptidase activity of this enzyme.

Experimental

Materials. Thermolysin was obtained from Daiwa Kasei (Osaka), (lot. T8CA81), and its active concentration was determined as described previously.[5] Four chromophoric substrates, 3-(2-furyl)acryloylglycyl-L-phenylalanyl-L-alanine (FuaGlyPheAla), FuaGlyLeuAla, FuaGlyPheNH_2 and FuaGlyLeuNH_2 were the same samples as used in the previous studies.[5,9] Good's buffers (Mes and Hepes) were purchased from Dojindo Laboratories (Kumamoto). Other chemicals were of reagent grade and used without further purifications. Deionized and distilled water was used in the present work.

Methods. The steady-state kinetics were followed by an ultraviolet/visible spectrophotometer (Union SM401, Union Giken, Hirakata). The difference spectrum between the chromophoric substrate(FuaGlyPheAla) and the products (FuaGly and PheAla) was referred to in the previous paper.[9]

The second-order rate constant (k_{cat}/K_m) was obtained from the initial velocity of the pseudo-first order rate at a low substrate concentration. The k_{cat} and K_m values were evaluated from Eadie-type plot of the velocities at various substrate concentrations. At lower pH the K_m for FuaGlyPheAla was so low that its concentration could not be reduced enough to evaluate K_m and k_{cat}/K_m. In such a case, the velocity devided by initial enzyme concentration at saturated substrate condition ([S]>0.3 mM) was considered as the k_{cat} (for details see result). The reactions were studied in 0.1 M buffer solution containing 0.01 M $CaCl_2$ unless otherwise mentioned.

The presteady state of the reaction was measured on a stopped-flow spectrophotometer (Union RA1100, Union Giken, Hirakata). The dead-time of this apparatus attached with a 10 mm cell has been determined as 3.0 ms.[11] The strong back ground absorbance of the substrate at high concentration limited observation at wavelengths higher than 330 nm. The temperature of the solution was controlled by circulating thermostated water (Neslab RTE-8) through the cell compartments of the spectrophotometers and occasionally checked with a thermister(±0.1 °C).

Results and Discussion

Steady State Kinetics. In Fig. 1, the pH dependences of k_{cat}/K_m, k_{cat}, and K_m[12] for FuaGlyPheAla are shown. At pH lower than 6, where the K_m becomes very low, we show only the apparent second-order rate constant at $[S]_0=10\mu M$ (which is the lowest concentration to get a reliable rate) in Fig. 1-a, the rate constant at $[S]_0>0.3$ mM in Fig. 1-b and the ratio of the above two quantities in Fig. 1-c. In Fig. 1-a the left half of

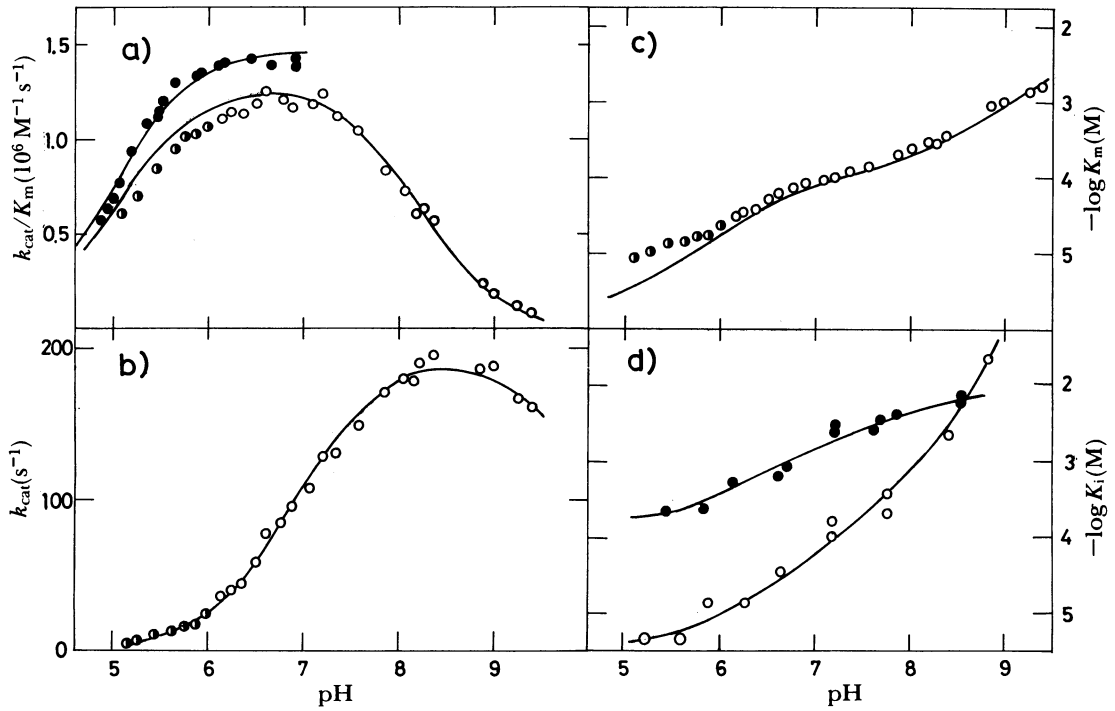
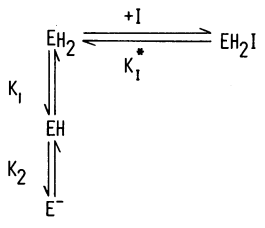


Fig. 1. pH-dependence of k_{cat}/K_m, k_{cat}, and K_m for FuaGlyPheAla and FuaGlyLeuAla at 25°C.
a) k_{cat}/K_m (○, ◑) FuaGlyPheAla; (●) FuaGlyLeuAla. ○, obtained from Eadie plot; ◑, ● rate at [substrate]=10 μM. b) k_{cat} for FuaGlyPheAla. (○) obtained from Eadie-plot with [substrate]=20 μM–2 mM; (◑) velocity at [substrate]>0.3 mM. c) K_m for FuaGlyPheAla. (○) from Eadie-plot; (◑) ratio of velocity at [substrate]=10 μM to that at [substrate]>0.3 mM. Due to the insufficiently low concentration of the substrate for the determination of k_{cat}/K_m, K_m data deviated downward from the curve calculated on the pK_1 obtained from FuaGlyLeuAla especially at low pH. d) pH dependence of two inhibitors for comparison. (○) ZPhe; (●) ZLeuGly. Both are taken from Ref. 5.

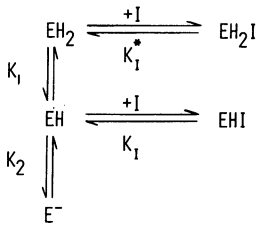
the pH profile of the second-order rate for FuaGlyLeuAla at $[S]_0=10\,\mu M$, which satisfies the condition of $[S] \ll K_m$ for this substrate, was also given. The pH dependence is apparently explained by assuming two pK_a's (pK_{a1} for the lower side and pK_{a2} for the higher side) and one limiting rate constant. The pK_{a1} was evaluated from the data of FuaGlyLeuAla to be 5.00 ± 0.03 and pK_{a2} was from those of FuaGlyPheAla as 8.18 ± 0.02, both of which are consistent to the values obtained for the neutral dipeptide substrates, FuaGlyPheNH$_2$ and FuaGlyLeuNH$_2$.[5] Compared with this pK_{a1}, the pH profile of k_{cat} value gave a high apparent pK_a. Accordingly K_m showed a very characteristic pH dependence, the value being approximately proportional to the 1/2 power of $[H^+]$. This resembles the pH dependence of the K_i of dipeptide substrate analogue[5] (Fig. 1-d, ●).

In the study of the pH dependence of K_i of *C-free N-benzyloxycarbonyl amino acids*, such as ZPhe and ZLeu,[5] it was made clear that these inhibitors are bound only to the enzyme form which is protonated at catalytic site with the carboxylato group coordinating on the active site zinc and they show a very strong pH dependence of K_i as an example given in Fig. 1-d, (○ for ZPhe). This result was formally represented by Scheme 1, where the cationic form of the enzyme is written as EH_2. Kn's and K_I* denote the proton dissociation constants and the dissociation constant for the inhibitor(I)-enzyme complex, respectively. The pH profile for *dipeptide inhibitor* (● for ZLeuGly) is, as well, explained by Scheme 2, where two binding modes are assumed. A K_I/K_I* ratio of 22 gave a satisfactory curve as fitted to the data in Fig. 1-d.

Based on these schemes, the presently observed pH dependence of K_m of the *tripeptide substrates* can be explained by Scheme 3, where S written in the left-hand side of E (SEH_2) indicates that the substrate is bound on the active site in a different manner. That is, this type of substrate can be bound on the enzyme



Scheme 1.



Scheme 2.

active site in two different manners. The one is a normal (productive) mode (K_s) in which the carbonyl carbon of the scissile bond is located on the zinc and the amino acid side residues are located in their respective subsites.[10] The other (K_s*) is considered similar to that seen in C-free *N*-blocked amino acids and the terminal carboxylate is coordinating on the zinc. In the kinetic formula, however, this scheme is identical with the one given for Scheme 4, where $1/K_3^* = 1/K_3 + K_S/K_S^*/K_1$. A nonlinear regression of the data in Fig. 1 gave the parameters for Scheme 4 as: $pK_1 = 5.3 \pm 0.1$, $pK_2 = 8.2 \pm 0.02$, $pK_3^* = 6.9 \pm 0.05$, $pK_4 = 10.0 \pm 0.1$. Insufficiently low concentration of FuaGlyPheAla gave slightly higher pK_1 for this substrate than the intrinsic value(5.0).[5] The solid curves in Fig. 1 are reconstituted on these parameters. When we *a priori* assume that the pK_a for the protonation of the productive enzyme-susbtrate complex is not so much different from that of the free enzyme, then we get the K_S/K_S^* ratio around $10-10^2$, which is not inconsistent to the value evaluated in Fig. 1-d.

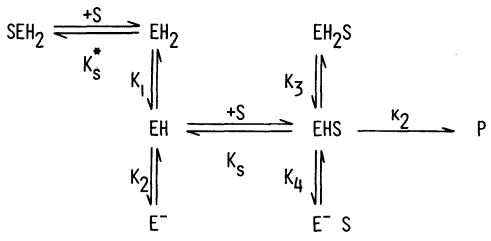
Presteady State Kinetics. Mixing FuaGlyPheAla with the enzyme in a stopped-flow apparatus, we observed the reaction traces as shown in Fig. 2. A presteady state relaxation process was observed before the steady state linear decrease in absorbance. To get clearer traces the temperature was lowered. The relevant parameters under some experimental conditions are summarized in Table 1. The inverse of the observed relaxation time of the presteady part is k_{burst} and the difference in the optical density during this burst process, which is obtained by an extrapolation of the steady state linear portion to time zero with consideration of the dead time of the apparatus, is the burst amplitude (ΔOD_{burst}). The steady state parameters obtained under this stopped-flow conditions ($v_{steady}/[E]_0$) were consistent with those measured by the conventional method. The strong background absorbance due to the substrate of higher concentrations, the small absorbance change accompanied by the large change of the steady state, and the rapidness of the process, all of these unfortunately limited the conditions and the accuracy of the observation of the presteady state process, but we can point the following features for the process detected here.

1) The apparent rate slowly increased with an increase in the substrate concentration,

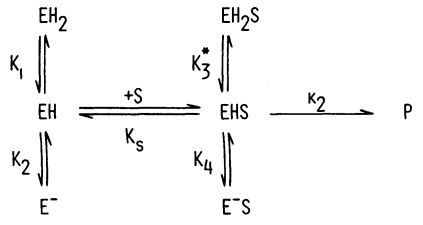
2) it increased only 2.5 times by an increase in the pH from 5.3 to 6.75, and,

3) the burst amplitude did not strongly depend on the substrate concentration or pH of the solution, at least within the range studied here.

By this observation of the presteady process we must modify the reaction Scheme of 4 or 3 into what allows the existence of a certain burst process. Morgan and Frouton observed a presteady process in thermolysin catalysis of the hydrolyis of fluorescent tripeptide or pentapeptide substrate[6] and explained the process as the binding of the substrate, having k_{bind} of 3×10^6 $M^{-1}s^{-1}$ and $k_{dissociate}$ of $55\,s^{-1}$ at 25 °C and pH 8.5. Lobb



Scheme 3.



Scheme 4.

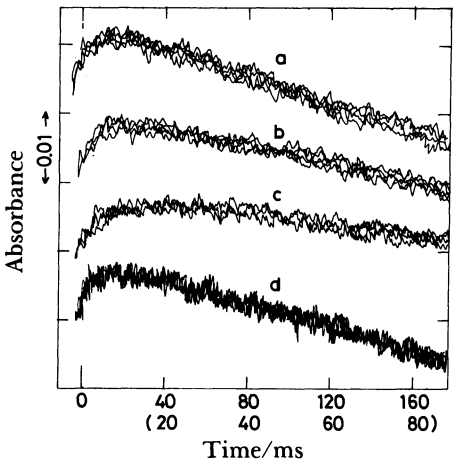


Fig. 2. Stopped-flow traces of the burst process observed in the hydrolysis of FuaGlyPheAla. Ordinate scale is 0.01 OD/scale.

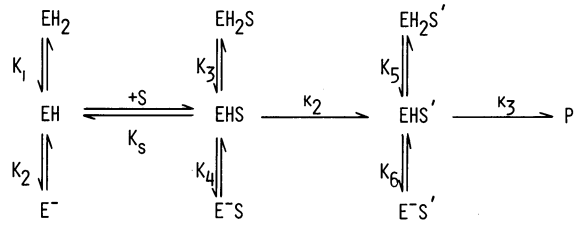
	Temp. (°C)	pH	[S]o mM	[E]o μM	abscissa scale(ms)
a)	25.0	5.35	0.1	10.1	40
b)	15.5	5.35	0.1	10.1	40
c)	7.5	5.35	0.1	10.1	40
d)	7.7	6.75	0.09	10.7	20

TABLE 1. PARAMETERS OF THE BURST PROCESS OF THERMOLYSIN CATALYSED HYDROLYSIS OF FuaGlyPheAla

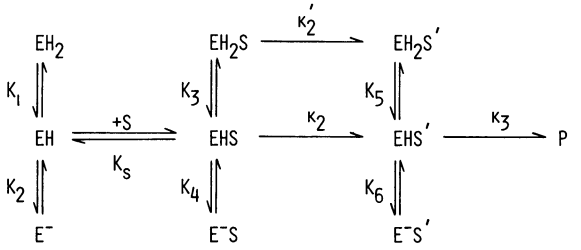
pH	Temp °C	[S]o mM	[E]o μM	ΔOD_{burst} $10^{-3}\,cm^{-1}$	k_{burst} s^{-1}	$v_{steady}/[E]_0$ s^{-1}
5.35	25.0	0.1	10.1	7	100	6.3
5.35	15.5	0.1	10.1	8	100	4.2
5.35	7.5	0.1	10.1	6	50	3.6
5.35	7.5	0.05	10.1	7	38	3.1
5.35	7.5	0.2	10.1	6	65	4.1
6.75	7.7	0.09	10.7	10	180	11.5

and Auld also observed a similar presteady state process for a *N*-dansylated tripeptide and an apparent rate constant was $50\,s^{-1}$ at 15 °C, pH 6.0 and [substrate] =0.045 mM.[7] If the presently observed burst process was the binding of the substrate, then k_{bind} would be calculated as around $2\times10^5\,M^{-1}s^{-1}$ and $k_{dissociate}$ around $30\,s^{-1}$. The former one is considerably small for a molecular complex formation process, even at a reduced temperature.[14] Furthermore in the case of chromophoric substrate such as the present one, binding on the enzyme active site generally results in a far smaller difference spectrum than the fluorescent one, as was exemplified for the Fua-substituted amino acid ester bound on α-chymotrypsin, where the difference extinction coefficient was less than a few hundreds.[14] The positive concentration dependence of the inverse of the relaxation time will discourage the possibility that the observed process is the conformational change of the enzyme protein prior to the binding of the substrate on it. Therefore we would like to conclude that the presently observed burst process is an isomerisation subsequent to the substrate binding. Since the observed process is clearly an upward change in the absorbance, it is neither due to a mere time lag of the final product appearance (see Appendix).

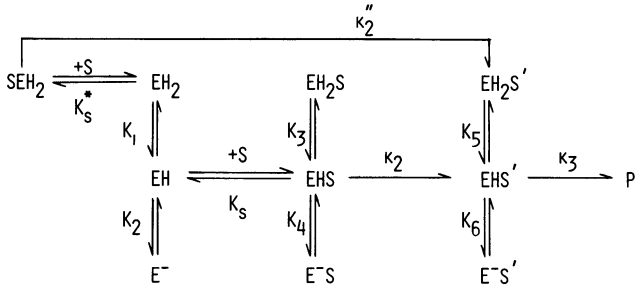
Possible Reaction Mechanism. The first possible and simplest mechanism we can postulate is such as Scheme 5. This scheme qualitatively explains the pH independence of the burst amplitude, by assuming that both EHS′ and EH_2S' have (positive) difference extinction coefficients against free substrate, and also



Scheme 5.



Scheme 6.



Scheme 7.

the pH dependences of steady state parameters. It cannot, however, explain the moderate pH dependence of the burst rate in the measured pH range. There could be, of course, a number of possible reaction schemes, which explain all of these quantities, when several reaction processes and kinetic parameters are assumed. The simplest and most reasonable one which is consistent to the present data is that given in Scheme 6, containing the second pathway from EH_2S to EH_2S'.

In contrast to the precise data regression for the steady state result in the former section, the limited number and rather high deviations of the presteady state data provided us only a semi-quantitative parametrization of the Scheme, and the calculated pH dependences of the several quantities from Scheme 6 are given in Fig. 3 for a plausible set of the parameters. In analogy to the relation of Scheme 3 and 4, Scheme 6 is formally equivalent to the reaction mechanism of Scheme 7, where the nonproductively bound substrate, as postulated in the steady state analysis, has a route to be converted to the productive mode and the actual reaction is likely to occur by such a mechanism. At relatively low pH the second route becomes important.

Then the problem is the molecular description of the EHS′ intermediate. Regarding several discussions so far given on the mechanism of zinc-containing proteases, at least three possibilities are to be considered;

1) conversion of the noncovalently bound substrate-enzyme complex to an intermediate covalently attached to the enzyme (such as acid anhydride acylenzyme),[15-17] 2) formation of the tetrahedral intermediate on the carbonyl carbon of the scissile bond, and 3) a conformational transition of the enzyme

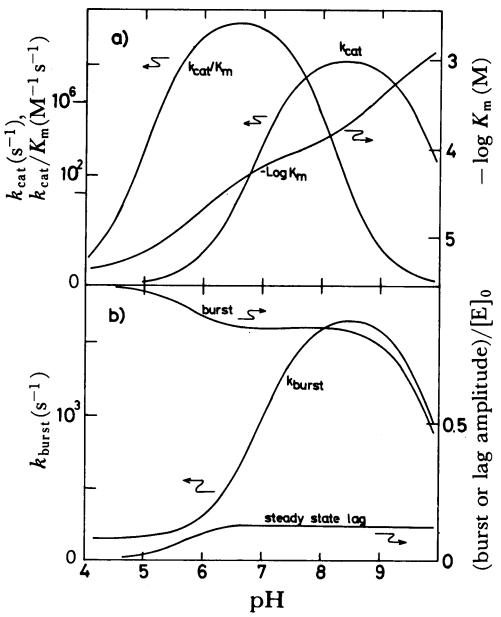


Fig. 3. Simulation of the pH dependences of several quantities at saturated substrate level calculated on the basis of the reaction Scheme 6 with following parameters, which are considered most plausible for the hydrolysis of FuaGlyPheAla at 25 °C.
$pK_1=5$, $pK_2=8.2$, $pK_3=7$, $pK_4=10$, $pK_5=6.8$, $pK_6=10$, $K_s=0.001$ M, $k_2=1500\,s^{-1}$, $k_{2'}=150\,s^{-1}$, $k_3=250\,s^{-1}$. (a) k_{cat}/K_m, k_{cat}, and K_m (b) k_{burst}, burst and the steady state lag.
Burst amplitude and lag are normalized to $[E]_0$ value.

protein or 3') that of the substrate.

The positive absorbance change due to the burst process will discourage reduction of the process to a purely conformational change of the protein. A change in the absorbance of the Fua-modified substrate due to the formation of an acyl intermediate have been observed in the case of serine proteases such as α-chymotrypsin[18] and trypsin[19] and have provided fruitful analyses of the presteady state kinetics for these enzymes. In those instances the difference extinction coefficients($\Delta\varepsilon$) of the acyl intermediate against the free substrate were around 3000—3500($M^{-1}cm^{-1}$) at 330nm and ≃3000 ($M^{-1}cm^{-1}$) at 335nm.[14] When we apply the reaction scheme 6 or 7 to the presently observed burst amplitude, the expected $\Delta\varepsilon_{335}$ values is calculated as only around 1000 ($M^{-1}cm^{-1}$) at saturated substrate concentration (see Appendix). Furthermore the k_{burst} expected at very large substrate concentration would be around $200\,s^{-1}$ at pH 6.75 and 7.5 °C and $>100\,s^{-1}$ at pH 5.15 and 25 °C. That at optimum conditions would be near $1000\,s^{-1}$, which is considerably large for an acyl transfer step from a peptide substrate. Even the rate constant for the acyl transfer from the ester to Glu270 of carboxypeptidase A under similar conditions[16] was estimated in the order of $10^1\,s^{-1}$ and the highest $k_{acylation}$ value observed for the acylation of Ser-195 of α-chymotrypsin[13] by normal alkyl ester of amino acid was no more than $10^3\,s^{-1}$.

On the basis of the temperature dependence of the pK_a values controlling the second-order rate constant of thermolysin catalysis and several experimental data given by other investigators,[10,20] we proposed the general-base mechanism including the zinc-coordinated water molecule for this enzyme. Recent X-ray studies on inhibitor or interaction with thermolysin also ruled out the anhydride mechanism.[21] Considering all these, the observed burst process is to be related to the possibility 2) or 3'). As for the hydrolysis of Fua-TrpNH_2 by α-chymotrypsin, Hess's group observed some presteady state process by using a temperature jump and stopped flow apparatus.[22] The k_{burst} was $31\,s^{-1}$ at pH 6.7 and 15 °C and the authors speculated that the observed intermediate is the tetrahedral intermdiate accumulated during the acylation reaction, since the acylation step is the rate limiting in the overall hydrolytic reaction of amide substrate catalyzed by this enzyme. Also in the cases of thermolysin and carboxypeptidase A, the general-base reaction can proceed *via* such an intermediate and for the specific substrates the accumulated amount of the intermediate could be significant.

On the other hand, Lipscomb[23] and Clealand[13] have postulated that in the carboxypeptidase A catalyzed hydrolysis of peptide the entry of the C-terminal residue into its subsite which places the carboxylato group on Arg145 and causes a strain in the peptide bond of the substrate, is rate-limiting. Though there is no evidently working electrostatic interaction in the case of thermolysin and the substrate binding force is mainly hydrophobic, the reactivity of the enzyme-bound substrate may also be raised by forcing the peptide bond to have a strained structure and to increase proton-acceptability of the imino group;[24] geometric destabilization, just we have seen in the α-chymotrypsin-catalyzed hydrolysis of activated peptide-model compounds.[25] In the case of thermolysin the destablization process might not be so slow to limit the overall reaction but it might limit the presteady process. The absorbance of the bound substrate could be altered with breakage of the resonance structure along the peptide bond to provide an observable burst process.

Though we cannot conclusively discriminate the two possibilities[26] given above, an isomerization process of the enzyme-bound substrate was observed as a presteady state process in the hydrolysis of tripeptide substrate and especially at lower pH, the conversion from the nonproductively (inversely) bound substrate to a productive one might be significant to this burst process.

Appendix

When we assume the reaction mechanism of Scheme 6, the time dependences of $EHS'+EH_2S'$ and P are given as follows;

$$[EHS'+EH_2S'] = (C_1/C_2)(1-\exp(-C_2t))(1+[H^+]/K_6), \quad (1)$$

$$[P] = k_3(C_1/C_2)t + k_3(C_1/C_2^2)(\exp(-C_2t)-1), \quad (2)$$

where

$$C_1 = (k_2+k_2'[H^+]/K_4)[E]_o/(A(1+[H^+]/K_6))$$

$$C_2 = (k_2+k_2'[H^+]/K_4)(1+[H^+]/K_6+K_5/[H^+])/(A(1+[H^+]/K_6))+k_3/(1+[H^+]/K_6)$$

$$A = 1+[H^+]/K_4+K_3/[H^+]+(1+[H^+]/K_2+K_1/[H^+])K_6/[S]_o.$$

The first term of Eq. 2 corresponds to the steady state change and the second term is the burst process. Assuming that the amount of ES is very small and stationary, the observed change in optical density is written

$$\Delta OD(t) = \Delta\varepsilon_{EHS'}[EHS'] + \Delta\varepsilon_{EH_2S}[EH_2S'] + \Delta\varepsilon_P[P]. \quad (3)$$

If the extinction coefficients of EHS' and EH_2S' can be considered equal($\Delta\varepsilon_x$), then Eq. 3 is reduced to

$$\Delta OD(t) = \Delta\varepsilon_x[EHS'](1+[H^+]/K_6) + \Delta\varepsilon_P[P]. \quad (4)$$

The change in the optical density as given in Fig. 2 means $\Delta\varepsilon_x>0$ and $\Delta\varepsilon_P<0$. $\Delta\varepsilon_P$ at 335 nm was evaluated as −1540 at 25 °C pH 5.35. The burst amplitude(ΔOD_{burst}), which is obtained experimentally by an extrapolation of the steady-state part to time zero, is derived from Eqs. 1, 2, and 4 as

$$\Delta OD_{burst} = \Delta\varepsilon_x(1+[H^+]/K_6)C_1/C_2 - \Delta\varepsilon_P k_3 C_1/C_2^2, \quad (5)$$

where the first term is from the formation of the intermediate(s) and the second term is due to the lag period of the product formation. According to Scheme 6, the amount of the intermediates formed at saturated substrate concentration becomes 90% of the total enzyme at acidic to neutral pH (Fig. 3) and therefore the fact that the burst amplitude(in O.D.) observed at saturated substrate concentration was the order of 0.01 at the present enzyme concentration means that the difference extinction coefficient of the intermediate is around 1000($M^{-1}cm^{-1}$).

We gratefully acknowledge Professor Norio Ise, Kyoto University, for his advice and encouragement throughout the work.

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26) In either case of tetrahedral intermediate or strained peptide bond, an apparent difficulty of the mechanism of Scheme 6 or scheme 7 is in the observed similarity in the pH dependence and the magnitude of K_i of the several inhibitors with those of K_m of the substrate. However, in many substrate-analogues, inhibition of the hydrolysis of the chromophoric substrate is as the result of the very slow reaction of the nonchromophoric substrate (considered as inhibitor) and therefore the evaluation of the K_i values always include the contribution from the (small fraction of) further reacting intermediate such as EHS'. Furthermore the significant contribution of inversely bound substrate is limited at lower pH. Therefore the discussion on the binding step of this enzyme in the previous paper[9] based on the pressure dependence of K_m is not influenced by the present finding of the presteady state process.