Observation of the pre-steady state process in thermolysin catalysis with a fluorescent displacement probe at low pH

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Abstract The pre-steady state process in the thermolysin-catalyzed hydrolysis of Cbz-Gly-Phe-Ala was observed at pH 4.5 by fluorescence stopped-flow method using Dns-Phe as a displacement probe. After the confirmation of the pre-equilibrium hypothesis for the binary interaction, the nonlinear substrate concentration dependence of the apparent kinetic constant for the pre-steady state process was analyzed and an existence of multi-intermediates was proposed.

Key words: Thermolysin; Pre-steady state kinetics; Stopped-flow method; Fluorescent inhibitor; Displacement method

1. Introduction

Many kinetic and structural studies have been made on thermolysin (E.C.3.4.24.4) [1–11], a thermostable microbial neutral protease containing zinc as a cofactor, but several questions on its reaction mechanism still remain unsolved [12]. As for the pre-steady state kinetics of this enzyme, a limited number of reports have been published and the results were not consistent. Some pioneering observations of pre-steady state processes for fluorescent depsipeptide and peptide substrates were reported [13,14], but they were considered as the binding process of the substrate [13]. Later, we reported that *N*-acyl-tripeptide substrates such as Fua-Gly-Phe-Ala showed some burst process at pH lower than 6 [10] and the analysis of the kinetic profiles implicated the existence of a new intermediate. The existence of multi-intermediates was also suggested by Izquierdo and Stein [15] through the measurement of the temperature dependence and the isotope effects of the steady-state rate.

One important reason for the insufficiency of the pre-steady state kinetic studies of thermolysin is in the lack of good reporting probes which can reversibly bind to the active site of the enzyme, as in the case of chymotrypsin-proflavin [16].

N-acyl amino acids are know to inhibit thermolysin [17] and a crystallographic structure of a binary complex of Cbz-Phe/thermolysin was reported [7]. We also found the interaction of *N*-acyl amino acids became very strong at lower pH [8]. Here we use such a competitive inhibitor as an displacement probe, namely Dns-Phe, in a stopped-flow observation of pre-steady state process of thermolysin reaction, since the energy transfer between the intrinsic tryptophane of the enzyme and the dansyl group of the probe can provide distinct fluorescence signal of close proximity of the two groups [15,12].

2. Experimental

Thermolysin was obtained from Daiwa Kasei (Osaka, Japan; lot T4DB191). Dns-Phe, and Cbz-Gly-Phe-Ala were purchased from Tokyo Kasei (Tokyo, Japan) and the Peptide Institute (Minoo, Japan), respectively.

The stopped-flow apparatus was made by Ohtsuka Electronics (Type Ra-401), which has a gas (N_2)-driven flow cell of a front-stop type and the dead-time was determined to be around 3 ms under usual conditions. Temperature-jump apparatus of Joule-heat type was also constructed by Ohtsuka Electronics Co. (Type RA-410). The cell volume is around 2 cm^3 and the capacitance of the coaxial cable is 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) were determined as 5 μs and 4.5°C, respectively. Static interactions of the fluorescent inhibitor with the enzyme were monitored by Hitachi (Tokyo, Japan) -850 or Shimadzu (Kyoto, Japan) -RF5000 fluorophotometer. Steady state kinetics and other spectrophotometric measurements were done on an ultraviolet/visible spectrophotometer SM401 (Union Giken; Hirakata, Japan) or UV-2200 (Shimadzu). Analysis of peptide hydrolysis by HPLC was done with Shimadzu LC10A-Cosmosil 5C-18P as described [18].

3. Results and discussion

When a solution of thermolysin (A) containing Dns-Phe was mixed with a solution of Cbz-Gly-Phe-Ala (B), containing Dns-Phe at the same concentration as in A, by a stopped-flow apparatus, the fluorescence intensity (λ_{ex} = 280 nm and λ_{em} = 550 nm) showed a time-dependent change as shown in Fig. 1. The whole trace was divided into three portions; (i) the first very rapid decrease in the fluorescence, (ii) the following almost flat level, and then (iii) the slow uprise of the signal. These are corresponding to (i) the pre-steady state process, (ii) the duration of the steady-state, and (iii) the breakdown of the steady-state leading to the complete hydrolysis of the substrate tripeptide, respectively. An independent analysis of the peptide hydrolysis by HPLC supported the interpretation of the latter two processes (Fig. 2).

The first pre-steady state portion was analyzed by a curve fitting method to give a single relaxation time constant ($\tau = k_{obs}^{-1}$). The concentration dependence of k_{obs} is shown in Fig. 3. Static measurements showed that Dns-Phe is bound to the enzyme stoichiometrically and it inhibited the enzyme reaction in a competitive manner. As described in detail by Brandt et al. [16], the displacement method is premising a rapid pre-equilibrium of the interaction between the reporting competitive inhibitor and the enzyme. A temperature-jump observation of this this binary system gave a relaxation curve such as shown

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Abbreviations: MES, 2-morpholinoethanesulfonic acid; Dns- or dansyl-, 5-(dimethylamino)naphthalenyl-1-sulfonyl-; mansyl-, 6-(*N*-methylanilino)-2-naphthalenyl-1-sulfonyl-; Fua-, 3-(2-furyl)-acryloyl-; DMSO, dimethyl sulfoxide.

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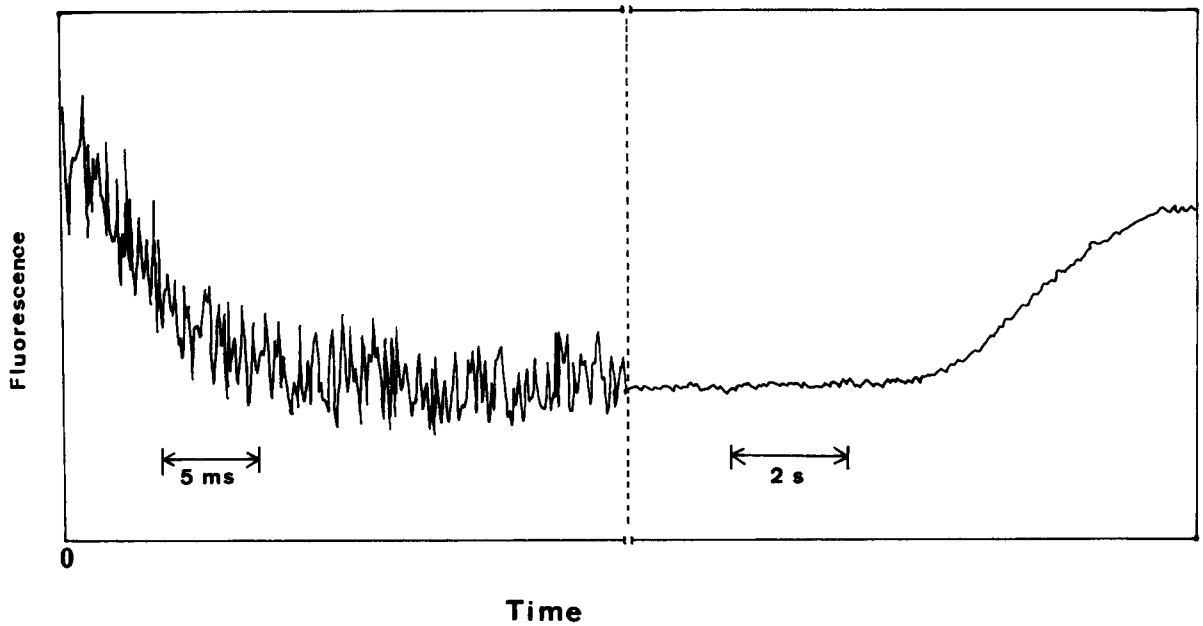

Fig. 1. A typical fluorescence stopped-flow trace of the reaction of thermolysin with Cbz-Gly-Phe-Ala in the presence of Dns-Phe at pH 4.5 (0.1 M MES/NaOH), 25°C, 0.1 M KBr, and 0.01 M $CaCl_2$. Excitation at 280 nm and observed at 550 nm. $[E]_o$ = 10.0 μM, $[S]_o$ = 0.25 mM, $[Dns\text{-}Phe]_o$ = 50 μM, 2.5% DMSO. Time scale is altered after the break.

in Fig. 4 and the relaxation time was found to be less than 1 ms under similar experimental conditions. (Detailed discussions on the thermodynamics and the kinetics of the binary interaction will be given elsewhere.) In the stopped-flow study, the concentrations of three species were set as $[S]_o \gg [E]_o < [D]_o$.

Unlike the case of Morgan and Fruton [14], who measured the reaction of thermolysin with mansylated tripeptides, Fig. 3 shows a clearly non-linear dependence with respect to the substrate concentration, which implicates an occurrence of an additional substrate–enzyme complex. When the hydrolysis of Cbz-Gly-Phe-Ala by thermolysin involves an isomerization process between the substrate binding and the catalytic (chemical) processes as in Eq. (1) and when the observed process is mostly reflecting this iso-merization, then the obtained pre-steady state relaxation time will be described as Eq. (2).

$$E + S \underset{K_s}{\rightleftharpoons} E \cdot S \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P \quad (1)$$

$$k_{obs} = \tau^{-1} = k_2[S]_o / \{[S]_o + K_s(1 + [D]_o/K_d)\} + k_3 \quad (2)$$

The data in Fig. 3 was analyzed by an iterative method with three variables of k_2, K_s, and k_3, by using K_d and k_{cat} (= $k_2k_3/(k_2 + k_3)$) value determined independently by a fluorophotometric and spectrophotometric measurement under corre-

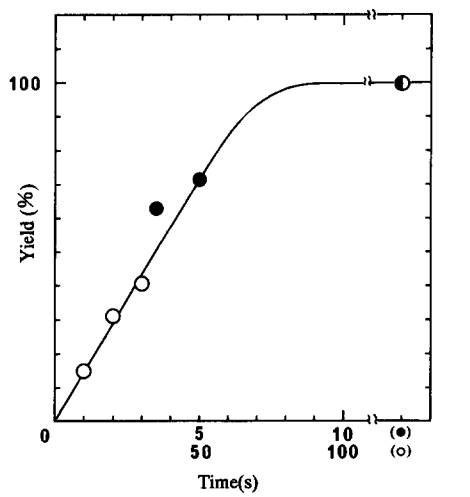

Fig. 2. HPLC observation of the product formation in thermolysin-catalyzed hydrolysis of Cbz-Gly-Phe-Ala.$[E]_o$ = 10.0 μM (●) and 1.0 μM (○). Note that the abscissa scale is changed for the lower enzyme concentration. Other conditions are as in Fig. 1. Solid curve is corresponding to the integration of the stopped-flow trace (area surrounded by the reaction curve and the extrapolated line from the signal after long time) in Fig. 1.

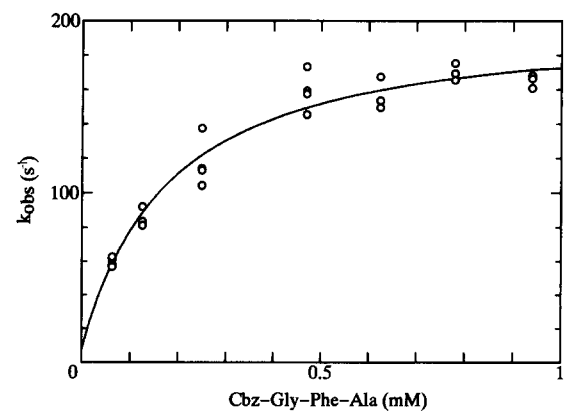

Fig. 3. Variation of the reciprocal of the observed rate constant ($k_{obs} = \tau^{-1}$) with the concentration of Cbz-Gly-Phe-Ala.Conditions are as in Fig. 1, except for $[S]_o$. The curve is drawn on the basis of eq. (2), with the parameters of K_d = 28.5 μM, K_s = 64 μM, k_2 = 195 s^{-1}, and k_3 = 7.7 s^{-1}.

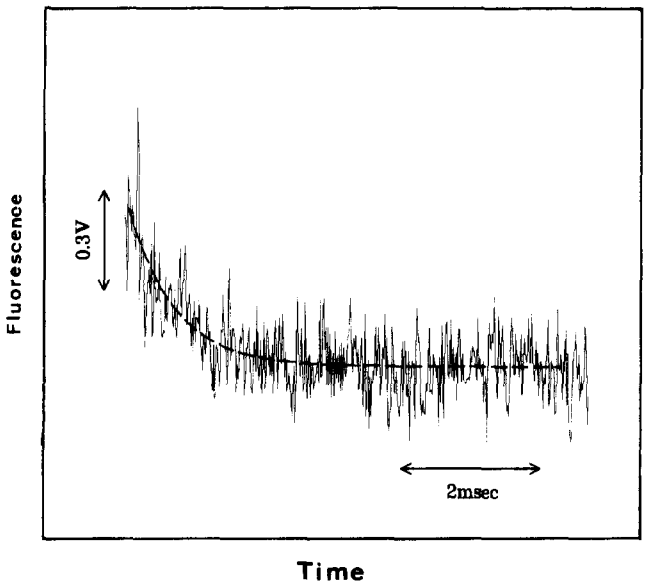

Fig. 4. A typical temperature-jump trace for the Dns-Phe/thermolysin interaction. pH 4.54, 25°C, 0.1 M KBr, 0.01 M $CaCl_2$, 2.5% DMSO. Excited at 280 nm and detected at 550 nm. $[TLN]_o$ = 10 μM, $[Dns\text{-}Phe]_o$ = 50 μM.The dotted curve is based on the non-linear regression of the obtained data assuming a single relaxation time, 0.71 ± 0.04s^{-1}.

sponding conditions (K_d = 28.5 ± 2.2 μM and k_{cat} = 7.4 ± 0.2 s^{-1}, at pH 4.5, 25°C). The evaluated values are; K_s = 64 ± 4 μM, k_2 = 195 ± 4 s^{-1}, and k_3 = 7.7 ± 0.4 s^{-1}.

Thus, at least in lower pH, thermolysin catalysis seems to involve multiple intermediates. Though a further study on the pH or temperature dependences of the parameters are necessary for an sufficient understanding of the characteristics of the intermediate and the process, which is in progress in our laboratory, the k_2 process will be explained by a mechanism that we proposed for the hydrolysis of Fua-Gly-Phe-Ala before [10], since the k_{obs} values measured here are comparable to those obtained for the latter substrate (ca. 100 s^{-1}).

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