J. Biol. Macromol. **8**(2), 38-47 (2008)

Article

Kinetic study on the dissociation of a dimeric protein, *Streptomyces* Subtilisin Inhibitor

Keiko Momma[1,2], Ben'ichiro Tonomura, and Keitaro Hiromi

Department of Food Science and Technology, Faculty of Agriculture,

Kyoto University, Kyoto 606-8502

Received August 31, 2007 ; Accepted March 18, 2008

Summary: There have been few kinetic studies on the dissociation of stable dimer proteins of which monomer subunit is not detectable under normal condition. Proteinaceous proteinase inhibitor, *Streptomyces* subtilisin inhibitor (SSI) exists as a stable homodimer. Modified SSI (SSI*) in which the reactive site is cleaved has also inhibitory activity and separated from SSI on native PAGE. When SSI and SSI* were mixed, a heterodimer was observed. By following the formation of the heterodimer, the dissociation rate constant of dimer into monomers was determined to be 3.2 x 10^{-3} s^{-1} at pH 7.0, 25°C. The activation energy for the dissociation was calculated to be 23.8 kcal/mol. The dissociation of SSI dimer was also observed for subtilisin BPN'-SSI complex. The rate constants and activation parameters for the dimer dissociation of the complex were similar to those of SSI dimer. These results indicated that subtilisin BPN' bound SSI resembled free SSI in the mechanism of dimer dissociation.

Keywords: dimer dissociation, *Streptomyces* subtilisin inhibitor, rate constant

There are many stable dimer proteins consisting of non-covalently bound subunits. However, there have been rather few kinetic studies on the dissociation of those stable dimer proteins. Koren and Hammes [1] reported the rate constants of dimer formation and dissociation of insulin, β-lactoglobulin, and α-chymotrypsin by monitoring the relaxation process of dimer-monomer equilibrium changed by temperature jump or dilution. But it is difficult to obtain the rate constants under the condition in which monomer subunit is not detectable in an equilibrium state.

Streptomyces subtilisin inhibitor (SSI, I, or I_2) is a proteinaceous proteinase inhibitor produced by *Streptomyces albogriseolus* S-3253 and strongly inhibits bacterial alkaline serine proteinases such as subtilisin BPN' (S-BPN', E) [2 - 4]. SSI exists in dimeric form (MW 23000) under the mild condition in the concentration range of 0.01 - 10 mg/ml (0.87

---

[1]Present address: Department of Social Design Studies, Faculty of Human Studies, Kyoto Bunkyo University, Uji, Kyoto, 611-0041, Japan

[2]Corresponding author

μM – 870 μM as a monomer) [5] and β-sheets of two subunits face each other to make a core of globular dimer SSI [6]. The monomer form of SSI has been detected only in unfolded conditions; e.g., caused by the addition of sodium dodecyl sulfate [7], heating [8], or acidification [9].

One dimeric SSI binds and inhibits two molecules of S-BPN' forming a tetrameric complex, E_2I_2, that has been shown by X-ray crystallography [10], fluorometric titration [11], inhibition [12], and sedimentation [5]. Monomer S-BPN'-SSI complex has not been detected in any conditions.

Modified SSI (SSI*, I*, or I^*_2) in which the reactive site peptide bond, Met73-Val74, was cleaved enzymatically also has strong inhibitory activity against S-BPN'. SSI* is separated from SSI on native PAGE. When I_2 and I^*_2 were mixed, a hybrid species, II*, was clearly detected on native PAGE. This provides us a good probe for studying the dissociation of SSI dimer. In this study, we report the dissociation rate of SSI dimer. Further we examined the dissociation of SSI dimer bound with S-BPN'.

MATERIALS AND METHODS

Proteins - S-BPN' (EC 3. 4. 21. 14, lot No. 730953) was purchased from Nagase Sangyo Co. Ltd. The protein concentration was determined spectrophotometrically from the absorbance at pH 7.0 by using A_{278} (1 mg/ml) = 1.063 and its molecular weight 27500 [13]. The concentration of active enzyme was determined to be 75% as described previously [12].

SSI was prepared as described previously [14]. SSI* was prepared as follows [4]: S-BPN'-SSI complex was isolated by gel filtration at pH 7.0 and subjected to pH jump to pH 2.7 at 0 ^oC. After 2 hours incubation at 0 ^oC, the pH of the solution was neutralized. SSI* was isolated by QAE-Toyopearl (Tosoh) column chromatography at pH 9.4. The protein concentration was determined spectrophotometrically from the absorbance at pH 7.0 by using A_{276} (1 mg/ml) = 0.829 and the molecular weight of 11500 (monomer) [12].

Determination of the dissociation rate constants - I_2 and I^*_2 solutions (13 μM each as monomer) equal in volume were mixed and incubated at pH 7.0 and at various temperatures. After appropriate periods, the sample was put on iced water, then quickly loaded on native PAGE [15] at pH 9.4 and 0 ^oC. Proteins were stained with Coomassie Brilliant Blue R-250 and the density of the bands were measured at 590 nm with a Shimadzu dual-wavelength TLC scanner CS-900. A normalized value of [II*] (%) (= [II*] x 100/ ($[I_2]$ + $[I^*_2]$ + [II*])) was analyzed as a first-order reaction to determine the dissociation rate constant.

As for the dissociation rate constant of SSI dimer of EI complex, I_2 and E_2I_2 solutions (7 μM each as monomer) equal in volume were mixed and the time course of the amount of EI_2 was analyzed as a first order reaction.

Determination of activation parameters - The free energy of activation for the dissociation of SSI dimer was calculated from the dissociation rate constant

$$\Delta G^{\ddagger} = 2.303RT\{\log(k_BT/h) - \log k_{-1}\} \qquad (1)$$

where k_B is Boltzmann's constant and h, Plank's constant.

$$\Delta H^{\ddagger} = E_A - RT \qquad (2)$$

$$\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T \qquad (3)$$

RESULTS AND DISCUSSION

Assignment of II* an hybrid dimer of SSI and SSI* on native PAGE - Equal volume of I_2 and I^*_2 solutions (12 μM each as monomer) were mixed and incubated at pH 7.0 and room temperature for 1 h. The mixture was loaded on native PAGE at pH 9.4 and gave three bands (Fig. 1, lane 3). The top and the bottom bands are I_2 and I^*_2, respectively, but the central band appeared newly by the mixing. Protein of the central band was extracted from the gel, and subjected to native PAGE again. That yielded similar three bands (Fig. 1, lane 4). The proteins of these three bands in lane 4 were extracted separately from the gel, and they were loaded separately to the native PAGE; the top band gave I_2 (lane 5), the central band gave three bands (lane 6), and the bottom band gave I^*_2 (lane 7). These results indicated that the central band was the hybrid species of SSI and SSI*, namely II*, and that respective subunits are exchanged under the ordinary conditions at pH 7.0 and room temperature within 1 h.

Dissociation rate constant of SSI dimer - We tried to determine the dissociation rate constant of SSI dimer by following the II* formation. The equilibria between monomer and dimer are shown as follows:

$$\underset{k_1}{\overset{I_2}{\uparrow\downarrow}}\ \underset{}{k_{-1}} \quad 2I \quad + \quad \underset{k_1'}{\overset{I^*_2}{\uparrow\downarrow}}\ \underset{}{k_{-1}'} \quad 2I^* \quad \underset{k_{-1}''}{\overset{k_1''}{\rightleftarrows}} \quad 2II^* \qquad (4)$$

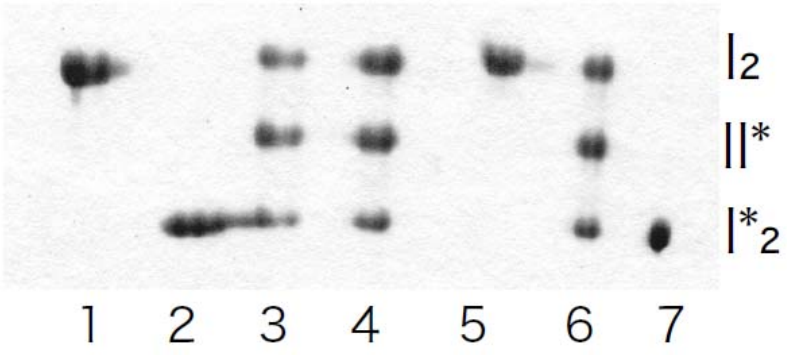

Fig. 1. Native PAGE profile of I_2, II*, and I^*_2 at pH 9.4.
lane 1, I_2; lane 2, I^*_2; lane 3 mixture of I_2 and I^*_2; lane 4, proteins extracted from the central band of lane 3; lane 5, proteins extracted from the top band of lane 4; lane 6, proteins extracted from the central band of lane 4; lane 7, proteins extracted from the bottom band of lane 4.

where I_2, I^*_2, I, and I* indicate SSI dimer, SSI* dimer, SSI monomer, and SSI* monomer, respectively; k_1, k_1', and k_1" are the association rate constants and k_{-1}, k_{-1}', and k_{-1}" are the dissociation rate constants at the respective steps indicated. The velocity of change of the three species can be expressed in the linear differential equations:

$$d[I_2]/dt = k_1[I]^2 - k_{-1}[I_2],$$
$$d[I^*_2]/dt = k_1'[I^*]^2 - k_{-1}'[I^*_2], \quad (5)$$
$$d[II^*]/dt = k_1''[I][I^*] - k_{-1}''[II^*].$$

$[I_2]_0 = [I_2] + [I] + [II^*]/2$, $[I^*_2]_0 = [I^*_2] + [I^*] + [II^*]/2$, where $[I_2]_0$ and $[I^*_2]_0$ represent the initial concentration of those species. Since the equilibria shown in Eq. 4 are extremely in favor for the dimeric species under the conditions of present experiments, the concentrations of monomers, [I] and [I*], can be regarded negligibly small compared with those of dimeric species. Accordingly, the amount of $[I_2] + [I^*_2] + [II^*] \fallingdotseq [I_2]_0 + [I^*_2]_0$. The rate constants for I_2, I^*_2, and II* are assumed to be same, $k_1 = k_1' = k_1''$ and $k_{-1} = k_{-1}' = k_{-1}''$, because there is no significant structural change at the dimer interface between I_2 and I^*_2 [16] and of overall structure [4, 17] between I_2 and I^*_2, and I^*_2 shows strong enzyme inhibitory activity similar to I_2 [4]. Solution of the above equations under the initial condition, $[I_2]_0 = [I^*_2]_0$, yields,

$$[II^*] = [I_2]_0(1-\exp(-k_{-1}t)) \quad (6)$$

Figure 2a shows the time dependence of the formation of II*. Figure 2b shows the time course of the normalized value of [II*], which was fitted to Eq. 6 by non-linear least squares method. The value of the dissociation rate constant k_{-1} was determined to be 1.6×10^{-4} s^{-1} at

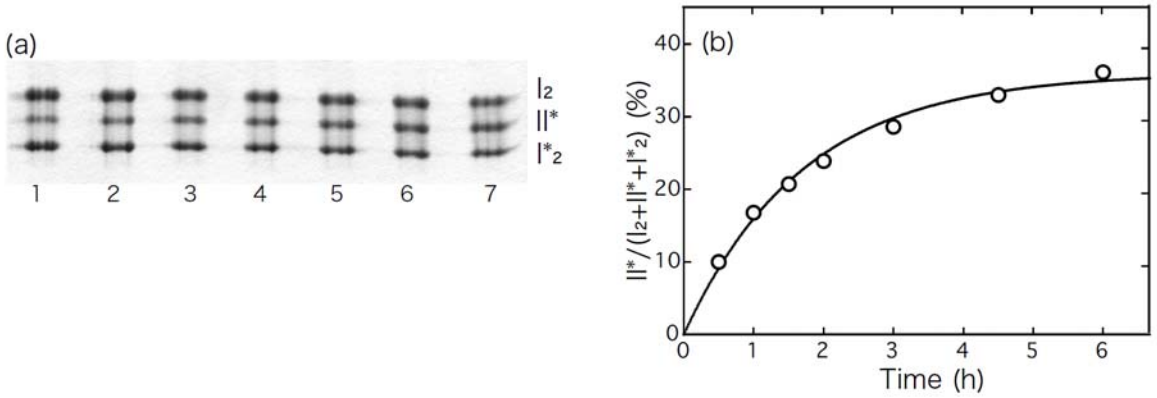

Fig. 2. Time course of II* formation with mixing of I_2 and I^*_2.
(a) Native PAGE profile at pH 9.4. The mixture of I_2 and I^*_2 (13 μM each) were incubated at 4.3 °C and pH 7.0. lanes 1 – 7, the incubation times were 0.5, 1.0, 1.5, 2.0, 3.0, 4.5, and 6.0 hours, respectively. 4.2 μg protein/lane was loaded. (b) Time course of ratio of the II* to total inhibitor density. The solid line is the theoretical curve drawn based on Eq. 6 with $k_{-1} = 1.6 \times 10^{-4}$ s^{-1}.

Table 1. Effect of temperature on the dissociation rate constant of SSI dimer at pH 7.0.

Temperature (°C)	k_{-1} (s^{-1})	$t_{1/2}$ (min)
1	0.8×10^{-4}	139
4.3	1.6×10^{-4}	70.8
9.5	3.8×10^{-4}	30.7
14.1	6.2×10^{-4}	18.6
20.0	16.5×10^{-4}	7.0
25.0	31.5×10^{-4}	3.6

4.3 °C, pH 7.0, accordingly, the half-life time of this reaction was 71 min (Table 1).

The maximum ratio of [II*] to the total inhibitor concentration obtained in this experiments were 35 to 40% against the theoretical value of 50%, but the reason was not clear. The ratio of II* would be lower than 50% if one monomer protein (for example I) prefers to bind with same protein (I) than the other (I*), however it has no effects on dissociation. The experiment using SSID68N, a mutant SSI without nick, instead of I* gave the similar dissociation rate constant and the final hybrid species ratio (data not shown). So the final ratio would not be the results of the system using of SSI*.

Akasaka et al. reported that the dissociation rate constant of I_2 was 1.6×10^{-6} s^{-1} (half life time c.a. 120 h) at pH 8.7, 4 °C by using chemically modified SSI (nitorotyrosyl -SSI) with HPLC (ion exchange column) [18]. The reason of the large difference between the report and this study is not specified. The differences of pH, protein (chemically modified or enzymatically modified), and the method for detection might be the cause.

The association rate constant of SSI monomer to SSI dimer ($2I \rightarrow I_2$) has not been obtained experimentally. It may be estimated by assuming a diffusion-controlled association. The encounter rate constant for this size of proteins can be assumed as 6×10^9 $M^{-1}s^{-1}$ [19]. The steric factor which determines the probability of occurrence of specific association among random encounter of two subunits is assumed to be 10^{-2}, based on the consideration that about 10% of whole the surface of the monomer is covered by the dimer formation [20]. Thus, the rate constant for the diffusion-controlled association of SSI monomer would be 6×10^7 $M^{-1}s^{-1}$. Accordingly, the dissociation equilibrium constant of the dimeric SSI, $K_d = k_{-1}/k_1$, is calculated to be 5×10^{-11} M at 25°C.

The dissociation rate constants were determined at 1 – 25 °C (Table 1). The activation energy, E_A, for the dissociation process of I_2 to 2I was obtained to be 23.8 kcal/mol from the Arrhenius plot (Fig. 6). $\Delta G^{\ddagger}$ was 20.9 kcal/mol, $\Delta H^{\ddagger}$ was 23.2 kcal/mol, and $\Delta S^{\ddagger}$ was 7.7 kcal/(mol·K).

Assignment of enzyme-inhibitor complexes, EI_2 and EII* - S-BPN' and an excess amount of I_2 were mixed at pH 7.0, 25 °C, and applied to native PAGE. Three bands, E_2I_2, X, and I_2 from the top were observed (Fig. 3, lanes 1 - 5). E_2I_2 indicates the enzyme inhibitor complex of SSI dimer and two molecules of S-BPN'. Protein of X extracted from the gel was loaded to native PAGE and gave the bands corresponding to E_2I_2, X, and I_2 again (data not shown). So the band X was presumed to be EI_2 in which one enzyme molecule binds

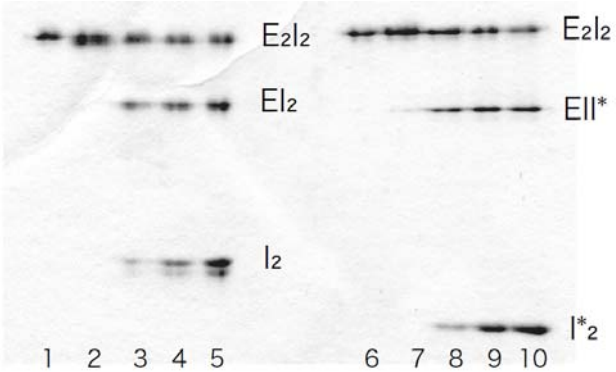

Fig. 3. Native PAGE profile of E_2I_2, EI_2, EII*, I_2, and I^*_2 at pH 9.4.
S-BPN' (7.5 μM) was mixed with I_2 or I^*_2 at various molar ratio and incubated for 10 min at room temperature; lanes 1 – 5 for I_2, lanes 6 –10 for I^*_2. The molar ratio ([I]/[E] or [I*]/[E]) is 0.5 (lanes 1 and 6), 1.0 (lanes 2 and 7), 2.0 (lanes 3 and 8), 3.0 (lanes 4 and 9), 4.0 (lanes 5 and 10). The sub-bands of I_2 in lanes 3 – 5 were observed because of the heterogeneity of the N-terminal. The N-terminus amino acid of I^*_2 and the top band of I_2 is Tyr7.

with one SSI dimer. When S-BPN' and an excess amount of I^*_2 were mixed, three bands, E_2I_2, Y, and I^*_2 were observed (Fig. 3, lanes 6 - 10). The band Y migrated a little further than EI_2, which was assumed to be EII*. The interaction between S-BPN' and SSI is thought to follow the standard mechanism of serine proteinase and proteinaseous inhibitor [21] proposed as follows: .

$$E + I \rightleftarrows EI \rightleftarrows EI^* \rightleftarrows E + I^* \qquad (7)$$

The stable complex is generally in the form of Michaelis complex, EI. S-BPN'-SSI complex was also revealed in the form of Michaelis complex by X-ray crystallography [10]. NMR study revealed that the reactive site peptide bond of SSI* was resynthesized within a measuring time (about 3 hours) [16], and we have observed that the resynthesis occurs within 15 s at room temperature (Momma et al., unpublished result). Thus, E_2I_2 and EII* would be observed instead of $E_2I^*_2$ and EI^*_2, respectively.

When E_2I_2 complex was mixed with I^*_2, EII* was observed (Fig. 4a, lane 2). Let us consider the case of mixing E_2I_2 and I^*_2. If the dissociation occurs at the enzyme-inhibitor interface, EI_2 and EII* should be observed (Fig. 4b). However, only EII* was observed, that means dissociation occurred at the SSI dimer interface (Fig. 4c) in this time scale.

Dissociation rate constant of SSI dimer in enzyme-inhibitor complex - Then we tried to determine the dissociation rate constant of SSI dimer in the enzyme-inhibitor complex by analyzing the EI_2 formation with mixing of E_2I_2 complex and I_2. The equilibria are shown as follows:

$$\underset{k_1 \uparrow\downarrow k_{-1}}{\overset{I_2}{2I}} + \underset{k_1' \uparrow\downarrow k_{-1}'}{\overset{E_2I_2}{2EI}} \underset{k_{-1}''}{\overset{k_1''}{\rightleftarrows}} 2EI_2 \qquad (8)$$

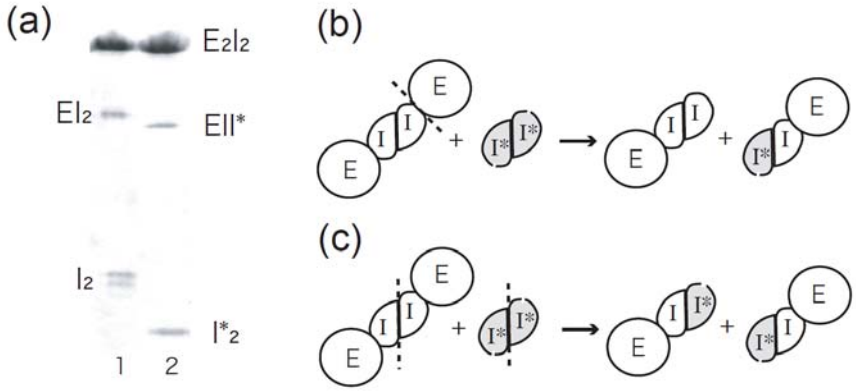

Fig. 4. The EII* formation by mixing of E_2I_2 and I^*_2.
(a) Equal volume of S-BPN'-SSI complex was mixed with I_2 or I^*_2 (7.5 μM each as monomer) and incubated for 10 min at room temperature. lane 1, $E_2I_2 + I_2$; lane 2, $E_2I_2 + I^*_2$, The sub-bands of I_2 were observed because of the heterogeneity of the N-terminal. (b) Model of the case if the dissociation occurs at the enzyme-inhibitor interface. When S-BPN' binds to I*, EII* is observed instead of EI^*_2 because of the reverse reaction of S-BPN'. (c) Model of the case if dissociation occurs at the SSI dimer interface.

By the similar calculation to the equilibria for I_2 and I^*_2, Eq. 9 was obtained under the initial condition, $[I_2]_0 = [E_2I_2]_0$,

$$[EI_2] = [I_2]_0(1-\exp(-k_{-1}t)) \qquad (9)$$

Figure 5a shows the time course of the formation of EI_2 by mixing of equal volume of E_2I_2 and I_2 solutions (7 μM each as monomer) and the density of spots on native PAGE was monitored. The rate constant of the dissociation was determined to be 3.0×10^{-3} s^{-1} at 26 °C, pH 7.0 (Fig. 5b). The rate constants for other temperatures and for the system of $E_2I_2 + I^*_2$,

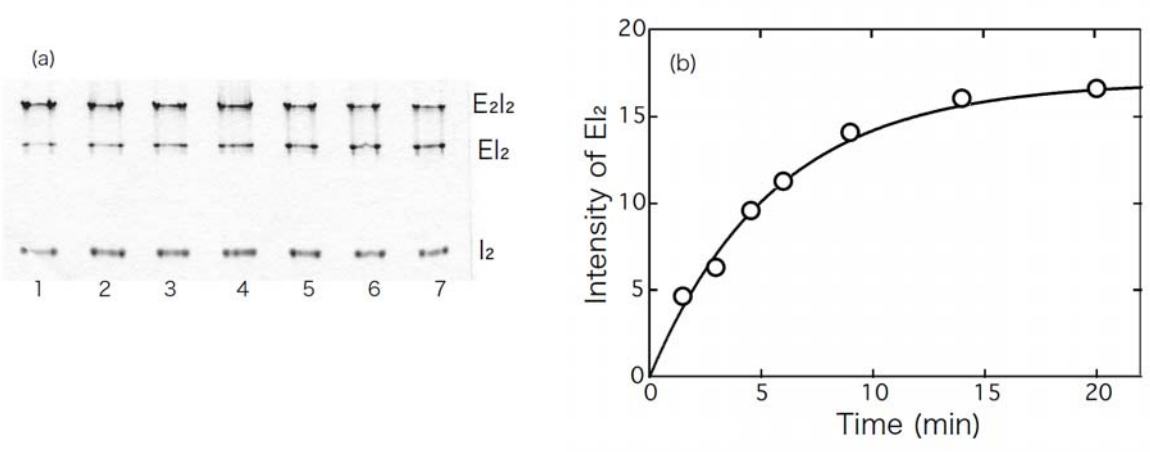

Fig. 5. Time course of EI_2 formation after mixing of E_2I_2 and I_2.
(a) Native PAGE at pH 9.4. The mixture of E_2I_2 and I_2 (7 μM each) were incubated at 26 °C and pH 7.0. lanes 1 – 7, the incubation times were 1.5, 3, 4.5, 6, 9, 14, and 20 min, respectively. 9.5 μg protein/lane was loaded. (b) Time course of ratio of the EI_2. The solid line is the theoretical curve drawn based on Eq. 9 with $k_{-1} = 3.0 \times 10^{-3}$ s^{-1}.

Table 2. Effect of temperature on the dissociation rate constant of SSI dimer bound with enzyme at pH 7.0.

Temperature (°C)	$E_2I_2+I_2$ k_{-1} (s^{-1})	$E_2I_2+I^*_2$ k_{-1} (s^{-1})
5	1.3×10^{-4}	1.3×10^{-4}
10	3.5×10^{-4}	2.5×10^{-4}
15	5.6×10^{-4}	6.0×10^{-4}
20	17×10^{-4}	14×10^{-4}
26	30×10^{-4}	36×10^{-4}

detection of the formation of EII*, are listed on Table 2. Arrhenius plots of all the data are shown in Fig. 6. The dissociation rate constants were equal each other for the system of I_2 + I^*_2, E_2I_2 + I_2 and E_2I_2+I^*_2. It was reported that the β-sheets structure at dimeric interface of SSI has little change between I_2 and E_2I_2 by X-ray crystallographic study [10]. The bound enzymes would have no influence on the SSI dimer dissociation. After 6 h of incubation of the mixture of S-BPN' and SSI*, not only EII* but also EI_2 was detected (data not shown). The results indicated that the dissociation at the interface between SSI and S-BPN' also took place. The dissociation rate constant of SSI and S-BPN' was determined to be 0.9×10^{-4} s^{-1}, at pH 7.0, 25°C [14]. So we neglected the dissociation of SSI and S-BPN' in this study.

The subunit-subunit interaction of SSI dimer is dominantly hydrophobic and the entire SSI molecule as a dimer forms a single egg-shaped structure [6]. SSI monomer has been detected only in the denatured form, so it has been thought that the dimerization is necessary to exist as a stable protein. The actual structure of the free monomer SSI under physiological conditions has not been known. And the structure of monomer S-BPN'-SSI complex has not been observed. However the formation of EII* with mixing of E_2I_2 and I^*_2 supports the existence of monomer S-BPN'-SSI complex in a short time.

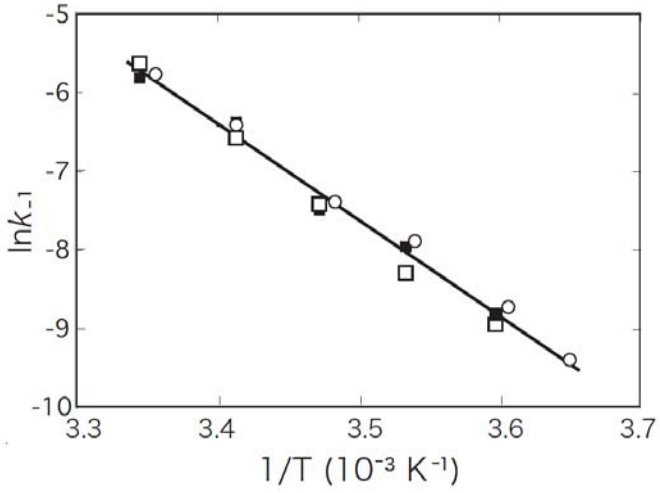

Fig. 6. Arrhenius plots of the dissociation rate constant of SSI dimer at pH 7.0.
○, I_2 + I^*_2; □,E_2I_2 + I_2; ■E_2I_2 + I^*_2.

REFERENCES

1. Koren, R. and Hammes, G. G. (1976) A kinetic study of protein-protein interactions. *Biochemistry* 15, 1165-1171.
2. Murao, S. and Sato, S. (1972) S-SI, a new alkaline protease inhibitor from *Streptomyces albogriseolus* S-3253. *Agric. Biol. Chem.* 36, 160-163.
3. Ikenaka, T., Odani, S., Sakai, M., Nabeshima, Y., Sato, S. and Murao, S. (1974) Amino acid sequence of an alkaline proteinase inhibitor (*Streptomyces* subtilisin inhibitor) from *Streptomyces albogriseolus* S-3253. *J Biochem (Tokyo)* 76, 1191-1209.
4. Hiromi, K., Akasa, K., Mitsui, Y., Tonomura, B. and Murao, S. (1985) Protein Protease Inhibitor – The Case of *Streptomyces* Subtilisin Inhibitor (SSI), Elsevier, Amsterdam.
5. Inouye, K., Tonomura, B., Hiromi, K., Kotaka, T., Inagaki, H., Sato, S. and Murao, S. (1978) The determination of molecular weights of *Streptomyces* subtilisin inhibitor and the complex of *Streptomyces* subtilisin inhibitor and subtilisin BPN' by sedimentation equilibrium. *J Biochem (Tokyo)* 84, 843-853.
6. Mitsui, Y., Satow, Y., Watanabe, Y. and Iitaka, Y. (1979) Crystal structure of a bacterial protein proteinase inhibitor (*Streptomyces* subtilisin inhibitor) at 2.6 Å resolution. *J Mol Biol* 131, 697-724.
7. Inouye, K., Tonomura, B. and Hiromi, K. (1979) The effect of sodium dodecyl sulfate on the structure and function of a protein proteinase inhibitor, *Streptomyces* subtilisin inhibitor. *Arch Biochem Biophys* 192, 260-269.
8. Takahashi, K. and Sturtevant, J. M. (1981) Thermal denaturation of *Streptomyces* subtilisin inhibitor, subtilisin BPN', and the inhibitor-subtilisin complex. *Biochemistry* 20, 6185-6190.
9. Uehara, Y., Tonomura, B., Hiromi, K., Sato, S. and Murao, S. (1976) An usual florescence spectrum of a protein proteinase inhibitor, *Streptomyces* subtilisin inhibitor *Biochim. Biophis. Acta* 453, 513 - 520.
10. Takeuchi, Y., Satow, Y., Nakamura, K. T. and Mitsui, Y. (1991) Refined crystal structure of the complex of subtilisin BPN' and *Streptomyces* subtilisin inhibitor at 1.8 Å resolution. *J Mol Biol* 221, 309-325.
11. Uehara, Y., Tonomura, B. & Hiromi, K. (1978). Direct fluorometric determination of a dissociation constant as low as 10^{-10} M for the subtilisin BPN' - protein proteinase inhibitor (*Streptomyces* subtilisin inhibitor) complex by a single photon counting technique. *J Biochem (Tokyo)* 84, 1195-202..
12. Inouye, K., Tonomura, B., Hiromi, K., Sato, S. and Murao, S. (1977) The stoichiometry of inhibition and binding of a protein proteinase inhibitor from *Streptomyces* (*Streptomyces* subtilisin inhibitor) against subtilisin BPN'. *J Biochem (Tokyo)* 82, 961-967.
13. Markland, F. S. and Smith, E. L. (1967) Subtilisin BPN. VII. Isolation of cyanogen bromide peptides and the complete amino acid sequence. *J Biol Chem* 242, 5198-5211.
14. Masuda-Momma, K., Hatanaka, T., Inouye, K., Kanaori, K., Tamura, A., Akasaka, K., Kojima, S., Kumagai, I., Miura, K. and Tonomura, B. (1993) Interaction of subtilisin BPN' and recombinant *Streptomyces* subtilisin inhibitors with substituted P1 site residues. *J Biochem (Tokyo)* 114, 553-559.

15. Davis, B. J. (1964) Disc electrophoresis. II. Method and application to human serum proteins. *Ann NY Acad Sci.* 121, 404-427.
16. Kainosho, M. and Miyake, Y. (1988) *Nippon Nogeikagaku Kaishi* 12, 1822-1827.
17. Akasaka, K. (1983) Conference on Biomolecular Structure, Abstracts, pp. 2-3.
18. Akasaka, K., Fujii, S., Hayashi, F., Rokushika, S., and Hatano, H. (1982) A novel technique for the detection of dissociationassociation equilibrium in a highly associable macromolecular system. *Biochem Int* 5, 637 – 642.
19. Hiromi, K. (1979) Kinetics of Fast Enzyme Reaction. Halsted Press, New York pp.256.
20. Satow, Y., Watanabe, Y. and Mitsui, Y. (1980) Solvent accessibility and microenvironment in a bacterial protein proteinase inhibitor SSI (*Streptomyces* subtilisin inhibitor). *J Biochem (Tokyo)* 88, 1739-1755.
21. Laskowski, M., Jr. and Sealock, R. W. (1971) in The Enzymes 3rd. ed. vol III Hydrolysis: peptide bond (Boyer, P. D. Ed.) Academic Press, New York pp. 375 – 473.

Communicated by Ohnishi Masatake