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Kinetic study of the effects of solvation on the dimerization process of α-chymotrypsin

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The dimeric association process of α-chymotrypsin has been studied with the aid of a stopped-flow spectrophotometer at various temperatures and pH values. From the temperature dependences of the forward reaction rate constant (k_f) and the equilibrium dimerization constant (K_D), the reaction system observed here is concluded to be entropy-driven. The increase in entropy can be attributed to the release of water molecules from both the active site and the surface part of the protein molecule during the course of dimerization. From the pH dependences of the reaction rate constants and the equilibrium constant, the reaction is concluded to depend strongly on the dissociations of the site between the carboxyl group of the aspartic acid and imidazolyl group of the histidine residues (in the higher pH region), and the site between the imidazolyl group of the histidine and the carboxyl group of the tyrosine residue (in the lower pH region), respectively.

1. Introduction

Interactions between protein molecules are of great importance in biological phenomena such as enzyme-substrate, enzyme-inhibitor and antibody-antigen reactions [1]. Kinetic studies of these reactions, however, have not been performed often because of the relatively greater difficulty in the detection of these reactions than of small molecule-protein reactions [2]. Considering the present situation, in which only large amounts of static data without supporting evidence from kinetics are being accumulated, it is desirable to investigate association kinetics of protein molecules in detail.

The dimerization of α-chymotrypsin has been extensively studied by Timasheff et al. [3,4] using an ultracentrifugation technique. X-ray crystallographic studies showed that Tyr-146 of one molecule was bound to the active site of another unit [5,6]. Previously, Ikeda et al. [7] investigated the influence of dimerization on the catalytic activity of α-chymotrypsin. They strictly evaluated the dimerization constant and determined the amino acid residues participating in the dimerization. However, kinetic data on the dimerization of α-chymotrypsin have not been reported except by Koren and Hammes [8], which comprised only one pair of forward (k_f) and backward (k_b) reaction rate constants ($k_f = 3700\ M^{-1}\ s^{-1}$, $k_b = 0.68\ s^{-1}$, $K_D = k_f/k_b = 5500\ M^{-1}$, at pH 4.3, 25 °C, in 0.05 M KNO_3). Considering that this dimerization reaction is slow and clear enough to observe using a conventional spectrophotometric stopped-flow technique, it is worthwhile to investigate the reaction kinetics in detail. Here we studied this dimerization reaction at various pH values and temperatures in order to clarify various factors which affect protein-protein interactions.

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2. Experimental details

2.1. Materials

α-Chymotrypsin (crystallized 3 times) was purchased from Worthington and used without further purification. Bromophenol blue (BPB) of the highest grade was obtained from Nakarai Chemicals (Kyoto, Japan). Other reagents were commercially available. Deionized water was distilled just prior to use.

2.2. Kinetic measurements

The dimerization process of α-chymotrypsin was observed with the help of a probe (BPB) using a stopped-flow spectrophotometer (RA-1100, Union Engineering, Hirakata, Osaka, Japan). BPB, an acid-base indicator, was used to monitor small pH changes (< 0.1 pH unit) caused by the difference in proton binding between chymotrypsin monomers and dimers. The details of the apparatus have been described elsewhere [9]. The solution which contains various amounts of α-chymotrypsin (150–450 μM) and a fixed amount of BPB (10 μM) + KCl (9.8 mM) was mixed with an equal volume of BPB (10 μM) + KCl (9.8 mM) solution, and the relaxation curve at 590 nm induced by the dilution of α-chymotrypsin was followed. pH values of the two kinds of solutions were strictly adjusted to equal each other using 0.01–0.1 N HCl or NaOH before mixing. A Hitachi-Horiba F-7ss pH meter was used for pH measurements. The quartz observation cell of the stopped-flow apparatus was thermostatted using a Lauda water bath (15–35 °C). The concentration of α-chymotrypsin was determined spectrophotometrically at 282 nm (molecular extinction coefficient, $\epsilon = 2.05\ cm^{-1}\ mg^{-1}$ ml, molecular weight = 24 500) [10] using a high-sensitivity spectrophotometer (SM-401, Union Engineering, Hirakata, Osaka).

2.3. Circular dichroism

Circular dichroism of α-chymotrypsin solutions was recorded on a Jovin-Yvon dichrograph mark III-J CD spectrophotometer (Union Engineering).

3. Results and discussion

3.1. Effect of pH on k_f and k_b values

By the dilution of α-chymotrypsin solution, we could observe a clear relaxation curve as exemplified in fig. 1. Koren and Hammes found that two relaxation processes existed in the α-chymotrypsin-BPB system: one with a relaxation time of about 100 μs, the other with a relaxation time of about 0.5 s. They assigned the first relaxation process to the interaction of α-chymotrypsin with BPB and the second to the dimerization of α-chymotrypsin [8]. BPB affects the catalytic activity of α-chymotrypsin [8] and we assumed here that BPB binds specifically to the active site of monomer α-chymotrypsin and that the dimerization of α-chymotrypsin occurs in competition with the formation of a BPB-α-chymotrypsin complex. The second relaxation time did not depend on the concentration of BPB but was dependent on that of α-chymotrypsin [8], showing that the relaxation reflects solely the change in the situation of the dimerization of α-chymotrypsin. The relaxation process we measured corresponded to the second process that Koren and Hammes observed.

From the reciprocal of the relaxation time (τ^{-1}) at various concentrations of α-chymotrypsin, we estimated both forward and backward reaction rate constants (k_f, k_b) using scheme 1 and eqs. 1–3 [11], where M, D, [M], [D] and $[M]_0$ denote the monomeric and dimeric forms of α-chymotrypsin, the concentration of monomeric and dimeric α-chymotrypsin in equilibrium and

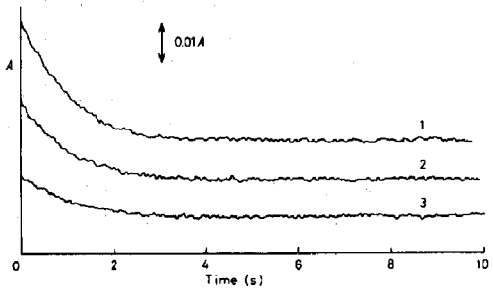


Fig. 1. Typical traces of the relaxation effect of dimerization of α-chymotrypsin at 25 °C; pH 3.7. Curve 1, $[M]_0 = 218$ μM; curve 2, $[M]_0 = 159$ μM; curve 3, $[M]_0 = 85$ μM.

$$2M \underset{k_b}{\overset{k_f}{\rightleftharpoons}} D$$

Scheme 1.

the total concentration of α-chymotrypsin, respectively [8].

$$\tau^{-1} = 4k_f[M] + k_b \quad (1)$$

$$K_D = k_f/k_b = [D]/[M]^2 = ([M]_0 - [M])/2[M]^2 \quad (2)$$

$$(\tau^{-1})^2 = 8k_f k_b[M]_0 + k_b^2 \quad (3)$$

Typical plots of $(\tau^{-1})^2$ vs. $[M]_0$ are shown in fig. 2. A linear relationship was observed between $(\tau^{-1})^2$ and $[M]_0$ as expected from eq. 3. The k_f and k_b values obtained from the Y-intercept and the slope of plots at various pH values are shown in fig. 3. Both plots for k_f and k_b values exhibit so-called 'bell-shaped' behavior. The equilibrium constant K_D ($= k_f/k_b$) also displays a bell-shaped curve (fig. 4). These types of behavior with respect to pH can be explained by the change in electrostatic interactions between two monomer molecules induced by protonation or deprotonation of amino acid residues in the active site, and not by the conformational change of protein molecules as mentioned below (section 3.2). Our k_f and K_D values are lower than those reported by Koren and Hammes, probably because of the difference

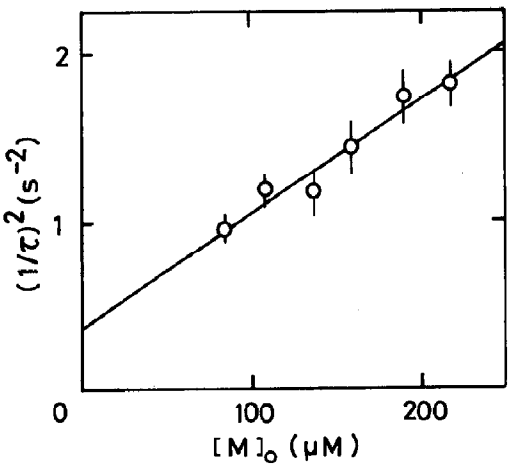


Fig. 2. τ^{-2} as a function of $[M]_0$ at 25 °C; pH 4.3.

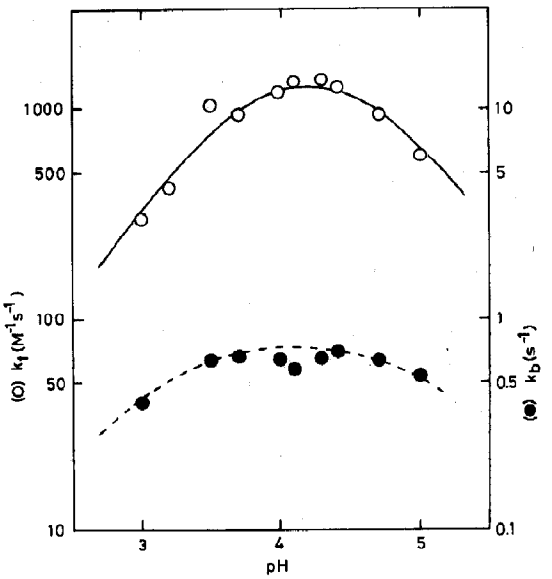


Fig. 3. k_f (○) and k_b (●) as a function of pH at 25 °C. Theoretical curve obtained using eq. 3 with $k_{f,1} = 0$ M^{-1} s^{-1}, $k_{f,2}$ $(K_{M,2}/K_{M,1}) + k_{f,3} = 2500$ M^{-1} s^{-1}, $k_{f,4} = 0$ M^{-1} s^{-1}, $pK_{M,1} = 3.8$ and $pK_{M,2} = 4.6$. (------) Theoretical curve obtained using eq. 4 with $k_{b,1} = 0$ s^{-1}, $k_{b,2} = 0.85$ s^{-1}, $k_{b,3} = 0$ s^{-1}, $pK_{D,1} = 3.0$ and $pK_{D,2} = 5.2$.

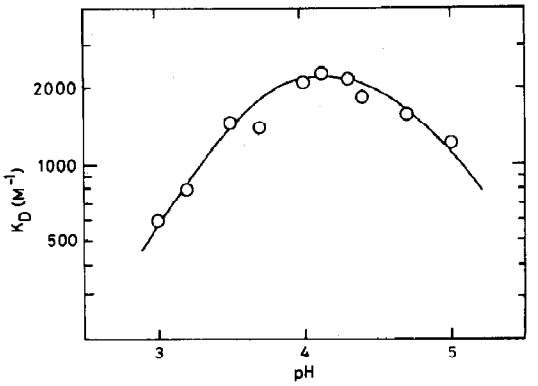


Fig. 4. K_D as a function of pH at 25 °C. (———) Theoretical curve calculated using the equation below with $pK_{M,1} = 3.6$, $pK_{M,2} = 4.6$, $pK_{D,1} = 2.8$, $pK_{D,2} = 5.2$, $K(pH = \infty) = 330$ M^{-1}:

$$K = K(pH = \infty)\frac{(1 + a_H/K_{D,1})(1 + a_H/K_{D,2})}{(1 + a_H/K_{M,1})(1 + a_H/K_{M,2})}$$

where a_H denotes the activity of H^+.

in experimental conditions (this work, in 9.8 mM KCl; Koren and Hammes, in 0.05 M KNO_3).

We tried to fit the experimental results of the pH dependences of k_f and k_b values with the theoretical values calculated from eqs. 4 and 5, which were derived for the rapid equilibrium between protonated and unprotonated species of α-chymotrypsin. Ikeda et al. [7] determined the protonation equilibrium constants for the dimerization process and assigned those values to several residues of the enzyme (fig. 5) from the pH dependence of the catalytic activity of dimeric α-chymotrypsin [7]. Using those molecular species ((1)–(7) in fig. 5), we assumed four and three elementary processes for the forward and backward reactions, respectively (subscripts M and D denote the monomeric and dimeric forms of α-chymotrypsin) (scheme 2 and fig. 5). k_f and k_b are given by eqs. 4 and 5, respectively.

$$k_f = \frac{k_{f,1}\frac{[H^+]}{K_{M,1}} + k_{f,2}\frac{K_{M,2}}{K_{M,1}} + k_{f,3} + k_{f,4}\frac{K_{M,2}}{[H^+]}}{\frac{[H^+]}{K_{M,1}} + \frac{K_{M,2}}{K_{M,1}} + 1 + \frac{K_{M,2}}{[H^+]}} \tag{4}$$

$$k_b = \frac{k_{b,1}\frac{[H^+]}{K_{D,1}} + k_{b,2} + k_{b,3}\frac{K_{D,2}}{[H^+]}}{\frac{[H^+]}{K_{D,1}} + 1 + \frac{K_{D,2}}{[H^+]}} \tag{5}$$

Using the curve fitting method with experimental data for k_f and eq. 4, two kinds of dissociation constants for monomeric α-chymotrypsin were

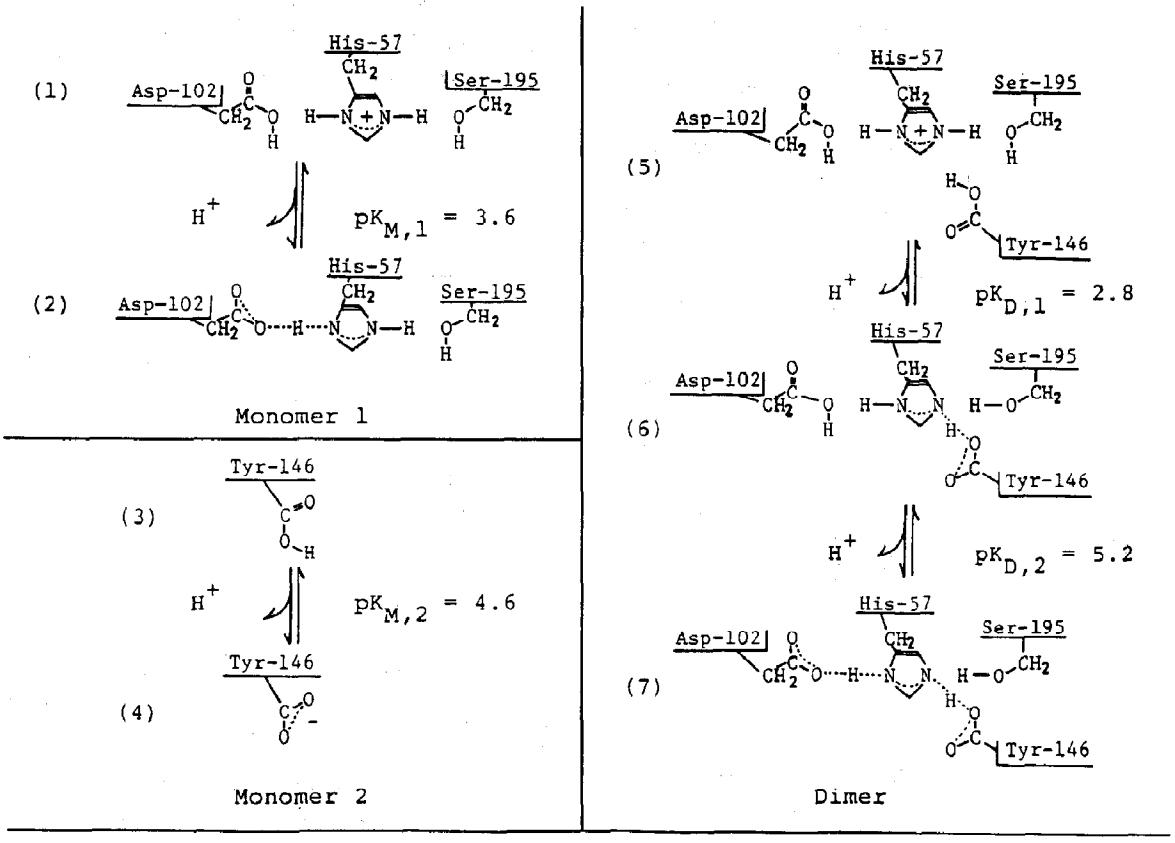
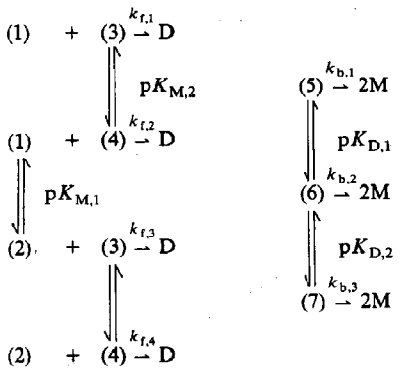


Fig. 5. Groups relating to the dimerization process and assignment of pK values.



Scheme 2.

estimated as $pK_{M,1} = 3.8$ and $pK_{M,2} = 4.6$. We could also evaluate four kinds of rate constants for the forward reaction. The $k_{f,2}$ and $k_{f,3}$ values are given by the equation $k_{f,2}(K_{M,2}/K_{M,1}) + k_{f,3} = 2500\ M^{-1}\ s^{-1}$, but they could not be separated because the concentration ratio [(1)][(4)]/[(2)][(3)] (equal to $K_{M,2}/K_{M,1}$) was constant within every pH region. The rate constants $k_{f,1}$, $k_{f,4}$ could not be determined either, since the corresponding plateau was not reached over the pH region in which measurements were made. It can only be stated that $k_{f,1}$, $k_{f,4} < 100\ M^{-1}s^{-1}$ and these values were set to be equal to zero in eq. 4 when the theoretical function in fig. 3 was calculated.

The pH dependence of k_b was in good agreement with the scheme and eq. 5 when two kinds of dissociation constants for dimeric α-chymotrypsin and three kinds of backward rate constants were set to be $pK_{D,1} = 3.0$, $pK_{D,2} = 5.2$, $k_{b,1} = 0\ s^{-1}$, $k_{b,2} = 0.85\ s^{-1}$ and $k_{b,3} = 0\ s^{-1}$, respectively. These two pK values for the monomeric enzyme ($pK_{M,1}$, $pK_{M,2}$) and two for the dimer ($pK_{D,1}$, $pK_{D,2}$) are in good agreement with those reported by Ikeda et al. (3.6, 4.6, 2.4 and 5.5, respectively) [7].

Vandlen and Tulinsky found that in crystalline, dimeric α-chymotrypsin, the terminal carboxyl group of the B chain Tyr-146 of one molecule closely approached His-57 in the active site of another molecule (4.5–5 Å to the center of the imidazolium ring) [5]. Their crystallographic study suggested hydrogen bond formation between the protonated α-carboxyl group of Tyr-146 and the carbonyl oxygen of His-57, although the possibility of an electrostatic interaction between the imidazole ring of His-57 and the ionized carboxyl group of Tyr-146 was not excluded [5]. On the other hand, Gorbunoff et al. [12] found that methylation of $N^{\epsilon 2}$ of His-57 and removal of the Tyr-146 residue inhibited the dimerization of α-chymotrypsin in solution and that this inhibition was not due to a steric effect but resulted from removal of the ionizable site. Thus, Tyr-146 and His-57 were believed to be necessary for dimer formation of α-chymotrypsin in both crystalline and solution states, although the roles of the these residues differed.

The pH dependence of dimerization observed here could be readily explained in terms of electrostatic interaction, but was inconsistent with hydrogen bond formation between the carbonyl oxygen of His-57 and the α-carboxyl of Tyr-146. It is reasonable to suppose that Tyr-146 in the crystalline dimer is located somewhat differently from its position in solution, in order to permit interaction between the phenolic hydroxyl group of Tyr-146 and SO_4^{2-} derived from ammonium sulfate used for crystallization [5] which was totally excluded in solution. Thus, we concluded that electrostatic interaction between the imidazole ring of His-57 and the α-carboxyl group of Tyr-146 was the most important for dimer formation in solution.

The active site of dimeric α-chymotrypsin can be depicted as shown in fig. 5, where the values of $pK_{M,1}$, $pK_{M,2}$, $pK_{D,1}$ and $pK_{D,2}$ listed were evaluated from the K_D vs. pH curve in fig. 4. In this system dimerization reflects the charge state of the catalytic system, including the α-carboxyl group of Tyr-146. The four pK_a values determined from the pH dependence of dimer formation might be attributed to this catalytic system.

In the monomeric enzyme, Ikeda et al. attributed the first dissociation constant ($pK_{M,1}$) to ionization of the site between the Asp-102 carboxyl and His-57 $N^{\delta 1}$ and the second ($pK_{M,2}$) to ionization of the Tyr-146 α-carboxyl group. The $pK_{M,1}$ value was in agreement with the pK value determined from the pH dependence of the catalytic activity of monomeric α-chymotrypsin [7]. It

is reasonable to suppose that $pK_{M,1}$ is attributable to the catalytic triad in the monomeric enzyme.

In the dimeric enzyme, they attributed the first ionization to the site between the Tyr-146 α-carboxyl group and His-57 $N^{\epsilon 2}$, and the second to that between His-57 $N^{\delta 1}$ and the Asp-146 carboxyl, respectively. At the first deprotonation (from state (5) to (6)), a hydrogen bond is formed between $N^{\epsilon 2}$ of His-57 and the α-carboxyl of Tyr-146, and this bond stabilizes the dimeric enzyme. At the second deprotonation (from (6) to (7)) the dimeric enzyme is destabilized because of the repulsive electrostatic interaction between the catalytic triad and Tyr-146.

Aune and Timasheff [3] previously declared that the four pK values of ionizable groups were 3.6 and 4.0 in the monomer and 2.4 and 6.2 in the dimer and attributed the first ionizable groups of both monomeric and dimeric enzymes solely to the carboxyl group of Tyr-57 ($pK = 3.6$ in the monomer, $pK = 2.4$ in the dimer) and the second to the imidazole ring of His-57 ($pK = 4.0$ in the monomer, $pK = 6.2$ in the dimer). They suggested that dimerization was accompanied by shifts in the pK values of those groups [3]. The experimental conditions used by Aune and Timasheff (0.1 M NaCl, 0.01 M acetate buffer, 25°C) differ slightly from ours. Nevertheless, the pK values that they estimated statically are very different from those evaluated kinetically by us and by Ikeda et al. Since protonation and deprotonation of ionizable groups in the protein molecule occur cooperatively with the neighboring side chains, we consider the assignments of ionizable groups by Ikeda et al. to be more appropriate than those of Aune and Timasheff.

3.2. *Effect of temperature on k_f and k_b values*

Fig. 6 demonstrates the temperature dependences of both k_f and k_b, fig. 7 depicting that of K_D. It is evident that the k_f and K_D values do not show a linear relationship at high temperatures. This means that the activation and thermodynamic parameters are temperature-dependent and that the heat capacity of dimerization (ΔC_p) is negative. From Arrhenius plots of k_f and van 't Hoff plots of K_D at low temperature, we were

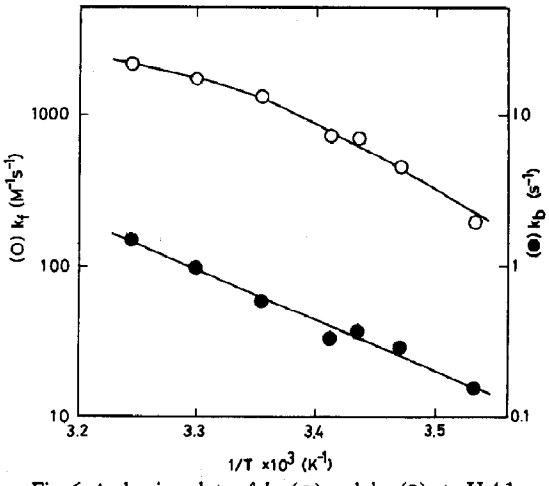


Fig. 6. Arrhenius plots of k_f (○) and k_b (●) at pH 4.1.

able to evaluate the thermodynamic and activation parameters of this reaction system, the results obtained being compiled in table 1.

The enthalpy of activation $\Delta H^{\ddagger}$ for k_f (19.5 kcal mol^{-1}) is much greater than that of a simple diffusion-controlled reaction (3–5 kcal mol^{-1}) [13], which suggests that there exists a process demanding a high activation energy. In low-temperature regions, all four thermodynamic and activation parameters (ΔH, ΔS, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$) were positive, suggesting that the driving force for the complexation of α-chymotrypsin is the entropy gain.

The largely positive values of the entropy of association ΔS and the entropy of activation $\Delta S^{\ddagger}$ are attributed to the hydrophobic interaction which is caused by the release of water molecules in and

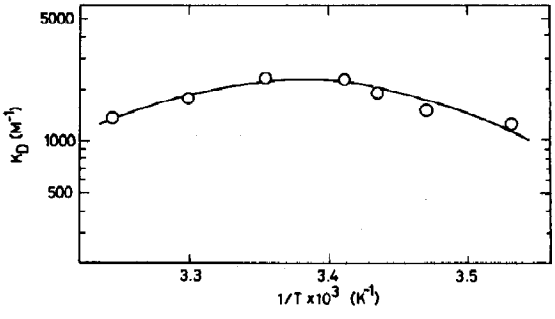


Fig. 7. Van 't Hoff plots of K_D at pH 4.1.

Table 1

Activation and thermodynamic parameters for the dimerization of α-chymotrypsin at 20 °C and pH 4.1

	$\Delta G^{\ddagger}$ (kcal mol^{-1})	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (e.u.)
k_f	13.3±0.2	19.5±0.2	21±2
k_b	17.7±0.2	14.9±0.2	−9±2
	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (e.u.)
K_D	−4.4±0.4	4.6±0.4	30±4

around the binding site of the α-chymotrypsin molecule as a result of association [14]. 40–70 water molecules have been reported to be bound to an α-chymotrypsin molecule [15]. In an X-ray crystallographic study Blevins and Tulinsky reported that the specific site for α-chymotrypsin is occupied by ordered water molecules in a similar manner to γ-chymotrypsin and other proteins [6]. Some of these water molecules are likely to be displaced by the binding site of another α-chymotrypsin molecule during the dimerization reaction. By this displacement the hydrogen bonds between the water molecules might also be broken to give rise to the positive ΔH.

Similar tendencies of an increase in entropy and entropy of activation have been observed in the association of soybean trypsin inhibitor with α-chymotrypsin ($\Delta S = 70$ e.u., $\Delta S^{\ddagger} = 29$ e.u. [16]; $\Delta S = 48$ e.u. [17]) and that of *Streptomyces* subtilisin inhibitor with subtilisin ($\Delta S = 56.7$ e.u., $\Delta S^{\ddagger} = 2.41$ e.u. [18,19]). Paladini and Weber [20] observed the pressure-induced reversible dissociation of enolase. Their static results ($\Delta V = -65 \pm 8$ ml mol^{-1}) for the dissociation step also support the release of large amounts of water on association [20]. Recently, Thompson and Lakowicz [21] reported a large positive volume change ($\Delta V = 150$ ml mol^{-1}) for the self-association of melittin and attributed this to the release of water molecules at the time of association driven by the hydrophobic interaction between melittin molecules [21].

It should be noted here that ΔH, ΔS, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ decreased with rising temperature. From the CD measurements, we were unable to detect any influence of temperature on the conformation of the α-chymotrypsin molecule at 10–40 °C, which excludes the possibility of a conformational change participating in the decrease of these parameters, although an asymmetric conformational change at the time of dimerization was reported in an X-ray crystallographic study (1.67 Å resolution) [10]. We consider the water structure around the α-chymotrypsin molecules at high temperatures to be different from that at low temperatures. The formation of an ordered water structure by the dissolution of nonpolar groups in water is strongly promoted at low temperature and, as a result, the tendency for the hydrophobic association is increased [14]. In contrast, at high temperature, as the solvent structure is disturbed, the contribution of hydrophobic interactions to positive values of ΔS and $\Delta S^{\ddagger}$ is diminished while the enthalpy term dominates the stability of the dimer [14].

In addition to the hydrophobic interaction mentioned here, at least one type of specific electrostatic interaction occurs on dimerization as mentioned in section 3.1. We interpret the thermodynamics of dimerization of α-chymotrypsin in terms of the following conceptual model;

(1) Two hydrated monomers interact hydrophobically with each other and induce the release of water molecules. This process is accompanied by positive ΔH and ΔS values [14,22].

(2) Hydrophobically associated species interact more strongly by ionic interaction which at least induces the interaction between the imidazole ring of His-57 and the α-carboxyl group of Tyr-106. This process is accompanied by a slightly positive ΔH and a positive ΔS [14,23].

To clarify further the role of solvation in the dimerization reaction, we performed a differential pressure-jump experiment [24]. However, the conformational change undergone by α-chymotrypsin molecules on application of high pressures markedly perturbed the relaxation curves [25] and it was very difficult to estimate the volume change and the volume of activation in the dimerization reaction at that moment.

In conclusion, we were able to observe the essential role of solvation in the dimerization reaction of α-chymotrypsin molecules in terms of the kinetics. The ionic dissociation constants estimated from the kinetic data support the cooper-

ative ionization of the amino acid residues in the active site during the dimerization process.

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