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Large $\Delta H^{\neq}$ and $\Delta S^{\neq}$ Values Obtained in Hydrolysis of α-Chymotrypsin or Catalase with α-Chymotrypsin

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α-Chymotryptic hydrolyses of globular proteins, α-chymotrypsin (Chym) and catalase (Cata), were examined kinetically at pH 7.8 and temperatures between 30 and 50 °C. In both cases, the reactions followed second-order rate equations; $-\mathrm{d[Chym]}/\mathrm{d}t = k(\text{chy-chy})[\mathrm{Chym}]^2$ for the autolysis of Chym and $-\mathrm{d[Cata]}/\mathrm{d}t = k(\text{chy-cat})[\mathrm{Chym}]\,[\mathrm{Cata}]$ for the chymotryptic hydrolysis of Cata. From the Arrhenius plots, $\Delta H^{\neq}$/kJ mol^{-1} and $\Delta S^{\neq}$/J K^{-1} mol^{-1} were obtained as follows: for k(chy-chy)/dm^3 mol^{-1} s^{-1}, 270 and 681 at 30—44 °C, and for k(chy-cat)/dm^3 mol^{-1} s^{-1}, 72.0 and 2.32 at 30—42 °C, respectively. At higher temperatures than 45 °C, the Arrhenius plots showed upward-concave curvatures in both cases. These extremely large activation parameters obtained for Chym autolysis and moderately large activation parameters obtained for Cata proteolysis are discussed in terms of deformation of the three-dimensional structures and the sites of proteolysis in the globular structures of the two enzymes.

Keywords——α-chymotrypsin; catalase; proteolysis; kinetics; activation parameter; protease autolysis; peptide chain unfolding

Introduction

Hydrolysis of α-chymotrypsin (Chym) catalyzed by Chym, namely the autolysis of Chym, is reported to exhibit second-order kinetics with respect to the concentration of Chym in phosphate buffer at 25 °C.[1,2] The cleavage of peptide linkages of Chym was confirmed by the difference in electrophoretic separations before and after the reaction. The finding[3] that immobilized Chym does not degrade under the same reaction conditions supports the proposition that a bimolecular collision mechanism between two globular protein molecules is involved in this autolysis.

One of the results of the present study is that second-order kinetics was valid at higher temperatures of 30—45 °C for the autolysis of Chym, and that the α-chymotryptic hydrolysis of catalase (Cata) also showed second-order kinetics with respect to [Chym] and [Cata] at 25—45 °C in phosphate buffer.

The activation parameters, $\Delta H^{\neq}$ and $\Delta S^{\neq}$, for the second-order rate constants of the two reactions, autolysis of Chym and proteolysis of Cata, were also determined. Extremely large $\Delta H^{\neq}$ and $\Delta S^{\neq}$, 270 kJ mol^{-1} and 681 J K^{-1} mol^{-1} were obtained for the former, while for the latter the values were somewhat smaller, 72.0 kJ mol^{-1} and 2.32 J K^{-1} mol^{-1}, respectively. Mihalyi[4] summarized values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for the enzymatic hydrolysis of protein substrates, classifying the substrates into three categories, as listed in Table I. The autolysis of Chym belongs to the last category, while the reaction between Chym and Cata belongs to the second category. The origin of these abnormal or normal activation parameters is related to the existence or non-existence of a preliminary unfolding process of the globular structure of the protein for the hydrolysis. The reason for the difference in activation parameters between autolysis of Chym and proteolysis of Cata will be discussed in relation to the three-dimensional structures of the two protein substrates.

TABLE I. Classification of Enzymatic Hydrolyses of Protein Substrates with Regard to Activation Parameters[4)]

Category of reaction (Protein substrates)	$\Delta G^{\ddagger}$/kJ mol^{-1}	$\Delta H^{\ddagger}$/kJ mol^{-1}	$\Delta S^{\ddagger}$/J K^{-1} mol^{-1}
Denatured proteins	46—78	22—59	−92—0.0
Native proteins with normal parameters	72—85	44—77	−92—−17
Native proteins with abnormal parameters	63—80	130—240	170—460

Experimental

Material——α-Chymotrypsin from bovine pancreas (Chym) was purchased from Sigma as a three-times-recrystallized powder. Catalase from bovine liver (Cata) was also obtained from Sigma as a purified powder. A solution of Chym was prepared daily as a 1.21×10^{-5} or 1.92×10^{-5} mol dm^{-3} phosphate-buffered solution at pH 7.8 (0.03 mol dm^{-3} KH_2PO_4/0.03 mol dm^{-3} Na_2HPO_4) and was cooled in an ice-water bath. A stock solution of Cata was prepared as a 2.1×10^{-6} mol dm^{-3} solution in the same buffer and stored in a cool box at 4 °C to be used for α-chymotryptic hydrolysis of Cata. Concentrations of active enzymes, [Chym] and [Cata], were assayed according to the methods reported in the literature.[1,5)]

p-Nitrophenyl acetate (pNPA) was recrystallized three times from a hexane–chloroform (1 : 1) solution (mp 79.0—80.0 °C). A stock solution of pNPA was prepared as a 1.38×10^{-3} mol dm^{-3} solution in dry acetonitrile. Hydrogen peroxide was diluted with distilled water, adjusted to 1.18 mol dm^{-3} and stored in a cool box at 4 °C.

Procedure——A solution of Chym at 1.92×10^{-5} mol dm^{-3} buffered at pH 7.8 was incubated at temperatures between 30 and 50 °C. An aliquot was taken from the solution at suitable time intervals to measure the remaining [Chym]. Measurement of [Chym] was performed indirectly by measurement of the rate of chymotryptic hydrolysis of pNPA.

The hydrolysis of pNPA was followed in terms of the release of *p*-nitrophenoxide anion using a Shimadzu UV-210A double-beam spectrophotometer at 400 nm. A cuvette containing 3.45×10^{-3} dm^3 of phosphate buffer was temperature-equilibrated, and 150×10^{-6} dm^3 of a pNPA stock solution was added. Non-enzymic slow hydrolysis of pNPA was observed. Enzymic hydrolysis of pNPA was initiated by addition and prompt mixing of the sample solution of Chym in an amount of 100×10^{-6} dm^3. The reaction was followed for about 20 min. The net rate of enzymic hydrolysis of pNPA was calculated by subtracting the rate of non-enzymic hydrolysis rate from the measured total hydrolysis rate. [Chym] in the sample solution was determined from this net hydrolysis rate based on the kinetic analysis of the reaction below.[1)]

$$\text{Chym} + \text{pNPA} \underset{}{\overset{K_s}{\rightleftharpoons}} \text{Chym-pNPA} \xrightarrow{k_2} \text{acetyl Chym} \xrightarrow{k_3} \text{Chym} + \text{acetic acid} \quad (1)$$
$$+$$
$$p\text{-nitrophenol}$$

$$v_0 = \frac{k(\text{chy-pNPA})[\text{Chym}]_0[\text{pNPA}]_0}{K_m(\text{chy-pNPA}) + [\text{pNPA}]_0}, \quad \text{where} \quad k(\text{chy-pNPA}) = \frac{k_2 k_3}{k_2 + k_3} \fallingdotseq k_3 \quad (2)$$

Assay temperatures of the remaining [Chym] were adjusted to those of the autolysis reaction conditions, except for reactions conducted at higher temperatures than 35 °C, for which the assay temperature was fixed at 35 °C. Arrhenius plots for *k*(chy-pNPA) and van't Hoff plots for K_m (chy-pNPA) have been reported at pH 7.4[6)] and were reexamined at pH 7.8 by ourselves.[7)] Both measured values coincided well between temperatures 25 and 35 °C, possibly due to the rather broad bellshaped pH profile characteristics of chymotryptic hydrolysis.

The molar extinction coefficient (ε) of *p*-nitrophenoxide anion at 400 nm was 18000 when measured in the completely dissociated state in a 0.01 mol dm^{-3} solution of sodium hydroxide. For the determination of hydrolysis rate in the present experiments, however, an experimentally obtained ε_{400} was adopted as listed below at various temperatures, and in the presence of the phosphate buffer (0.03 mol dm^{-3} KH_2PO_4/0.03 mol dm^{-3} Na_2HPO_4) and 4% acetonitrile.

temperature (°C)	25.0	30.0	35.0
ε_{400}	15900	16200	16400

Mixed solution of Cata and Chym, the initial concentrations of 2.1×10^{-7} and 6.07×10^{-6} mol dm^{-3}, respectively, was buffered at pH 7.8 and incubated at temperatures between 30 and 50 °C. Aliquots were taken at suitable time intervals to determine the remaining [Cata] from measurements of the rate of decomposition of H_2O_2.

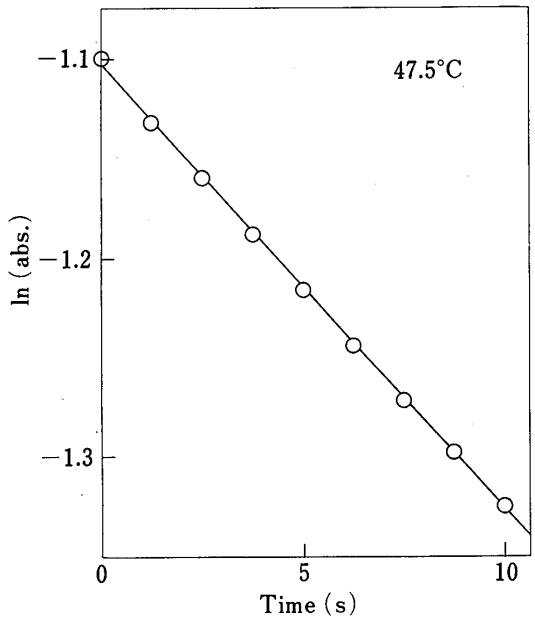

Fig. 1. First-Order Kinetics for Enzymatic Decomposition of H_2O_2 at 47.5 °C

$[H_2O_2]$ was monitored in terms of the UV absorbance at 250 nm.

The rate of enzymic decomposition of H_2O_2 was measured by monitoring the change of ultraviolet (UV) absorbance at 250 nm with a Hitachi UV-228 spectrophotometer. Phosphate buffer solution (2.70×10^{-3} dm^3) was placed in a cuvette and temperature-equilibrated, then 200×10^{-6} dm^3 of a H_2O_2 solution at 2.36×10^{-1} mol dm^{-3} was mixed and stirred for about 2 min. Enzymic reaction was initiated by adding 100×10^{-6} dm^3 of sample Cata solution into the cuvette. Enzymic decomposition of H_2O_2 shows first-order reaction kinetics based on the two-step mechanism below.[5]

$$\text{Cata} + H_2O_2 \xrightarrow{k_1} \text{Cata-}H_2O_2$$

$$\text{Cata-}H_2O_2 + H_2O_2 \xrightarrow{k_2} \text{Cata} + H_2O + O_2 \quad (3)$$

$$-\frac{d[H_2O_2]}{dt} = \frac{2k_1k_2[\text{Cata}]}{k_1+k_2}[H_2O_2] = k(\text{cat-}H_2O_2)[H_2O_2] \quad (4)$$

The first-order kinetics was confirmed by ourselves as exemplified in Fig. 1 for a mixed solution of Cata and H_2O_2 incubated at 47.5 °C for 120 s. The assay temperature of Cata was fixed at 25 °C, namely, enzymic decomposition of H_2O_2 was followed for about 30 s at 25 °C, and [Cata] of the sample solution was determined from the measured k(cat-H_2O_2) and the numerical value of $2k_1k_2/(k_1+k_2)$ at 25 °C reported in the literature.[5]

Another experiment to examine the temperature dependency of enzymic decomposition of H_2O_2 was conducted at temperatures of 24.5—52.2 °C and at the initial [Cata] of 4.2×10^{-7} mol dm^{-3}. In this experiment the first-order rate constant was measured at the same temperatures to the respective temperatures of the decomposition reactions, *i.e.*, 24.5—52.2 °C.

Results and Discussion

Reaction between Chym and Chym (Autolysis of Chym)

Buffered solutions of Chym at pH 7.8, the initial concentration of 1.92×10^{-5} mol dm^{-3}, were incubated at temperatures between 30.0 and 50.2 °C. Concentrations of active Chym assayed from the hydrolysis rate of pNPA decreased with increasing incubation time, t (Fig. 2). The second-order kinetics defined by Eqs. 5 and 6 fitted the obtained results within the temperature range of the present experiments (Fig. 3).

$$-\frac{d[\text{Chym}]}{dt} = k(\text{chy-chy})[\text{Chym}]^2 \quad (5)$$

$$\frac{1}{[\text{Chym}]} - \frac{1}{[\text{Chym}]_0} = k(\text{chy-chy})t \quad (6)$$

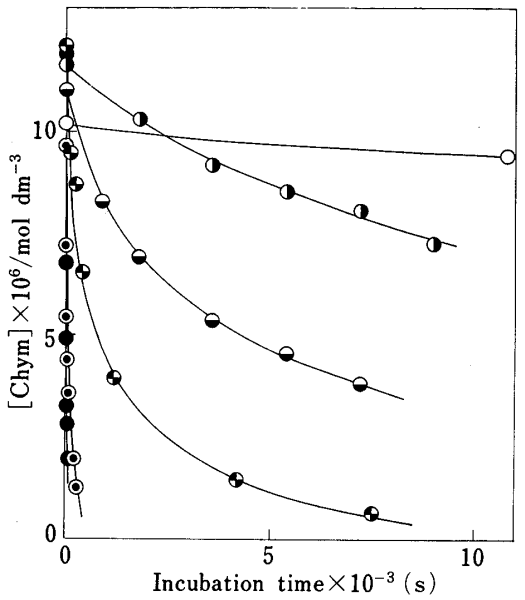

Fig. 2. Deactivation of α-Chymotrypsin (Chym) at pH 7.8

Temperatures (°C): ○, 30.0; ◑, 34.6; ◒, 39.6; ◔, 45.0; ⊙, 47.5; ●, 50.2.

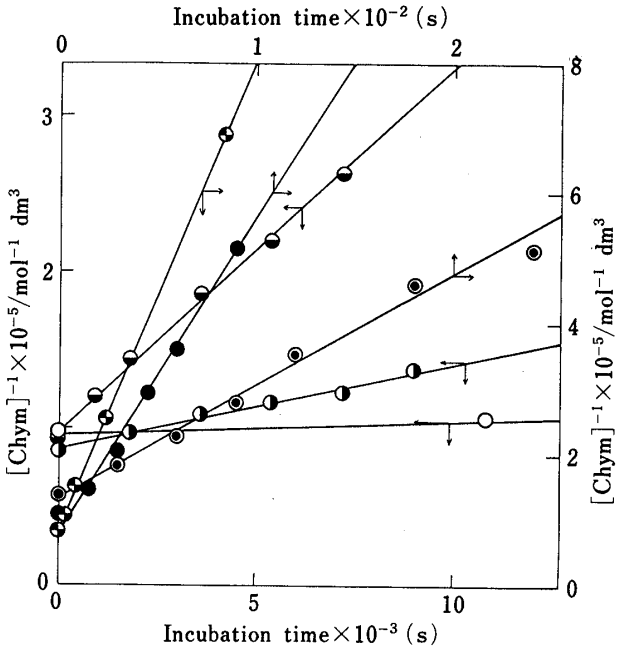

Fig. 3. Second-Order Kinetics for Deactivation of α-Chymotrypsin (Chym)

Symbols and temperatures: see Fig. 2.

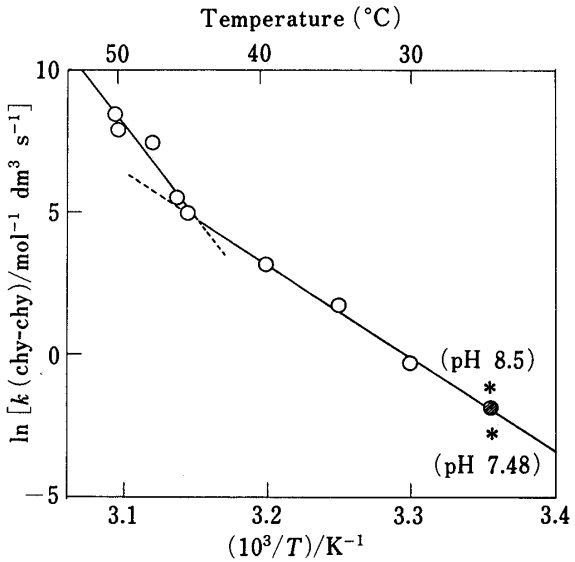

Fig. 4. Arrhenius Plots for the Second-Order Rate Constant k (chy-chy) Defined in Eq. 5

The shaded circle at $10^3/T=3.36$ (25 °C) is an interpolation of the asterisked data taken from the literature.[3]

The second-order kinetics may approximately be deduced from the Michaelis–Menten rate equation for the chymotryptic hydrolysis of a substrate S, by taking S to be identical with Chym and assuming K_m to be sufficiently larger than [S]. As a matter of fact, the obtained rate of the autolysis was much smaller than, for example, that of the chymotryptic hydrolysis of pNPA by a factor of 10^{-3} at 30 °C. K_m(chy-pNPA) in Eq. 2 is as small as 10^{-5}—10^{-6} mol dm^{-3} at 30 °C (Fig. 9). A larger K_m value implies poorer incorporation of the substrate Chym into the active site of the catalyst Chym.

Figure 4 illustrates Arrhenius plots of k(chy-chy). The values of k(chy-chy) at 25 °C were reported to be 0.071 and 0.35/dm^3 mol^{-1} s^{-1} at pH 7.48 and 8.50,[3] respectively, which, if interpolated at pH 7.8, gives a value which does not conflict with the present results. The plots show a break at around 44 °C, and the reaction is sharply accelerated at higher temperatures.

A kinetic analysis according to the transition state rate theory was carried out to obtain activation parameters for the reaction below 44 °C.

The results are listed in Table II. Gibb's free energy of activation calculated at 298 K, $\Delta G^{\neq}$, is a positive value of 64.0 kJ mol^{-1} indicating that the reaction is a rather slow one. At the same time, it is found that the components of $\Delta G^{\neq}$, *i.e.*, $\Delta H^{\neq}$ and $\Delta S^{\neq}$, both have abnormally large positive values, that is, 270 kJ mol^{-1} and 681 J K^{-1} mol^{-1} at 30—44 °C.

Abnormally large activation parameters obtained in the present autolysis of Chym could be placed in the third category in the classification proposed by Mihalyi,[4] as seen in Table I. It should be noted that the experimental results adopted by Mihalyi in Table I[8-12] were obtained with most of the substrates at concentrations close to the K_m value of the enzyme–substrate complex formation. The present experiments were performed at very low protein concentrations of below 10^{-5} mol dm^{-3} order. Therefore, the data in Table II include the thermodynamic quantities arising from the factor $1/K_m$, almost explicitly. This thermodynamic quantity associated with the formation of the interprotein complex formation is in general characterized by $\Delta G \fallingdotseq -21$ kJ mol^{-1}, $\Delta H \fallingdotseq 0$, $\Delta S \fallingdotseq +84$ J K^{-1} mol^{-1},[10,13] which gives a K_m value of around 2×10^{-4} mol dm^{-3}. For a more reasonable comparison with the values in Table I, these figures should be subtracted from the data in Table II. The origin of these abnormally large $\Delta H^{\neq}$ and $\Delta S^{\neq}$ values obtained in autolysis of Chym might be the unfolding of the three-dimensional structure of Chym. This is supported by the results at higher temperatures, where unfolding of the enzyme molecule is unequivocally taking place.

Reaction between Chym and Cata

Aqueous solutions of Cata at 2.1×10^{-7} mol dm^{-3}, buffered at pH 7.8, were incubated at 47.5 and 50.0 °C in the presence or absence of Chym at the concentration of 6.07×10^{-6} mol dm^{-3}. Percentages of remaining active Cata were plotted against the in-

Table II. Activation Parameters for α-Chymotryptic Hydrolyses of α-Chymotrypsin and Catalase

k/dm^3 mol^{-1} s^{-1}	Temp./°C	$\Delta G^{\neq}$/kJ mol^{-1}	$\Delta H^{\neq}$/kJ mol^{-1}	$\Delta S^{\neq}$/J K^{-1} mol^{-1}
k (chy-chy)	25—44	64.0	270	681
k (chy-cat)	30—42	71.2	72.0	2.32

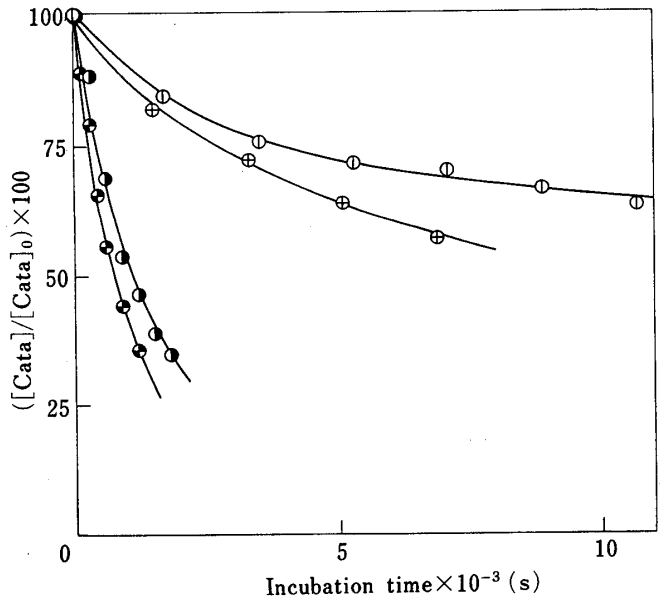

Fig. 5. Plots of the Deactivation of Catalase in the Presence or Absence of α-Chymotrypsin against Incubation Time

	47.5	50 (°C)
presence of Chym	◑	◔
absence of Chym	⊕	⊕

cubation time as illustrated in Fig. 5. A substantial difference is observed in the rate of deactivation of Cata between the experiments in the presence and absence of Chym. Undoubtedly, the deactivation of Cata is enhanced by the presence of Chym even at 47.5—50.0 °C, where the catalytic action of Chym is diminishing. This acceleration arises from the chymotryptic hydrolysis of Cata.

Second-order kinetics was postulated for the deactivation of Cata in the presence of Chym.

$$-\frac{d[\text{Cata}]}{dt}=k(\text{chy-cat})[\text{Chym}][\text{Cata}] \tag{7}$$

In this case, the differential equations, Eq. 7, was solved in combination with Eq. 5 to get the integrated equation shown below.

$$\ln\frac{[\text{Cata}]_0}{[\text{Cata}]}=\frac{k(\text{chy-cat})}{k(\text{chy-chy})}\ln(1+k(\text{chy-chy})[\text{Chym}]_0 t) \tag{8}$$

In Eq. 8, $[\text{Cata}]_0$ and $[\text{Chym}]_0$ are the initial experimental conditions and values of k(chy-chy) are known at temperatures 30—50 °C from the experiments described in the preceeding section. Accordingly k(chy-cat) can be obtained by plotting ln[Cata] against $\ln(1+k(\text{chy-chy})[\text{Chym}]_0 t)$. The versatility of Eq. 8 was examined in Fig. 6 at the incubation temperature of 35 °C and in Fig. 7 at 45 °C for various $[\text{Cata}]_0$ levels. In these figures, ln[Cata] is replaced with $\ln k(\text{cat-}H_2O_2)/s^{-1}$ due to the proportionality of [Cata] and $k(\text{cat-}H_2O_2)$. (See the experimental section, Eq. 4.)

Arrhenius plots of k(chy-cat) are shown in Fig. 8. Again in this case, a clear discontinuity appeared at around 42 °C. Kinetic parameters, $\Delta G^{\neq}$, $\Delta H^{\neq}$ and $\Delta S^{\neq}$, were calculated at 298 K for the lower temperatures than 42 °C. Results are listed in Table II together with those for k(chy-chy). The value of $\Delta G^{\neq}$ for k(chy-cat) is 71.2 kJ mol^{-1} and is comparable with that for k(chy-chy), 64.0 kJ mol^{-1}. Namely, Cata is hydrolyzed slightly more slowly than Chym.

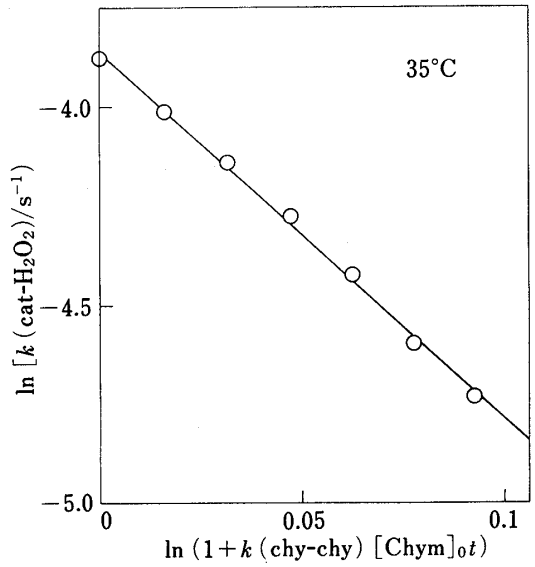

Fig. 6. Validity of Eq. 8 Examined at 35 °C and pH 7.8

See the text, k (cat-H_2O_2) is proportional to [Cata] as seen in Eq. 4. $[\text{Chym}]_0=6.07\times10^{-6}$ and $[\text{Cata}]_0=2.1\times10^{-7}$ mol dm^{-3}.

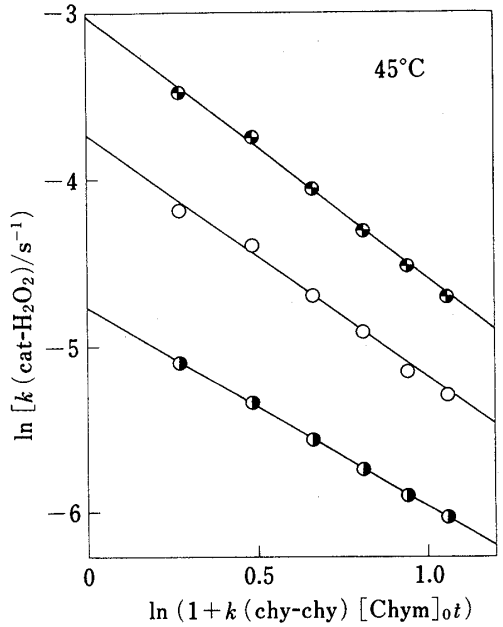

Fig. 7. Validity of Eq. 8 Examined at 45 °C and pH 7.8 with Various Initial Concentrations of Cata

$[\text{Chym}]_0=6.07\times10^{-6}$ mol dm^{-3}. $[\text{Cata}]_0=4.2$ (◒), 2.1 (○), 1.05 (◑), $\times10^{-7}$ mol dm^{-3}.

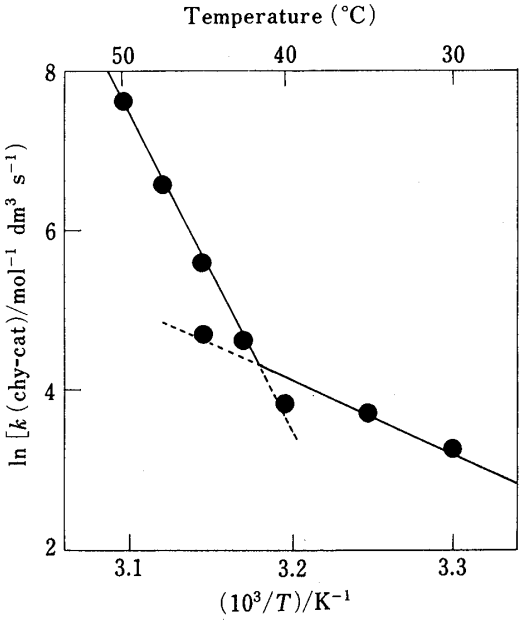

Fig. 8. Arrhenius Plots for the Second-Order Rate Constant k (chy-cat) Defined in Eq. 7

Values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$, 72.0 kJ mol^{-1} and 2.32 J K^{-1} mol^{-1}, are not so large as those of k(chy-chy), which are 270 kJ mol^{-1} and 681 J K^{-1} mol^{-1}, respectively. A mechanism involving preliminary thermal deformation of the enzyme on hydrolysis, as in the case of the autolysis of Chym, might not be operating for the reaction between Chym and Cata, presumably either because Cata is more refractory than Chym, or because the site of proteolysis may be near the surface layer of the globular Cata. In this connection, however, the large positive value of $\Delta G^{\neq}$, 71.2 kJ mol^{-1}, implies that the site is not easily accessible to Chym.

Discussion on the Mechanism

The considerably larger values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for k(chy-chy) than for k(chy-cat) may originate from the difference in thermal stability bwtween the two enzymes, as mentioned above; in other words, K(chy) is larger than K(cat) in the equations below.

$$\text{Chym (native)} \underset{\longleftarrow}{\overset{K(\text{chy})}{\longrightarrow}} \text{Chym' (unfolded)} \tag{9}$$

$$\text{Cata (native)} \underset{\longleftarrow}{\overset{K(\text{chy})}{\longrightarrow}} \text{Cata' (unfolded)} \tag{10}$$

As a matter of fact, kinetic analysis of chymotryptic hydrolysis of pNPA reveals a deviation from linearity at around 35 °C in the van't Hoff plots of ln K_m *vs.* $1/T$ as shown in Fig. 9. After correction for the deactivation of Chym due to autolysis, the deviation from the straight line still remains at around 37 °C. On the other hand, kinetic analysis of the decomposition of H_2O_2 with Cata reveals deviation from the straight line at around 44 °C in the Arrhenius plots of ln k(cat-H_2O_2) *vs.* $1/T$, as shown in Fig. 10.

Based on the above considerations, the bimolecular reaction of two globular proteins expressed as rate equations in Eqs. 5 or 7, k(chy-chy) or k(chy-cat) could be replaced by more detailed rate constants involving collisions between Chym(native) and Chym' (unfolded) or Chym (native) and Cata' (unfolded), as below.

$$-\frac{\text{d[Chym]}}{\text{d}t} = k(chy\text{-}chy)[\text{Chym}]^2 + k(chy\text{-}chy')[\text{Chym}][\text{Chym}']$$
$$= \{k(chy\text{-}chy) + k(chy\text{-}chy') \cdot K(\text{chy})\}[\text{Chym}]^2 \tag{11}$$

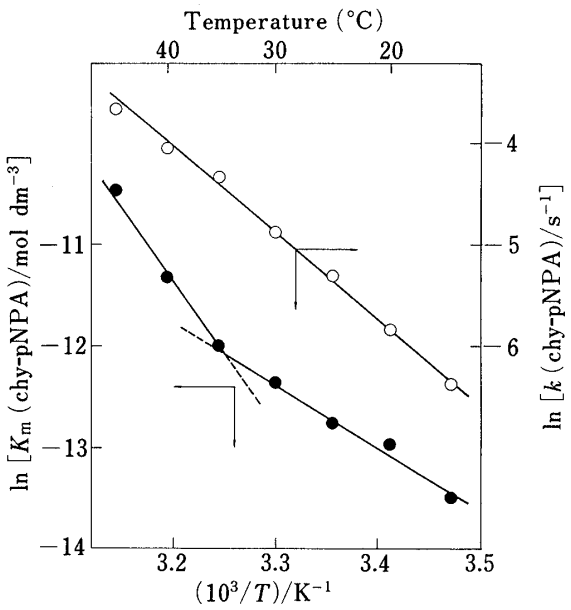

Fig. 9. Arrhenius Plots for k (chy-pNPA) and van't Hoff Plots for K_m (chy-pNPA) Defined in Eq. 2

Chylotryptic hydrolysis of pNPA was conducted at pH 7.8 and 15—45 °C.

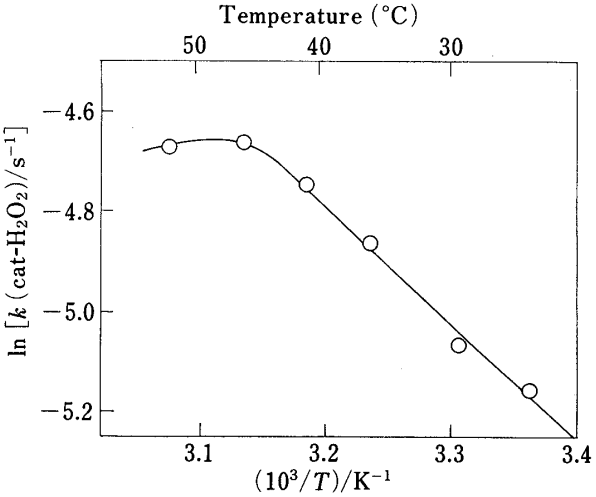

Fig. 10. Arrhenius Plots for the First-Order Rate Constant k (cat-H_2O_2) Defined in Eq. 4

Decomposition of H_2O_2 by catalase was conducted at pH 7.8 and 25—52 °C.

$$-\frac{d[Cata]}{dt}=\{k(chy\text{-}cat)+k(chy\text{-}cat')\cdot K(cat)\}[Chym][Cata] \quad (12)$$

Large values in $\Delta H^{\neq}$ and $\Delta S^{\neq}$ must arise from the second term of the overall rate constant. It is known that structural deformations of biomolecules often involve large enthalpy and entropy changes.[14,15] Consequently, the autolysis of Chym, Eq. 11, should proceed mainly through the second term of the overall rate constant, *i.e.*, $k(chy\text{-}chy')\cdot K(chy) \gg k(chy\text{-}chy)$, while the proteolysis of Cata, Eq. 12, should proceed *via* the first term, *i.e.*, $k(chy\text{-}cat) \gg k(chy\text{-}cat')\cdot K(cat)$.

One may anticipate postulation of equilibrium in Eqs. 9 and 10, because, in general, thermal deformation is either reversible or irreversible. But the proposed peptide chain unfolding in Eqs. 9 and 10 should be reversible so as not to conflict with the second-order kinetics obtained experimentally. Otherwise, irreversible bimolecular processes, such as Chym'+Chym', Chym'+Cata', or Cata'+Cata'→ entangled nonactive enzymes, should exist. Elucidation of the involvement of these processes remains to be done.

Another possible cause of the difference of rate-controlling step between the two reactions above is related to the three-dimensional structures of the two enzymes. For the catalyst Chym, there is no amino acid residue such as Tyr, Phe or Trp, which is susceptible to chymotryptic hydrolysis, on the accessible surface of the substrate Chym molecule, unless the peptide chain of the globular Chym is partly unfolded, or unless some relaxation takes place in the tertiary structure of Chym. On the other hand, the large refractory globular protein, Cata, has such cleavable sites near the surface layer, so that Chym could access the sites without peptide chain unfolding. At present, it is difficult to discriminate and estimate the contributions of the two causes quantitatively.

A further study is now under way to provide a basis for the above speculative discussions, that is, a study to get precise information about the site of initial cleavage in the three-dimensional structure of the protein substrates, combined with activation energy measurements.

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