Conformational Effect (Induced-Fit) on Catalytic Activity of α-Chymotrypsin

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The kinetics for the hydrolyses of *p*-nitrophenyl esters of acetic acid and certain amino acid derivatives mediated by α-chymotrypsin have been studied. The kinetics are a function of the medium viscosity, which indicate that the enzyme must change its conformation during the reaction. Detailed analysis of the dependence of kinetic rate constants on the medium viscosity has revealed that (1) the reaction is associated with the induced-fit conformational adjustment of the enzyme: When the enzyme-substrate (ES) complex is converted into the corresponding acyl enzyme, the conformation of the enzyme is changed in order to accommodate the acyl group in the pocket of the enzyme at an appropriate position. (2) The conformationally distorted α-chymotrypsin recovers its conformation when it releases the acid part of the substrate ester. (3) The formation of an ES complex is independent of any induced-fit movement. The induced-fit conformational adjustment of the enzyme plays a crucial role in its catalytic activity.

As a typical representative of serine proteinases, α-chymotrypsin has been subjected to many studies of the mechanism of enzymatic catalysis. At present, no one casts doubt on the proposal that the active center of α-chymotrypsin consists of the catalytic triad: Ser^{195}, His^{57}, and Asp^{102}.[1] However, after an initial claim by Rogers and Bruice,[2—4] controversial evidence and ideas have been proposed for the contribution of these three functional groups to the catalytic activity of the enzyme. The mechanism that is accepted today by the majority of chemists and biochemists is that the proton relay extends to the imidazolyl group of His^{57} and the carboxylate anion in Asp^{102} plays a role in stabilizing the imidazolium cation thus formed in proximity.[5] The presence of an oxyanion hole has also been proposed.[6]

A study on the kinetic solvent isotope effect based on the proton inventory technique has provided controversial results:[7] Only one proton is involved in the rate-determining step when *p*-nitrophenyl acetate is subjected to hydrolysis mediated by α-chymotrypsin, whereas two protons participate in the rate-determining step of hydrolysis when *p*-nitrophenyl 3-phenylpropanoate or *N*-acetyl-L-tryptophanamide is employed as a substrate.

The difference in reaction mechanism may be understood in terms of the induced-fit conformational adjustment:[8] The conformation of an enzyme changes depending on the structure of a substrate when it forms an enzyme-substrate (ES) complex with the substrate, so that the catalytic functional groups in the active site of the enzyme are arranged according to their most appropriate positions for the chemical reaction. Thus, the modified conformation is maintained (or continues to change) during the chemical reaction until the enzyme releases the reaction product(s) completely.

Although the above idea clearly explains the observation, there has been no report on the participation of conformational change in the reactions catalyzed by α-chymotrypsin, and the mechanistic interests have been focused mainly on the transformations of functional groups.

When the fluctuation of solvent molecules is fast enough compared with the chemical reaction at the rate-determining step, the rate constant, k_{TST}, which is defined by Eyring's absolute rate theory[9,10] is the rate constant that describes the overall reaction rate. On the other hand, when the medium viscosity becomes high and the rate constant for the fluctuation of solvent molecules, k_f, becomes low, then k_{TST} is not the sole index for describing the overall reaction rate. Instead, Eq. 1 should be applied for elucidating the overall rate constant, k, and it is known that k_f is correlated with viscosity, η, of the medium according to Eq. 2.[11,12]

$$k^{-1} = k_f^{-1} + k_{TST}^{-1} \quad (1)$$

$$k_f \propto \eta^{-\alpha} \qquad (0 < \alpha \leq 1) \quad (2)$$

For a substrate in an ES complex, the framework of the enzyme is the reaction medium, and the fluctuation of the backbone of the enzyme is equivalent to the fluctuation of solvent molecules for a reaction occurring in a homogeneous solution. Normally, the induced-fit conformational adjustment of an enzyme is a fast process, but it is anticipated that a slow rate for conformational adjustment of the enzyme may affect the net rate of certain enzymatic reactions. The rate for conformational change can be controlled by changing the viscosity of the medium. Indeed, there are several examples where the change in medium viscosity affects the rate for conformational change of an enzyme and, consequently, changes the overall reaction rate.[13—18]

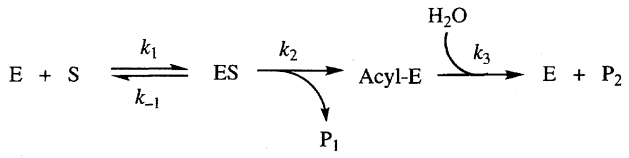
If the conformational change is associated with the reaction of α-chymotrypsin, as expected from proton inventory studies,[7,19] a series of reaction kinetics as a function

of medium viscosity will surely contribute to endorse the proposed process. Thus, we studied the kinetics of a series of reactions of α-chymotrypsin with *p*-nitrophenyl acetate and certain amino acid derivatives as substrates, and found that the formation of an acyl enzyme as an intermediate of the reaction requires the change in conformation of the enzyme in order to achieve a smooth reaction. The process of releasing carboxylic acid as the secondary product also requires the conformational change. The simplified reaction scheme is illustrated in Scheme 1, which will be revised as the discussion proceeds.

Results

Circular Dichromism Spectra. The effect of medium viscosity on the secondary structure of the enzyme were investigated by circular dichromism (CD) spectroscopy. CD spectra in the wavelength of 200—300 nm were recorded for the enzyme dissolved in water and in 45 wt% aqueous glycerol. The spectra shown in Fig. 1 are virtually identical, indicating that the secondary structure of the enzyme remains intact in a highly viscous medium.

Kinetic Analysis. The kinetics monitored by using a stopped-flow spectrophotometric apparatus reveal that the appearance of *p*-nitrophenolate anion, P_1, does not obey first-order kinetics even at the initial state of the reaction. Thus, the observed kinetics for the formation of P_1 are composed of initial fast and the following slow processes.[20—22] Since we measure the increase in the concentration of P_1, $[P_1]$, there is no doubt that the observed kinetics correspond to the formation of acylated α-chymotrypsin, AcE. It is well documented that the formation of acylated α-chymotrypsin takes place faster than its decomposition.[22,23] In other words, the acylated α-chymotrypsin is accumulated during the reaction.



P_1 : First product (from alcohol part)
P_2 : Second product (from acid part)

Scheme 1.

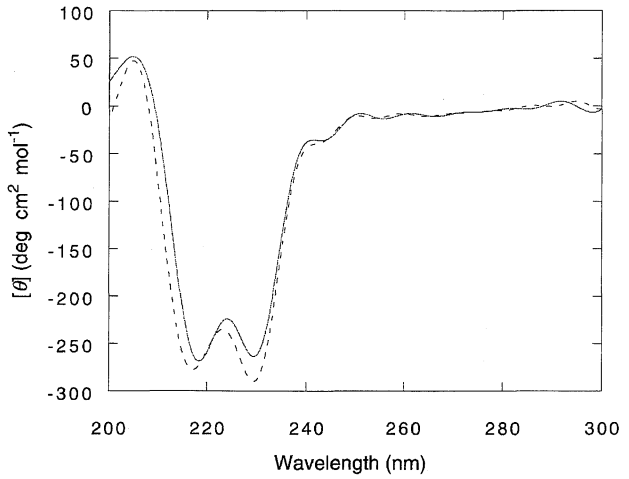


Fig. 1. CD spectra of α-chymotrypsin in water at pH 7.50 (solid line) and in 45 wt% glycerol in water at pH 7.50 (dotted line).

Thus, the experimentally available Michaelis constant, K_m^{ex}, obtained from the analysis of the second slow process does not correspond to the *true* Michaelis constant on the basis of ES complex, K_m, because, experimentally, we observed the increase in the concentration of AcE instead of that of ES. Therefore, it is necessary to elucidate a mathematical relationship between true and apparent Michaelis constants for the exact analysis of the present kinetic results.

When the steady-state condition for [ES] under $[S] \approx [S]_0$ is assumed, $[P_1]$ is given by Eq. 3.[24]

$$[P_1] = At + B(1 - e^{-Ct}), \tag{3}$$

where

$$A = \frac{k_{cat}}{1 + K_m^{ex}/[S]_0}[E]_0 \tag{4}$$

$$B = \frac{(k_{cat}/k_3)^2}{(1 + K_m^{ex}/[S]_0)^2}[E]_0 \tag{5}$$

$$C = k_3 + \frac{k_2}{1 + K_m/[S]_0} \tag{6}$$

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \tag{7}$$

and

$$K_m^{ex} = \frac{k_3}{k_2 + k_3} K_m. \tag{8}$$

When the decomposition of the acyl enzyme is slow, then total turnover becomes slow and we can observe initial fast kinetics corresponding to the single turnover process and the succeeding slow kinetics corresponding to the multi-turnover process independently. This is the case for *p*-nitrophenyl acetate (AcPNP, **1**). On the other hand, the initial fast process was unable to be detected with the other substrates, *p*-nitrophenyl *N*-benzyloxycarbonyl-L-alaninate (ZAlaPNP, **2**), *p*-nitrophenyl *N*-acetylglycinate (AcGlyPNP, **3**), *p*-nitrophenyl *N*-acetyl-L-alaninate (AcAlaPNP, **4**). Therefore, single turnover condition ($[E]_0 \approx 10[S]_0$) was employed for elucidating k_2 for these substrates. The observed rate constant k_{obsd} is correlated with k_2 by Eq. 9 under the conditions employed.[20]

$$\frac{1}{k_{obsd}} = \frac{1}{k_2} + \frac{K_m}{k_2} \cdot \frac{1}{[E]_0} \tag{9}$$

The k_2 step in the reaction with *p*-nitrophenyl *N*-acetyl-L-phenylalaninate (AcPhePNP, **5**) was so fast that it was impossible to evaluate the value even under the single turnover condition, and the rate constant k_3 for this substrate was calculated assuming $k_2 \gg k_3$.

Since K_m^{ex} and k_{cat} are experimentally observable quantities, and k_2 is thus available by plotting k_{obsd} against various concentrations of α-chymotrypsin, a combination of Eqs. 7 and 8 affords k_3 and K_m. Equation 8 predicts that K_m^{ex} approaches K_m when $k_2 \ll k_3$ holds.

Effect of Viscosity on Kinetic Parameters. The viscosity of the medium was changed by adding appropriate amounts of glycerol to buffer solutions of α-chymotrypsin as a viscogen. The kinetic parameters elucidated for the substrates **1** through **5** as functions of medium viscosity are summarized in Tables 1 through 5.

Figures 2 through 6 illustrate the dependence of K_m^{ex}, K_m, k_{cat}, k_2, and k_3, respectively, on medium viscosity, where the values at zero concentration of glycerol are employed as the standards and the ordinates are scaled by normalized values.

Discussion

Induced-Fit in Acylation Process. As expected from Eq. 8, it is proven that K_m^{ex} approaches K_m most closely at the viscosity where the difference between k_2 and k_3 is maximum, and K_m remains almost constant in all the substrates studied. This result, together with the unaltered CD spectrum of the enzyme with the change of medium viscosity, suggests that the association of the enzyme and a substrate, or the initial contact of the enzyme and a substrate, does not require appreciable changes in the conformation of the enzyme.

On the other hand, the rate constant k_2 decreases as the medium viscosity increases in all the substrates studied, which reveals that a chemical reaction that takes place between a substrate and the enzyme must be associated with the change in the conformation of the enzyme in order to accommodate an acyl group in its pocket appropriately to form the acyl enzyme. Then, Scheme 1 should be revised to Scheme 2, where E′ represents an enzyme conformationally different from E.

The possibility that the induced-fit conformational adjustment observed in the k_2 step is not related to the acyl-transfer process directly but is related to the release of *p*-nitrophenolate anion, P_1, will be discussed later in relation to the release of the second product, P_2, the acid part of the substrate. Our concept of the k_2 step includes both acyl-transfer

Table 1. Kinetic Parameters for the Hydrolysis of *p*-Nitrophenyl Acetate (AcPNP, **1**) Mediated by α-Chymotrypsin in Glycerol/Water Mixed Solvent[a,b]

Glycerol	Viscosity	K_m^{ex}	K_m	k_{cat}	k_2	k_3
wt%	mPa s	$\times 10^4$ M	$\times 10^3$ M	$\times 10\ s^{-1}$	s^{-1}	$\times 10\ s^{-1}$
0.00	0.801	0.091	2.2	0.34	8.0	0.34
		(0.002)	(0.2)	(0.01)	(0.2)	(0.01)
9.99	1.078	0.29	1.5	0.98	4.9	1.0
		(0.02)	(0.3)	(0.09)	(0.2)	(0.09)
20.1	1.489	0.86	1.4	3.1	3.4	3.5
		(0.04)	(0.2)	(0.1)	(0.3)	(0.1)
34.8	2.490	2.3	1.6	5.1	3.5	6.0
		(0.1)	(0.1)	(0.1)	(0.3)	(0.2)
41.3	3.039	3.1	1.7	5.4	2.9	6.7
		(0.2)	(0.2)	(0.1)	(0.1)	(0.1)

a) Reaction conditions: $T = 303 \pm 0.1$ K; pH = 7.50±0.02; $I = 0.2$ (KCl); CH_3CN 1.6% (v/v).
b) Standard errors are indicated in parentheses.

Table 2. Kinetic Parameters for the Hydrolysis of *p*-Nitrophenyl *N*-Benzyloxycarbonylalaninate (ZAlaPNP, **2**) Mediated by α-Chymotrypsin in Glycerol/Water Mixed Solvent[a,b]

Glycerol	Viscosity	K_m^{ex}	K_m	k_{cat}	k_2	k_3
wt%	mPa s	$\times 10^5$ M	$\times 10^4$ M	s^{-1}	s^{-1}	s^{-1}
0.00	0.801	2.04	2.2	2.4	26.3	2.6
		(0.08)	(0.1)	(0.1)	(0.1)	(0.1)
9.99	1.078	6.2	1.9	5.6	17.3	8.2
		(0.2)	(0.1)	(0.4)	(0.5)	(0.8)
25.1	1.492	5.0	0.68	8.2	11.0	32
		(0.1)	(0.08)	(0.6)	(0.5)	(5)
28.3	2.013	4.4	0.71	5.8	9.3	16
		(0.4)	(0.07)	(0.1)	(0.8)	(1)
34.8	2.490	2.7	0.86	2.5	7.9	3.6
		(0.4)	(0.07)	(0.1)	(0.8)	(1)
41.3	3.039	3.7	1.6	1.1	4.8	1.4
		(0.1)	(0.1)	(0.1)	(0.4)	(0.1)

a) Reaction conditions: $T = 303 \pm 0.1$ K; pH = 7.50±0.02; $I = 0.2$ (KCl); CH_3CN 1.6% (v/v).
b) Standard errors are indicated in parentheses.

Table 3. Kinetic Parameters for the Hydrolysis of *p*-Nitrophenyl *N*-Acetylglycinate (AcGlyPNP, **3**) Mediated by α-Chymotrypsin in Glycerol/Water Mixed Solvent[a,b]

Glycerol	Viscosity	K_m^{ex}	K_m	k_{cat}	k_2	k_3
wt%	mPa s	$\times 10^4$ M	$\times 10^3$ M	s^{-1}	s^{-1}	s^{-1}
0.00	0.801	0.58	1.7	0.560	16.2	0.560
		(0.06)	(0.2)	(0.002)	(0.2)	(0.003)
9.99	1.078	5.7	1.5	4.3	11.1	7.1
		(0.5)	(0.2)	(0.2)	(0.2)	(0.5)
25.1	1.492	10.0	1.2	7.6	9.0	45
		(0.1)	(0.2)	(0.7)	(0.2)	(5)
34.8	2.490	11.0	1.3	6.5	7.9	35
		(0.1)	(0.2)	(0.6)	(0.4)	(9)
42.0	3.474	6.3	1.4	3.1	6.7	5.5
		(0.2)	(0.1)	(0.9)	(0.5)	(0.6)

a) Reaction conditions: $T = 303 \pm 0.1$ K; pH = 7.50±0.02; $I = 0.2$ (KCl); CH_3CN 1.6% (v/v).
b) Standard errors are indicated in parentheses.

Table 4. Kinetic Parameters for the Hydrolysis of *p*-Nitrophenyl *N*-Acetylalaninate (AcAlaPNP, **4**) Mediated by α-Chymotrypsin in Glycerol/Water Mixed Solvent[a,b]

Glycerol	Viscosity	K_m^{ex}	K_m	k_{cat}	k_2	k_3
wt%	mPa s	$\times 10^4$ M	$\times 10^3$ M	$\times 10^{-1}$ s^{-1}	$\times 10^{-1}$ s^{-1}	$\times 10^{-1}$ s^{-1}
0	0.801	0.62	3.4	0.27	15.0	0.275
		(0.04)	(0.1)	(0.01)	(1.9)	(0.009)
9.90	1.074	6.1	6.7	1.12	11.6	1.25
		(0.6)	(0.4)	(0.05)	(1.3)	(0.08)
24.9	1.791	14.8	5.4	2.63	9.6	3.6
		(0.5)	(0.4)	(0.03)	(0.8)	(0.1)
34.1	2.435	27.0	4.9	3.8	6.9	8.6
		(0.9)	(0.4)	(0.1)	(0.6)	(0.5)
40.4	2.963	21.0	2.4	3.7	4.3	25.9
		(0.4)	(0.3)	(0.1)	(0.4)	(0.2)

a) Reaction conditions: $T = 303 \pm 0.1$ K; pH = 7.50±0.02; $I = 0.2$ (KCl); CH_3CN 1.6% (v/v).
b) Standard errors are indicated in parentheses.

Table 5. Kinetic Parameters for the Hydrolysis of *p*-Nitrophenyl *N*-Acetylphenylalaninate (AcPhePNP, **5**) Mediated by α-Chymotrypsin in Glycerol/Water Mixed Solvent[a,b]

Glycerol	Viscosity	K_m^{ex}	K_m	k_{cat}	k_2[c]	k_3
wt%	mPa s	$\times 10^5$ M	M	$\times 10^{-3}$ s^{-1}	s^{-1}	$\times 10^{-3}$ s^{-1}
0	0.801	0.35	—	0.101	—[d]	0.10[e]
		(0.03)		(0.003)		
9.90	1.074	2.4	—	1.01	—	1.01[e]
		(0.2)		(0.03)		
24.9	1.791	8.8	—	2.44	—	2.44[e]
		(0.3)		(0.02)		
34.1	2.435	7.5	—	1.74	—	—[f]
		(0.9)		(0.05)		
40.4	2.963	6.7	—	1.05	—	—[f]
		(0.6)		(0.03)		

a) Reaction conditions: $T = 303 \pm 0.1$ K; pH = 7.50±0.02; $I = 0.2$ (KCl); CH_3CN 1.6% (v/v).
b) Standard errors are indicated in parentheses. c) Too fast to be evaluated. d) $k_2 = 23700$ s^{-1} has been reported.[23] e) The condition of $k_2 \gg k_3$ was assumed in Eq. 7. f) It is uncertain whether the condition $k_2 \gg k_3$ can be assumed or not.

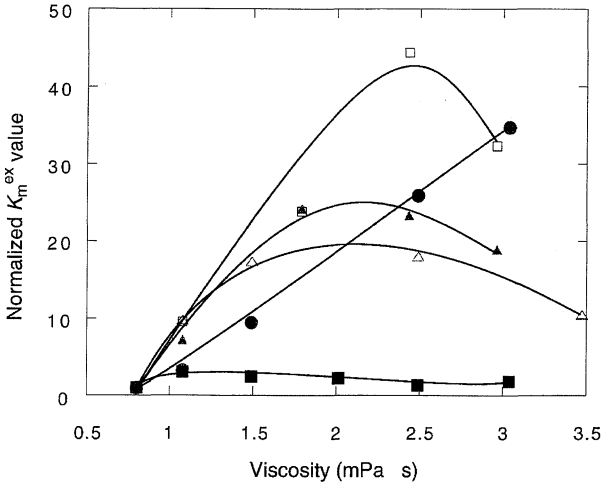


Fig. 2. Dependence of K_m^{ex} on medium viscosity. ●, **1**; ■, **2**; △, **3**; □, **4**; ▲, **5**.

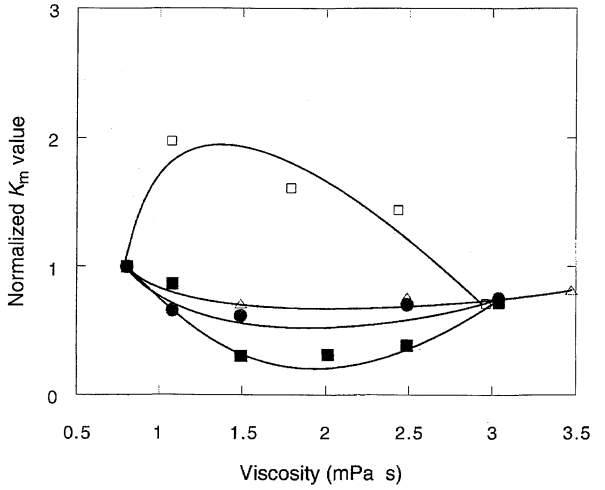


Fig. 3. Dependence of K_m on medium viscosity. ●, **1**; ■, **2**; △, **3**; □, **4**.

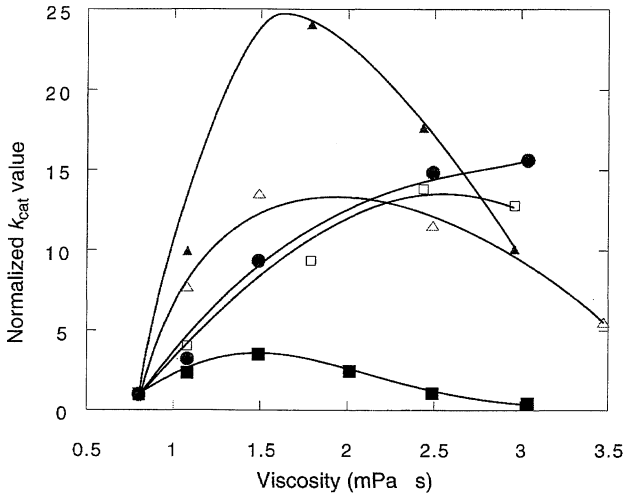


Fig. 4. Dependence of k_{cat} on medium viscosity. ●, **1**; ■, **2**; △, **3**; □, **4**; ▲, **5**.

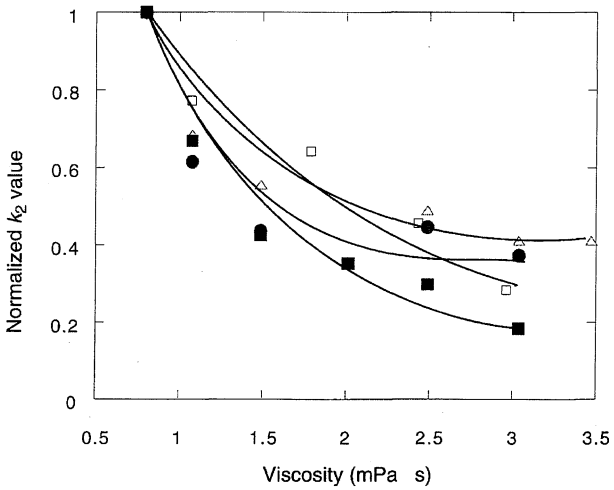


Fig. 5. Dependence of k_2 on medium viscosity. ●, **1**; ■, **2**; △, **3**; □, **4**.

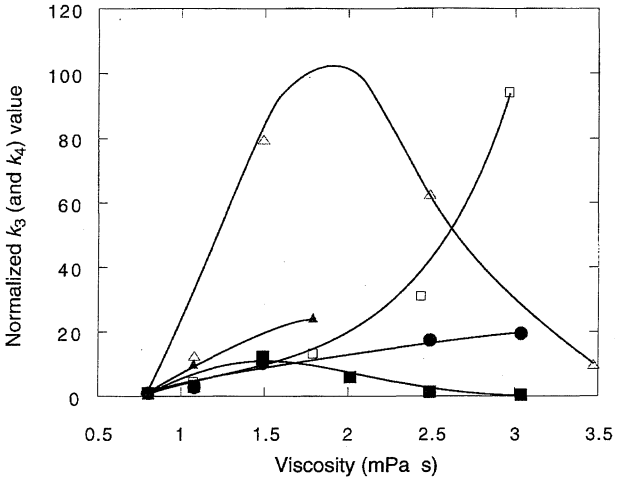
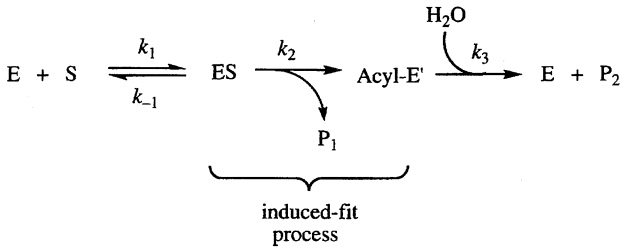


Fig. 6. Dependence of k_3 (and k_4) on medium viscosity. ●, **1**; ■, **2**; △, **3**; □, **4**; ▲, **5**.



Scheme 2.

and phenolate-releasing processes at this moment.

Dependence of k_2 on Medium Viscosity: The Value of α. The dependence of k_2 on medium viscosity was analyzed with respect to Eq. 2 and plots for each substrate are shown in Fig. 7.

Although α values for most of the substrates employed are reasonable ($0 < \alpha \leq 1$),[11,12] the value for **2**, which has a hydrophobic group in the molecule and is one of the most appropriate substrate for α-chymotrypsin hydrolysis among those analyzed,[25] is approximately 1.2, which is larger than unity. We believe that the discrepancy stems partly from

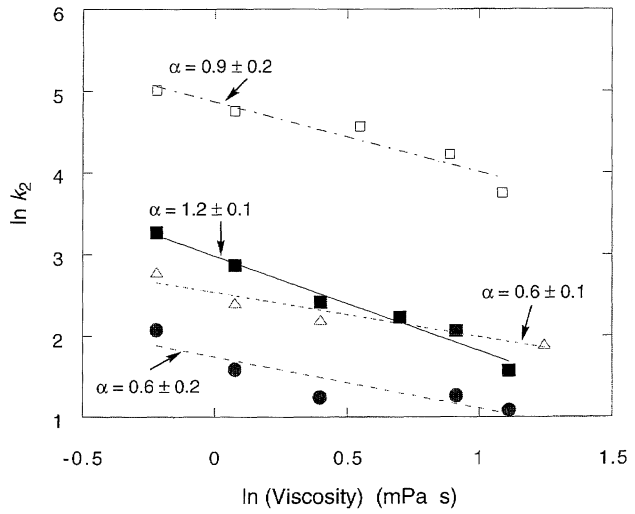


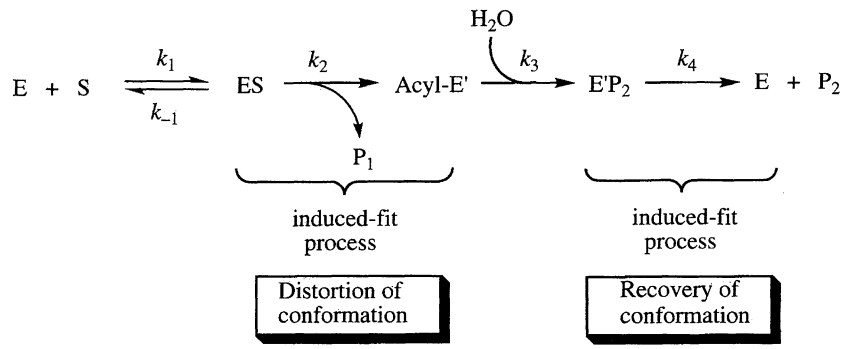
Fig. 7. Evaluation of α, the sensitivity of kinetic rate constant k_2 on the medium viscosity. ●, **1**; ■, **2**; △, **3**; □, **4**.

experimental error: The precision of the rate constant k_2 is unsatisfactory for discussing α in detail, because k_2 is included in a composite rate constant experimentally. There is, however, another reason for the ambiguity of α. As was mentioned in the introduction, the induced-fit movement plays a role in arranging the functional groups of the catalytic triad in the pocket of the enzyme in their correct positions by changing the total conformation of the enzyme in order to make them exert maximum catalytic activity. Therefore, if the conformation of an enzyme is inappropriate for catalysis, chemical reaction(s) that take place in the pocket must inevitably undergo a transition state higher in energy than those that proceed in a pocket of regular shape. In other words, the reaction scheme under the distorted conformation of the enzyme may be different from that obtained under normal conformation, and Eq. 2 cannot be applied to k_2 strictly. Thus, the complete separation of k_{TST} and k_f as defined by Eq. 1 is impossible experimentally in the present case, and the observed rate constant does not necessarily follow Eq. 2. This effect appears most significantly in the reaction of the most appropriate substrate, **2**. Unfortunately, because k_2 for **5** is too large to be measured, we cannot conclude the discussion without ambiguity at the moment.

In principle, the rate constant k_3 should also be correlated with α by Eq. 2. But the precision for this rate constant is worse, because of fewer experimental points (vide supra), than are available for k_2, and we wish to reserve the discussion on it.

Induced-Fit in Deacylation Process. An acyl enzyme with an unfavorable conformation, i.e., a conformation that was not adopted to accommodate the acyl group, tends to eject the acyl group from its pocket so as to stabilize itself as the free enzyme. Thus, the decrease in k_2 is coupled by an increase in k_3. However, the increase in k_3 with the increase in medium viscosity is not monotonic. Instead, it begins to decrease at about $\eta \approx 1.6$ mPa s. This is accounted for by the presence of an additional process in this step, which becomes unfavorable as the medium becomes more viscous. We assign this additional process to the product-releasing step with a rate constant of k_4. This assignment proposes that a conformation suitable for the acyl enzyme is also suitable for releasing P_2, the acid part of the substrate. In other words, the release of free acid from the enzyme of undistorted conformation is not a simultaneous process. The release of P_2 requires energy corresponding to the release of distortion energy that exists in the conformationally distorted enzyme, and Scheme 2 is now revised to Scheme 3 by taking into account the induced-fit conformational adjustment for the release of P_2. On one occasion, an EP_2 complex was isolated and subjected to X-ray crystallographic study.[6]

The shapes of the curves for k_{cat} shown in Fig. 4 are similar to those for k_3/k_4 shown in Fig. 6. This similarity stems from the facts that this step is more sensitive to the effect of induced-fit conformational adjustment than the k_2 step and the chemical (catalytic) reactivity of the enzyme is regulated by this process. It has been known that the rate-determining step in the enzymatic hydrolysis of an ester is the step in which the acyl enzyme is hydrolyzed into a free enzyme and an acid.[22] Absolute values for k_2 and k_3 listed in Tables 1 through 5, as well as substrate independence of the k_2 step shown in Fig. 5, also support the idea that the hydrolysis of acyl enzyme is (at least in part) the rate-determining step of the present reaction under normal conditions. However, interestingly, the rate-determining step is shifted to the k_2 step in the reactions occurring in highly viscous media for certain substrates. An exception is the reaction with **1**, where the rate-determining step is always in the k_3/k_4 step even in the most viscous medium studied.



Scheme 3.

Table 6. Kinetic Parameters for the Hydrolysis of *p*-Nitrophenyl Acetate (AcPNP, **1**) Mediated by α-Chymotrypsin in Sucrose Solutions Sucrose/Water Mixed Solvent[a,b]

Sucrose	Viscosity	K_m^{ex}	K_m	k_{cat}	k_2	k_3
wt%	mPa s	$\times 10^5$ M	$\times 10^3$ M	$\times 10\ s^{-1}$	s^{-1}	$\times 10\ s^{-1}$
0.00	0.801	0.91	2.2	0.34	8.0	0.34
		(0.02)	(0.2)	(0.01)	(0.2)	(0.01)
8.99	1.099	1.6	1.4	0.21	5.8	0.21
		(0.1)	(0.4)	(0.01)	(0.6)	(0.06)
12.2	1.295	6.4	2.5	0.32	9.0	0.32
		(1.4)	(0.2)	(0.02)	(0.5)	(0.03)
21.1	1.712	2.0	1.27	0.38	4.4	0.80
		(0.6)	(0.04)	(0.02)	(0.2)	(0.03)
26.5	2.213	0.91	1.4	0.31	4.8	0.33
		(0.1)	(0.2)	(0.36)	(0.3)	(0.01)
30.5	2.818	1.2	1.3	0.36	4.0	0.36
		(0.1)	(0.2)	(0.01)	(0.2)	(0.01)
34.2	3.437	1.1	1.0	0.45	4.0	0.46
		(0.1)	(0.1)	(0.01)	(0.3)	(0.01)

a) Reaction conditions: $T = 303\pm0.1$ K; pH = 7.50±0.02; $I = 0.2$ (KCl); CH_3CN 1.6% (v/v).
b) Standard errors are indicated in parentheses.

Table 7. Kinetic Parameters for the Hydrolysis of *p*-Nitrophenyl *N*-Benzyloxycarbonylalaninate (ZAlaPNP, **2**) Mediated by α-Chymotrypsin in Sucrose/Water Mixed Solvent[a,b]

Sucrose	Viscosity	K_m^{ex}	K_m	k_{cat}	k_2	k_3
wt%	mPa s	$\times 10^5$ M	$\times 10^4$ M	s^{-1}	s^{-1}	s^{-1}
0.00	0.801	2.04	2.2	2.4	26.3	2.6
		(0.08)	(0.1)	(0.1)	(0.1)	(0.1)
8.99	1.099	4.6	0.79	10.0	17.2	23.9
		(0.2)	(0.08)	(0.1)	(0.9)	(0.9)
21.6	1.790	5.3	0.7	9.16	11.5	45
		(0.5)	(0.1)	(0.05)	(0.6)	(6)
26.5	2.213	3.5	0.52	7.3	10.8	22.5
		(0.4)	(0.02)	(0.2)	(0.6)	(0.6)
30.5	2.810	1.4	0.71	5.8	7.0	12.6
		(0.1)	(0.07)	(0.1)	(0.4)	(0.5)
34.2	3.437	1.8	0.27	3.5	5.3	10.3
		(0.1)	(0.04)	(0.1)	(0.5)	(0.5)

a) Reaction conditions: $T = 303\pm0.1$ K; pH = 7.50±0.02; $I = 0.2$ (KCl); CH_3CN 1.6% (v/v).
b) Standard errors are indicated in parentheses.

The observation that the maximum position if any in the plot of k_3/k_4 step for **1**, shifts to higher viscosity than the other amino acid substrates indicates that the reaction of this substrate is less sensitive to induced-fit conformational adjustment than the reactions of other substrates. The result obtained herein agrees well with that obtained by proton inventory kinetics, where **1** was found to be associated by the movement of one proton, whereas other amino acid substrates were associated with the movement of two protons at their respective transition states.[7,19] In other words, **1** is not an appropriate substrate for studying the catalytic activity of α-chymotrypsin. Although, conventionally, **1** has been employed as a substrate in the study of catalytic activity of α-chymotrypsin, one should be very careful in extending the results obtained with **1** as a substrate to the discussion of the general mechanism of α-chymotrypsin hydrolysis.

If the product-releasing process for the first product P_1 is assumed to be similar to that for the second product P_2, then the reaction might be associated with an induced-fit conformational adjustment for the release of P_1. Since P_1 is *p*-nitrophenolate anion for all the substrates studied, it is quite reasonable that k_2 remains constant regardless of the substrate structure if the release of P_1 is responsible for the induced-fit conformational adjustment observed in the k_2 step. At the moment, we have no scientific evidence to determine whether the acyl-transfer reaction or the P_1-releasing process requires stronger assistance of induced-fit conformational adjustment. The substrate dependence of k_3, however, supports the idea that the stability of the acyl enzyme is dependent on the induced-fit conformational adjustment.

Activity of Proton and Water. Since the addition of the viscogen did not change the pH of the medium (7.50±0.02),

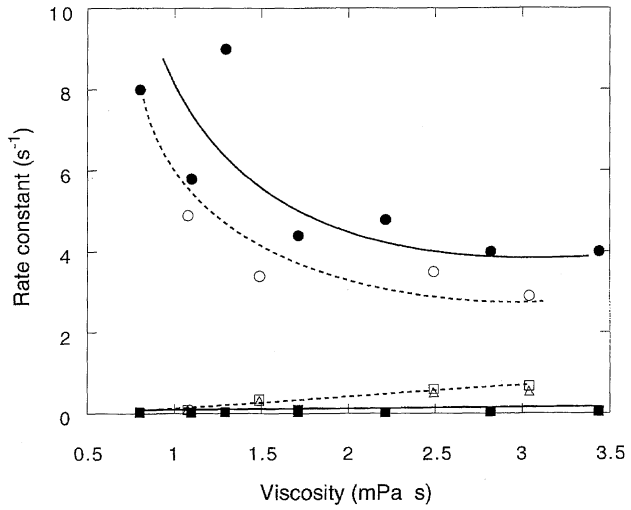


Fig. 8. Dependence of rate constants in hydrolysis of **1** on medium viscosity. ○, k_2; □, k_3; △, k_{cat} (in glycerol solutions); ●, k_2; ■, k_3; ▲, k_{cat} (in sucrose solutions).

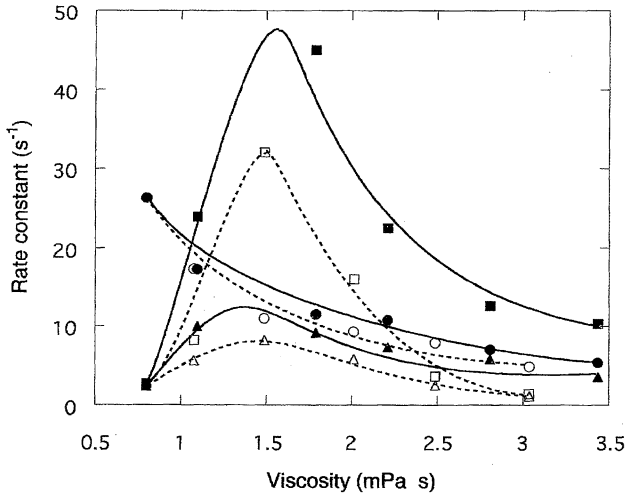


Fig. 9. Dependence of rate constants in hydrolysis of **2** on medium viscosity. ○, k_2; □, k_3; △, k_{cat} (in glycerol solutions); ●, k_2; ■, k_3; ▲, k_{cat} (in sucrose solutions).

we are convinced that the activity of the proton is not affected appreciably by the presence of the viscogen.

The plots shown in Figs. 1, 2, 3, 4, 5, and 6 should be corrected for the activity of water, which changes on addition of the viscogen to the reaction medium. Namely, the rate for deacylation (hydrolysis), the k_3-step, is a direct function of the activity of water. If we assume that the activity of water is proportional to its concentration,[26] then the activity of water is reduced roughly to 60% of the original in the most viscous medium so far studied. However, it has been reported that the activity of water is not affected appreciably by the addition of glycerol.[27—29] It should be noted that k_3 increases with the increase in viscosity. Thus, although the slight change in activity of water may affect the maximum point in the plot of k_3 vs. viscosity (Fig. 6), the discussion described above is not affected by the change in activity of water.

It has also been reported that all the proteins are preferentially hydrated on the addition of glycerol, which is the origin of the structure stabilization of a protein in aqueous glycerol.[30] Since the preference of hydration to a protein may relate to the property of viscogen, it is necessary to confirm that the effect observed herein is not specific to glycerol. In order to confirm the non-specific interaction between the viscogen and α-chymotrypsin, we also studied the viscosity-kinetics profile of the reaction with **1** and **2** as representative substrates by using sucrose as another viscogen (Tables 6 and 7). As shown in Figs. 8 and 9, almost identical profiles were obtained for two different viscogens, which reveals that the result and conclusion presently obtained are universal and do not depend on the nature of the viscogen.

Conclusion

Consequently, we now believe that both acylation/deacylation and release of products P_1/P_2 are dependent on the induced-fit conformational adjustment in the hydrolysis of an ester by α-chymotrypsin.[31] More results should be accumulated using substrates of various types before we reach a conclusion.

The effect of medium viscosity on the reaction rate reported herein is the first evidence, to our best knowledge, that enables one to assign the induced-fit conformational adjustment to a particular chemical step(s). Therefore, the induced-fit conformational adjustment as a metaphysical idea[8] has been armed with experimental evidence and has received a concrete physical meaning: The conformation of α-chymotrypsin in its ES complex changes to arrange the catalytic triad, and/or active functions in the oxyanion hole, at their appropriate positions in order to undergo acyl-transfer reactions from a substrate to the enzyme and from the acyl enzyme to water with reasonable velocity. The acyl group to be transferred is best accommodated in the conformationally distorted enzyme.

The distorted conformation of the enzyme in $E'P_2$ is contrasted to the ordinal conformation of the same enzyme in ES. If the equal (or similar) dependence of forward and reverse reactions on the induced-fit conformational adjustment can be assumed, we propose that the conformation E is suitable for the association of a material, whereas the conformation E′ stimulates the release of a material.

Experimental

Instruments. Circular dichromism (CD) spectra were recorded on a JASCO J-720W circular dichroism spectrophotometer. A Hitachi U-3210 spectrophotometer and a Union Giken RA-401 rapid reaction analyzer were used for kinetic measurements. Viscosity were measured by using an Ubbelohde viscometer at 303.0±0.1 K.

Materials. *p*-Nitrophenyl acetate (AcPNP, **1**) was purchased from Wako Pure Chemical Industries. Ltd. and recrystallized twice from ethyl acetate with a small amount of hexane. *N*-Benzyloxycarbonyl-L-alanine was prepared by the literature method[32] and converted into its *p*-nitrophenyl ester (ZAlaPNP, **2**) by the general method.[33] *p*-Nitrophenyl *N*-acetylglycinate (AcGlyPNP, **3**) was prepared from *N*-acetylglycine by the same method as described above. *p*-Nitrophenyl *N*-acetyl-L-alaninate (AcAlaPNP, **4**) and *p*-nitrophenyl *N*-acetyl-L-phenylalaninate (AcPhePNP, **5**) were pre-

pared by the literature methods, respectively.[34] Bovine pancreatic α-chymotrypsin (EC.3.4.21.1) was purchased from Sigma Chemical Co. (Type II grade, salt-free, lyophilized), and used without further purification. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, $pK_a = 7.41$ at 303 K), glycerol and sucrose were purchased from Nacalai Tesque. Inc. Glycerol was distilled under reduced pressure (bp 415 K, 399.9 Pa). Sucrose was used without further purification. Acetonitrile, which was used in order to improve solubility of the substrates to a buffer, was distilled on CaH_2 immediately before each use.

Medium Viscosity. Viscosity of the reaction medium was controlled by adding an appropriate amount of glycerol or sucrose to HEPES buffer (0.1 M, 1 M = 1 mol dm^{-3}).

CD Spectra. The effect of medium viscosity on the secondary structure of the enzyme was investigated by CD spectroscopy in the wavelength range of 200—300 nm. The enzyme was dissolved into Tris-HCl buffer at pH = 7.50±0.02 and ionic strength of 0.2 with or without 45 wt% glycerol.

Kinetic Measurements. A spectrophotometer and a rapid reaction analyzer were used for kinetic measurements. The former was used for steady-state measurements for **1**; it was equipped with an EPSON PC-286US personal computer. Data at every 6 s were input into the computer. The latter instrument was used for steady-state measurements for **2** through **5**, for the measurements of initial stage of the reactions (up to about 30 s after the scan) with **1**, and for the measurements under the conditions with large excess amounts of enzyme (single-turnover experiment) for **2** through **4**, respectively. The analyzer was equipped with an EPSON PC-286VE personal computer; data at 256 points during the scan were input into the computer. Both spectrophotometers were equipped with thermostated cell compartments so that the measurements could be done at desired temperatures.

Kinetics were measured by following the increase in absorbance at 400 nm, where the molar absorbance of *p*-nitrophenolate is 13800 at pH 7.50. Reaction conditions were kept constant over all kinetic measurements: temperature 303.0±0.1 K, concentration of the buffer 0.1 M, pH 7.50±0.02, ionic strength 0.2 (adjusted with KCl), and concentration of acetonitrile 1.6% (v/v).

As a typical procedure of these experiments, α-chymotrypsin (5 mg, ca. 2×10^{-7} mol) was dissolved into the buffer or to a mixture of glycerol and the buffer (2.0 cm^3), and the solution was diluted appropriately. A stock solution of a substrate was prepared by dissolving 0.5—0.8 mmol of the substrate to 10 cm^3 acetonitrile and diluted appropriately. The buffer or the mixture of glycerol and the buffer (3.0 cm^3) was placed in a curvet set in a thermostated compartment of the spectrophotometer. Then, after 50 mm^3 of a substrate solution was added, the mixture was shaken to make it homogeneous. Absolute absorbance of this solution was measured, and 80 mm^3 of the solution of enzyme was introduced. A measurement is composed of data with more than 6 different substrate concentrations. The initial concentrations of **1** for steady-state measurements are as follows: $[S]_0 = 8.30\times10^{-6}$—$1.20\times10^{-3}$ M and $[E]_0 = 8.53\times10^{-7}$—$6.22\times10^{-6}$ M. The concentrations of the substrate range from 0.5 to $5K_m^{ex}$. $[S]_0 \geq 10\ [E]_0$ was kept in each measurement.

A typical procedure of experiments with the stopped-flow apparatus is as follows: The stock solution of the enzyme was prepared by dissolving 20 mg of the enzyme into 50 cm^3 of the buffer or a mixture of viscogen and the buffer. A stock solution of a substrate was prepared in the same manner as that described for the measurements with the spectrophotometer. A solution for kinetic measurement was prepared by taking 1—16 mm^3 of the stock solution of a substrate and diluting it with incubated buffer at 303 K immediately before the measurement. About 3 cm^3 of the enzyme solution was incubated for 15 min in a reservoir on the stopped-flow apparatus, and mixed with the substrate solution at 303 K. Data for more than 6 different substrate concentrations were obtained for each measurement.

The initial concentrations of the substrates for steady-state measurements are as follows: $[S]_0 = 7.90\times10^{-6}$—$1.61\times10^{-4}$ M, $[E]_0 = 4.37\times10^{-7}$—$2.31\times10^{-6}$ M for **2**; $[S]_0 = 2.22\times10^{-5}$—$2.00\times10^{-3}$ M, $[E]_0 = 9.78\times10^{-7}$—$6.00\times10^{-6}$ M for **3**; $[S]_0 = 8.27\times10^{-5}$—$2.63\times10^{-3}$ M, $[E]_0 = 1.02\times10^{-6}$—$4.00\times10^{-6}$ M for **4**; $[S]_0 = 7.89\times10^{-7}$—$3.59\times10^{-4}$ M, $[E]_0 = 1.06\times10^{-7}$—$2.32\times10^{-6}$ M for **5**. $[S]_0 \geq 8\ [E]_0$ was kept in each measurement. The initial concentrations of the substrates in the measurement of initial stage of the reaction with **1** are as follows: $[S]_0 = 1.16\times10^{-4}$—$1.49\times10^{-3}$ M and $[E]_0 = 1.03\times10^{-5}$—$2.20\times10^{-5}$ M. $[S]_0 \geq 8K_m^{ex}$ was kept in each measurement. The initial concentrations of the substrates in single-turnover experiments are as follows: $[S]_0 = 3.51\times10^{-7}$—$6.70\times10^{-7}$ M, $[E]_0 = 3.50\times10^{-6}$—$4.50\times10^{-5}$ M for **2**; $[S]_0 = 6.29\times10^{-7}$—$1.00\times10^{-6}$ M, $[E]_0 = 6.28\times10^{-6}$—$4.15\times10^{-5}$ M for **3**; $[S]_0 = 6.65\times10^{-7}$—$8.10\times10^{-7}$ M, $[E]_0 = 6.79\times10^{-6}$—$5.07\times10^{-5}$ M for **4**. $[E]_0 \geq 10\ [S]_0$ was kept in each measurement.

The observed rate constants for substrates **1**, **3**, and **4** were corrected for spontaneous release of *p*-nitrophenolate anion. The correction was neglected for **2** and **5**, however, because the rates with these substrates were too slow compared with that of enzymatic hydrolysis.

Determination of Effective Concentration of the Enzyme. A solution of α-chymotrypsin was titrated by **1** following the method proposed by Bender et al.[22] In Eq. 5, $B \approx [E]_0$ when $k_2 \gg k_3$ and $[S]_0 \gg K_m^{ex}$. Hydrolysis of **1** was followed by the stopped-flow apparatus under the condition of $[S]_0 = 8K_m^{ex}$ to elucidate exact concentration of the enzyme, $[E]_0$, prior to the kinetic measurement.

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