Kinetic study on conformational effect in hydrolysis of *p*-nitroanilides catalyzed by α-chymotrypsin

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Received (in Cambridge, UK) 2nd September 1999, Accepted 4th February 2000

Effects of medium viscosity on kinetics for the hydrolysis of *p*-nitroanilides of certain amino acid derivatives catalyzed by α-chymotrypsin have been investigated. Observed data indicate that the overall rate constant, k_{cat}, is hardly affected by the medium viscosity in all the substrates employed and equals the rate constant at the acylation step, k_2, in the measured range of viscosity. By comparison with the data on *p*-nitrophenyl ester substrates reported previously, it is concluded that the formation of the tetrahedral intermediate in the course of the acylation of the enzyme is influenced by conformational change of the enzyme, whereas the breakdown of the intermediate is almost free from conformational effects.

Introduction

In recent years, chemists and biochemists have recognized that, without doubt, conformational change of an enzyme is essential for its catalytic activity. "Induced-fit theory" is one of the most popular hypotheses in mechanisms of an enzymatic reaction, which proposes conformational movement of an enzyme during the reaction. According to this theory, interactions between an enzyme and a suitable substrate induce conformational change of the enzyme so that the reactive groups at the active site are forced into an arrangement that results in crucial catalytic works.[1,2] Induced-fit hypothesis explains clearly the observed phenomena such as substrate selectivity and acceleration of a reaction. However, the theory has not been based upon direct experimental studies, but is merely hypothetical.

Although many papers report that conformational change of an enzyme controls enzymatic function,[3,4] the movement of an enzyme has been paid less attention than the catalytic work done by the reactive groups at the active site in studies on enzymatic reaction mechanisms.

α-Chymotrypsin is a representative of serine proteases and catalyzes, *in vivo*, the hydrolysis of an amide bond adjacent to a carboxylic group of an amino acid having a bulky and hydrophobic side chain (namely Phe, Trp or Tyr) which stems from the catalytic function of the reactive group composed of Ser^{195}, His^{57} and Asp^{102} (ref. 5). The mechanism of catalysis by these amino acid residues has almost been clarified.[6,7] This mechanism contains the formation of the acyl–enzyme, the general acid–base catalysis by Ser^{195} and His^{57}, and the stabilization of the imidazolium cation of His^{57} by the carboxylate anion of Asp^{102} both in acylation and deacylation of the enzyme. The presence of an oxyanion hole has also been proposed.[7] Spectrophotometric studies, *i.e.*, the difference spectrum between a free enzyme and an acyl–enzyme in the UV region,[8,9] the change in specific rotation[10] and so on, have suggested that conformational change of α-chymotrypsin takes place in the course of the reaction. A kinetic study using a competitive inhibitor, proflavin, also suggests reversible conformational change of the enzyme.[11] Furthermore, according to recent research by ^{1}H-NMR and X-ray crystallography,[12] binding of the peptidyl trifluoromethyl ketone as an irreversible inhibitor to the active site of α-chymotrypsin leads to shortening of the distance between $N^{\delta 1}$ on His^{57} and $O^{\gamma 2}$ on Asp^{102}, which acts as a "trigger" for the reaction. Based upon this research, we expect that some induced-fit processes exist in the course of an α-chymotrypsin-catalyzed reaction. However, since irreversible inhibitors or unusual reaction conditions were employed in these investigations, it is not obvious whether the results mentioned above can be extended to actual enzymatic reactions or not.

It has been reported that a dependence of the rate constant of a reaction in solution upon solvent fluctuation is observed as the viscosity of the medium becomes large.[13–16] Since the fluctuation of solvent molecules is relatively fast, compared with the chemical reaction at the rate-determining step under normal conditions, the rate constant, k_{TST}, which is defined by Eyring's absolute rate theory, 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, eqn. (1) should

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

be applied to elucidate the overall rate constant, k. It is known that k_f is related to the viscosity of the medium, η, according to eqn. (2) (refs. 15 and 16).

$$k_f \propto \eta^{-a} \quad (0 < a \leqslant 1) \quad (2)$$

For a substrate in an enzyme–substrate (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. In other words, the movement of an enzyme can be regarded as a kind of Brownian motion and it is influenced by solvent fluctuations. Therefore, the rate constant of a step where the conformational effect of an enzyme is significant must be affected by medium viscosity. Indeed, there are several examples where the rate of an enzymatic reaction or isomerization of a protein depends upon the medium viscosity.[17–19]

In a previous paper,[20] we reported the effect of medium viscosity on the hydrolysis of *p*-nitrophenyl esters catalyzed by α-chymotrypsin. As the medium viscosity increases, the rate of acylation of the enzyme decreases, whereas the deacylation rate increases and then begins to decrease at $\eta \approx 1.6$ mPa s. From these results, we proposed that induced-fit processes operate at

DOI: 10.1039/a907114h

Table 1 Kinetic parameters for the hydrolysis of *N*-succinyl-L-phenylalanine *p*-nitroanilide (Suc**F**-PNA, **1**) mediated by α-chymotrypsin in glycerol–water mixed solvent[a,b]

Glycerol (wt%)	Viscosity/mPa s	$K_M/10^{-3}$ M	$k_{cat}(\sim k_2)/10^{-2}$ s^{-1}
0.00	0.801	2.1 (0.2)	4.7 (0.2)
11.4	1.157	2.43 (0.09)	4.8 (0.1)
21.9	1.585	2.7 (0.3)	5.0 (0.2)
30.0	2.098	3.5 (0.2)	6.0 (0.2)
35.0	2.535	4.3 (0.3)	6.3 (0.2)
41.0	3.220	5.6 (0.4)	6.8 (0.3)

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

Table 2 Kinetic parameters for the hydrolysis of *N*-succinyl-L-prolyl-L-phenylalanine *p*-nitroanilide (Suc**PF**-PNA, **2**) mediated by α-chymotrypsin in glycerol–water mixed solvent[a,b]

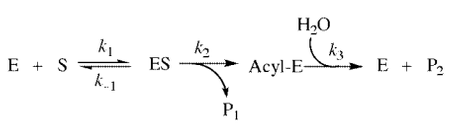
Glycerol (wt%)	Viscosity/mPa s	$K_M/10^{-3}$ M	$k_{cat}(\sim k_2)$/s^{-1}
0.00	0.801	1.9 (0.1)	0.72 (0.02)
6.68	0.993	2.3 (0.2)	0.75 (0.03)
10.1	1.100	3.3 (0.3)	0.85 (0.03)
17.4	1.372	1.6 (0.3)	0.68 (0.04)
28.9	2.011	2.2 (0.2)	0.57 (0.02)
34.8	2.491	3.5 (0.3)	0.72 (0.03)

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

the acylation of the enzyme and the release of the carboxylic acid part from the enzyme. It is also important to investigate the effect of medium viscosity on hydrolysis of an amide substrate, since this is the main function of α-chymotrypsin. Furthermore, a difference in the catalytic mechanism between an ester substrate and an amide substrate has been proposed in a study on the kinetic solvent isotope effect based on the proton inventory technique.[21] We have therefore studied the effect of medium viscosity on the hydrolysis of *p*-nitroanilides and would like to propose here that the formation of a tetrahedral intermediate in the course of the acylation of the enzyme is the subject of the conformational effect of the enzyme.

Results

The simple kinetic scheme is illustrated in Scheme 1. The analysis of kinetic data was performed according to this scheme.



Acyl-E : Acyl enzyme
P_1 : First product (from alcohol part)
P_2 : Second product (from acid part)

Scheme 1

The steady-state kinetics for the hydrolysis of a *p*-nitroanilide of an amino acid derivative were measured by following the appearance of *p*-nitroaniline, P_1, with a UV-VIS spectrophotometer or a stopped-flow apparatus. The kinetic parameters elucidated for *p*-nitroanilide substrates: *N*-succinyl-L-phenylalanine *p*-nitroanilide (Suc**F**-PNA, **1**), *N*-succinyl-L-prolyl-L-phenylalanine *p*-nitroanilide (Suc**PF**-PNA, **2**), and *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (Suc**AAPF**-PNA, **3**), are listed in Tables 1–3. Viscosity profiles of kinetic parameters, K_M and k_{cat} (normalized with the value at no viscogen), are illustrated in Figs. 1 and 2, respectively.

It has been reported that the step from ES to Acyl–E (k_2-step) is rate-determining in the hydrolysis of an amide mediated by α-chymotrypsin,[22–24] that is to say, $k_{cat} \approx k_2$. On the other hand, normally, the step for the hydrolysis of Acyl–E (k_3-step) is rate-determining in the hydrolysis of esters.

However, when the viscosity of the medium becomes higher, sometimes a reversal in the velocity of the k_2 and k_3 steps takes place and the k_2-step becomes rate-determining for some ester substrates.[20] Therefore, it is necessary to confirm that the k_2-step remains the rate-determining step throughout the media studied presently for the amide substrates.

For this purpose, we investigated the dependence of k_{cat} on viscosity for *p*-nitrophenyl *N*-succinyl-L-phenylalaninate (Suc**F**-PNP, **4**) and *p*-nitrophenyl *N*-succinyl-L-prolyl-L-phenylalaninate (Suc**PF**-PNP, **5**), having the same amino acid part as **1** and **2**, respectively, and compared it to that for amide substrates. The results are summarized in Tables 4 and 5.

The value of k_{cat} for the hydrolysis of these esters is at least three orders of magnitude larger than that for the hydrolysis of amides in the measured range of the medium viscosity, which proves that the rate-determining step in the hydrolysis of amides lies in the k_2-step.

As both amide and ester substrates afford the same Acyl–E, the k_3-step for both substrates should coincide. The fact that we

Table 3 Kinetic parameters for the hydrolysis of *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (Suc**AAPF**-PNA, **3**) mediated by α-chymotrypsin in glycerol–water mixed solvent[a,b]

Glycerol (wt%)	Viscosity/mPa s	$K_M/10^{-5}$ M	$k_{cat}(\sim k_2)$/s^{-1}
0.00	0.801	5.8 (0.5)	57 (2)
10.1	1.101	4.9 (0.3)	50.1 (0.3)
28.9	2.011	7.7 (0.2)	55.5 (0.4)
34.8	2.491	9.0 (0.6)	48.3 (0.9)
44.2	3.546	8.7 (0.5)	48.9 (0.8)

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

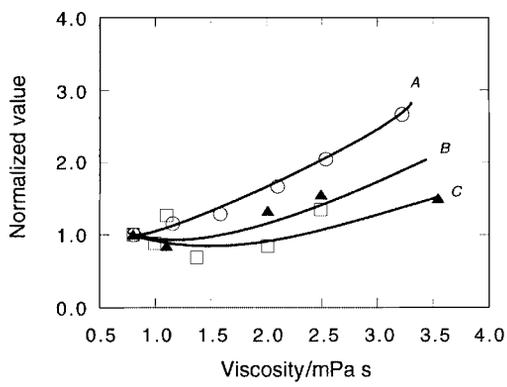


Fig. 1 Dependence of K_M for hydrolysis of *p*-nitroanilides on medium viscosity. (○) Suc**F**-PNA (**1**) (Curve *A*); (□) Suc**PF**-PNA (**2**) (Curve *B*); (▲) Suc**AAPF**-PNA (**3**) (Curve *C*).

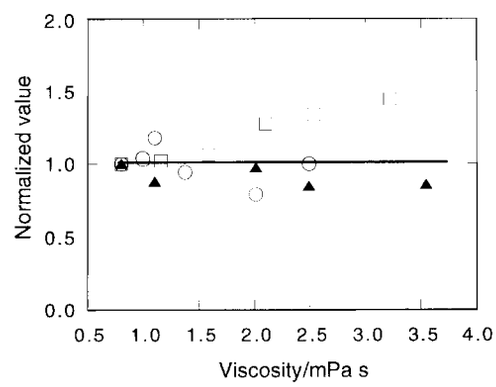


Fig. 2 Dependence of k_{cat} ($\sim k_2$) for hydrolysis of *p*-nitroanilides on medium viscosity. (○) Suc**F**-PNA (**1**); (□) Suc**PF**-PNA (**2**); (▲) Suc**AAPF**-PNA (**3**).

Table 4 Kinetic parameters for the hydrolysis of *p*-nitrophenyl *N*-succinyl-L-phenylalaninate (Suc**F**-PNP, **4**) mediated by α-chymotrypsin in glycerol–water mixed solvent[a,b]

Glycerol (wt%)	Viscosity/mPa s	$K_M^{ex}/10^{-4}$ M[c]	$k_{cat}/10^2$ s^{-1}
0.00	0.801	0.15 (0.01)	1.04 (0.02)
11.2	1.150	1.9 (0.1)	17.9 (0.1)
34.1	2.435	3.8 (0.4)	16.5 (0.8)
41.4	3.273	2.1 (0.2)	13.5 (0.5)

[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 error indicated in parentheses. [c] K_M^{ex} is the apparent Michaelis constant: $K_M^{ex} = k_3K_M/(k_2 + k_3)$.

Table 5 Kinetic parameters for the hydrolysis of *p*-nitrophenyl *N*-succinyl-L-prolyl-L-phenylalaninate (Suc**PF**-PNP, **5**) mediated by α-chymotrypsin in glycerol–water mixed solvent[a,b]

Glycerol (wt%)	Viscosity/mPa s	$K_M^{ex}/10^{-5}$ M[c]	$k_{cat}/10^2$ s^{-1}
0.00	0.801	0.50 (0.05)	0.69 (0.02)
10.8	1.130	3.3 (0.5)	3.3 (0.1)
21.0	1.862	9 (1)	4.4 (0.2)
32.9	2.357	14 (3)	4.3 (0.3)
43.6	3.048	25 (1)	4.9 (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 error indicated in parentheses. [c] K_M^{ex} is the apparent Michaelis constant: $K_M^{ex} = k_3K_M/(k_2 + k_3)$.

observed three orders of magnitude difference in reaction rates of ester and amide substrates indicates that the k_2-step is slow and rate-determining at least for the amide substrates throughout the range of medium viscosity studied.

In an α-chymotrypsin-catalyzed reaction, the Michaelis constant obtained in the steady-state measurement is the apparent value, K_M^{ex}, given by eqn. (3), where K_M is the *true* Michaelis constant given by eqn. (4).

$$K_M^{ex} = K_M \frac{k_3}{k_2 + k_3} \quad (3)$$

$$K_M = \frac{k_{-1} + k_2}{k_1} \quad (4)$$

However, in the case of the present system with amide substrates, since it has been confirmed that $k_2 \ll k_3$, then $K_M^{ex} = K_M$.

Discussion

Conformational effect on K_M

In all the *p*-nitroanilides studied, K_M increases slightly with the increase in viscosity, but the maximum variation is within 2.6 times the value at no viscogen. In our previous paper, we claimed that such a small variation is not at all significant when compared with other kinetic parameters; *e.g.* the variation of k_3 in hydrolysis of *p*-nitrophenyl esters is more than 50 times. That is, K_M is little affected by viscosity, and hence by conformational change of the enzyme.[20] It is concluded, therefore, that the association, or the initial contact of the enzyme and a substrate, does not require appreciable conformational change of the enzyme in the reaction of amides, as is also true for the reaction of esters.

Conformational effect on k_2-step

As described above, k_{cat} is identical to k_2 in the hydrolysis of *p*-nitroanilides catalyzed by α-chymotrypsin even in viscous media. The variation in k_{cat}, however, is so small that the value stays constant even at high viscosity. According to our previous paper,[20] k_2 for the hydrolysis of *N*-protected *p*-nitrophenyl esters

Table 6 Kinetic parameters for the hydrolysis of *N*-succinyl-L-leucine *p*-nitroanilide (Suc**L**-PNA, **6**) mediated by α-chymotrypsin in glycerol–water mixed solvent[a,b]

Glycerol (wt%)	Viscosity/mPa s	$K_M/10^{-3}$ M	$k_{cat}(\sim k_2)/10^{-4}$ s^{-1}
0.00	0.801	6.0 (0.4)	5.7 (0.2)
9.62	1.085	6.2 (0.5)	5.6 (0.2)
19.6	1.470	7.5 (0.6)	6.1 (0.3)
33.3	2.357	6.6 (0.4)	5.6 (0.2)
40.0	3.011	8.7 (0.4)	5.6 (0.2)

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

Table 7 Kinetic parameters for the hydrolysis of *p*-nitrophenyl *N*-succinyl-L-leucinate (Suc**L**-PNP, **7**) mediated by α-chymotrypsin in glycerol–water mixed solvent[a,b]

Glycerol (wt%)	Viscosity/mPa s	$K_M/10^{-4}$ M	k_2/s^{-1}
0.00	0.801	2.8 (0.4)	81 (11)
11.0	1.130	2.4 (0.3)	67 (7)
26.7	1.862	2.2 (0.3)	46 (4)
33.3	2.357	2.2 (0.3)	36 (4)
40.2	3.048	1.9 (0.3)	26 (3)

[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 error indicated in parentheses.

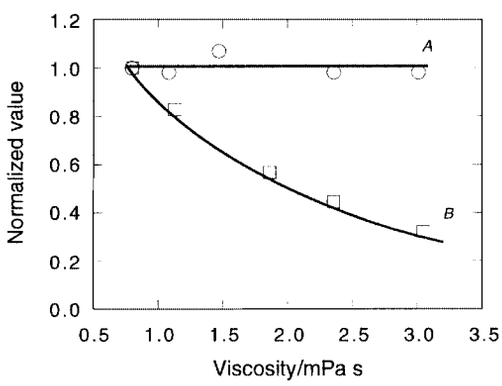


Fig. 3 Dependence of k_2 on medium viscosity. (○) Suc**L**-PNA (**6**) (Curve *A*); (□) Suc**L**-PNP (**7**) (Curve *B*).

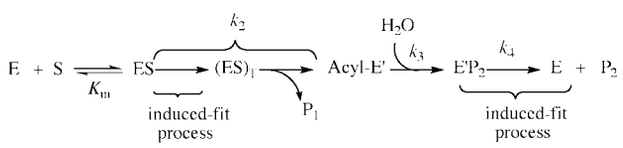
decreases monotonously with the increase in viscosity and falls to about one fifth of the value at no viscogen. Therefore, it is concluded that the k_2-step in the reaction of an ester substrate is significantly affected by conformational change of the enzyme, whereas that of an anilide substrate is hardly influenced at all.

To make sure of the tendency, we attempted to measure k_2 for a *p*-nitrophenyl ester and a *p*-nitroanilide which have the same amino acid part. Unfortunately, the k_2-step for *p*-nitrophenyl esters of phenylalanine derivatives is too fast to allow us to determine k_2 by our method (single-turnover kinetics). However, we were able to obtain the viscosity-profile of k_2 for L-leucine derivatives. In general, it is believed that α-chymotrypsin catalyzes hydrolysis of a peptide by recognizing the side chain of Phe, Tyr and Trp. Because L-leucine also has a bulky and hydrophobic side chain, this amino acid residue might also be recognized by α-chymotrypsin. The results are summarized in Tables 6 and 7. The viscosity-profile of k_{cat} (normalized with the value at no viscogen) is illustrated in Fig. 3.

The results represent the same trend as those described above and k_{cat} of the *p*-nitroanilide remained constant throughout the viscosity studied. Brouwer *et al.* reported that the k_{cat} for the hydrolysis of *N*-acetyltryptophan *p*-nitroanilide remains constant with an increase in the concentration of sucrose,[25] which is the same result as those observed in our research. Furthermore, it was reported and confirmed in the previous paper that the viscosity-profiles of the kinetic parameters for the hydrolysis of

p-nitrophenyl esters have the same trend both in glycerol and sucrose solutions.[20] Therefore, we have no doubt that the results and the discussions described in this paper are also universal and the kinetics do not depend on the nature of the viscogen.

A tetrahedral intermediate exists between an enzyme–substrate (ES) complex and an acyl–enzyme.[26–29] Furthermore, it is known that the formation of a tetrahedral intermediate is the step which determines k_2 in the case of *p*-nitrophenyl ester substrates, whereas it is the breakdown of the intermediate which determines k_2 in the reaction of *p*-nitroanilide substrates.[26,30] This change of the k_2-determining step depends on the pK_a of the leaving group (*p*-nitrophenol or *p*-nitroaniline in this research), which is the reason for the constancy of k_2 in the hydrolysis of *p*-nitroanilide substrates even in viscous medium: the formation of a tetrahedral intermediate requires more energy than its breakdown and catalytic assistance by the enzyme is more appreciated in the former than the latter. That is to say, conformational change of the enzyme plays a crucial role *not* in the breakdown of the intermediate *but* in the formation of it. Therefore, the reaction scheme from the viewpoint of the movement of the enzyme, which was proposed in the previous paper,[20] should be revised to become Scheme 2.



Scheme 2

In Scheme 2 $(ES)_T$ is the tetrahedral intermediate in the acylation of the enzyme and E′ represents an enzyme which is conformationally different from a native enzyme.

Conformational change of an enzyme during the formation of a tetrahedral intermediate was suggested recently by Cassidy *et al.*[12] According to their report, the formation of a low-barrier hydrogen bond between His[57] and Asp[102] is induced by substrate-induced conformational change of the enzyme, which plays a significant role in enhancing the basicity of $N^{\varepsilon 2}$ of the imidazole ring at His[57]. This report supports at least half of our result. However, our present conclusion has more significance, in that conformational change of the enzyme is confirmed *in the actual course of enzymatic reaction*, not in an inactive complex.

According to the research by Khoshtariya *et al.*, k_{cat} for the hydrolysis of ethyl *N*-acetyl-L-tyrosinate is independent of medium viscosity.[31] Although it is impossible to compare their results with ours directly, because k_{cat} is a composite of k_2 and k_3 in their system, their results seem to conflict with our previous results that k_{cat} or k_3 for the hydrolysis of *N*-protected amino acid esters increases with increasing viscosity and begins to decrease at about 1.6 mPa s (ref. 20). Difference in leaving P_1 (*p*-nitrophenolate or ethanol) cannot explain this disagreement, because the difference, if any, should appear in k_2. It should be noted that Khoshtariya's report is based on measurements at pH under 8.5. This pH is close to the pK_a of the amine group on Ile[16], which is located in the vicinity of the active site of the enzyme and contributes to maintaining the structure of the active site.[32] It was reported that the value of ellipticity at 230 nm in CD spectra increases over pH 8, which is considered to stem from the change in structure of the active site.[33,34] Hence, we believe that viscosity-profiles of kinetic parameters might be pH-dependent.

It has been believed that conformational change of an enzyme plays the most important role especially in the rate-determining step. Although our conclusion that induced-fit is not necessarily required in the rate-determining step, the decomposition of the tetrahedral intermediate, for the hydrolysis of amide sounds abnormal from the viewpoint of common sense, we consider that the induced-fit process in the formation of a tetrahedral intermediate induces the fixation of a proton on $N^{\varepsilon 2}$ of His[57] into a position ready to protonate the nitrogen of the leaving amine.

Conclusions

From the dependence of kinetic parameters for the hydrolysis of *p*-nitroanilides catalyzed by α-chymotrypsin upon medium viscosity, we propose that the formation of a tetrahedral intermediate in the acylation of the enzyme is more significantly subject to the conformational effect of the enzyme than the breakdown of the intermediate. After association with a substrate, the enzyme changes its conformation, which induces general base catalysis for the proton transfer between Ser[195] and His[57]. This movement results both in the formation of a tetrahedral intermediate and in the fixation of a proton on $N^{\varepsilon 2}$ of His[57] into a position ready to protonate the nitrogen of the leaving amine.

Experimental

Instruments

A Hitachi U-3210 spectrophotometer and a Union Giken RA-401 rapid reaction analyzer were used for kinetic measurements. The former was used in the measurements for substrates **1**, **2** and **6**, and the latter for the others. Viscosity was measured by using an Ubbelohde viscometer at 303.0 ± 0.1 K.

Materials

The enzyme, buffer, viscogen and cosolvent, which was used for improving the solubility of substrates, are described in the previous paper.[20] *p*-Nitrophenyl *N*-succinyl-L-phenylalaninate (Suc**F**-PNP, **4**) and *p*-nitrophenyl *N*-succinyl-L-leucinate (Suc**L**-PNP, **7**) were prepared from the corresponding *N*-benzyloxycarbonyl amino acid *p*-nitrophenyl ester by two steps: the deprotection by HBr in AcOH and the condensation with succinic anhydride. *N*-Benzyloxycarbonyl-L-phenylalanine *p*-nitroanilide was prepared from *N*-benzyloxycarbonyl-L-phenylalanine by the phosphoazo method[35,36] and was converted into *N*-succinyl-L-phenylalanine *p*-nitroanilide (Suc**F**-PNA, **1**) by the same method as that described above. *N*-*tert*-Butoxycarbonyl-L-leucine *p*-nitroanilide was also prepared by the phosphoazo method, then converted into L-leucine *p*-nitroanilide hydrochloride salt by HCl in AcOEt after deprotection of Boc group by trifluoroacetic acid. The salt was converted into *N*-succinyl-L-leucine *p*-nitroanilide (Suc**L**-PNA, **6**) by condensation with succinic anhydride. *N*-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (Suc-**AAPF**-PNA, **3**) was prepared by reference to the literature method.[37] *N*-Succinyl-L-prolyl-L-phenylalanine *p*-nitroanilide (Suc**PF**-PNA, **2**) was prepared from L-prolyl-L-phenylalanine *p*-nitroanilide hydrochloride salt which was obtained in the synthesis of Suc**AAPF**-PNA by condensation with succinic anhydride. *p*-Nitrophenyl *N*-succinyl-L-prolyl-L-phenylalaninate (Suc**PF**-PNP, **5**) was prepared from the corresponding *p*-nitrophenyl L-prolyl-L-phenylalaninate hydrochloride by condensation with succinic anhydride.

Medium viscosity

Viscosity of the reaction medium was controlled by adding an appropriate amount of glycerol to HEPES buffer (0.1 M).

Kinetic measurements

The procedure was almost the same as that described in the previous paper.[20] In the measurements for *p*-nitroanilide substrates, dimethyl sulfoxide was employed as the cosolvent (1.6% (v/v)) and kinetics were followed at 303.0 K by the increase in absorbance at 405 nm, where the molar absorbance

of *p*-nitroaniline is 9900 (HEPES 0.1 M, pH 7.50, ionic strength 0.2 and no glycerol). Since the molar absorbance of *p*-nitroaniline at 405 nm increases as glycerol is added to the buffer solution, we determined the absorbance for each mixture of glycerol and water. After the kinetic data were treated with repeated calculation by Taylor expansion, the best-fit kinetic parameters were obtained. In measurements for substrates **4**, **5** and **7**, the single-turnover condition ($[E]_0 \geqslant [S]_0$) was employed. In all kinetic measurements, the effective concentration of the enzyme was determined by the procedure described in the previous paper[20] and the references in it. In the measurements for **6**, after incubation of a solution of an enzyme (3.08 cm^3), a stock solution of a substrate (50 mm^3) was added to the solution, and the measurement of the absorbance was followed for about 30–60 min. The concentration of an enzyme in the measurements for **6** was *ca.* 9×10^{-5} M.

Initial conditions for steady-state measurements are as follows: $[S]_0 = 1.10 \times 10^{-3}$–$9.00 \times 10^{-3}$ M, $[E]_0 = 2.00 \times 10^{-6}$–$4.00 \times 10^{-6}$ M for **1**; $[S]_0 = 1.00 \times 10^{-3}$–$6.00 \times 10^{-3}$ M, $[E]_0 = 8.00 \times 10^{-7}$–$1.00 \times 10^{-6}$ M for **2**; $[S]_0 = 2.00 \times 10^{-5}$–$2.00 \times 10^{-4}$ M, $[E]_0 = 1.00 \times 10^{-6}$–$3.00 \times 10^{-6}$ M for **3**; $[S]_0 = 2.00 \times 10^{-5}$–$2.00 \times 10^{-4}$ M, $[E]_0 = 1.00 \times 10^{-6}$–$3.00 \times 10^{-6}$ M for **4**; $[S]_0 = 2.15 \times 10^{-5}$–$2.70 \times 10^{-4}$ M, $[E]_0 = 1.30 \times 10^{-6}$–$3.00 \times 10^{-6}$ M for **5**; $[S]_0 = 2.00 \times 10^{-3}$–$9.00 \times 10^{-3}$ M, $[E]_0 = 7.10 \times 10^{-5}$–$9.30 \times 10^{-5}$ M for **6**. $[S]_0 \geqslant 10\ [E]_0$ was kept throughout the measurement. Initial conditions of single-turnover measurement for **7** were $[S]_0 = 8.2 \times 10^{-7}$ M and $[E]_0 = 1.00 \times 10^{-5}$–$1.80 \times 10^{-4}$ M, where $[E]_0 \geqslant 10\ [S]_0$ was kept throughout the measurement.

The spontaneous hydrolysis was very slow compared with that of enzymatic reaction for all substrates employed, and the observed rates were not corrected for spontaneous hydrolyses.

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Paper a907114h