  Bull. Chem. Soc. Jpn., 59, 243—247 (1986)

Effect of Pressure on the Pre-Steady-State Kinetics of the Hydrolysis of *p*-Nitrophenyl Pivalate Catalyzed by α-Chymotrypsin

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(Received July 10, 1985)

The pre-steady-state of the hydrolysis of *p*-nitrophenyl pivalate (pNPT) catalyzed by α-chymotrypsin (α-CHT) in Tris buffer solutions was measured up to 2.4 k bar at 25 °C. The reaction was initiated by substrate binding, and followed by acylation and deacylation processes. From the pressure dependence on the dissociation constants (K_s), the volume change ΔV_K, was -14 ± 1 cm^3 mol^{-1}. From the pressure dependence on the rate constants (k_{acyl} and k_{deacyl}) of the acylation and deacylation processes, the activation volumes $\Delta V^{\neq}_{acyl}$ and $\Delta V^{\neq}_{deacyl}$ were -24 ± 1 and -2 ± 1 cm^3 mol^{-1}, respectively. The reaction mechanisms of α-CHT are discussed in terms of the reaction and activation volumes.

α-Chymotrypsin (α-CHT) is a well-known endoproteinase which hydrolyzes the peptide bonds at the C end of hydrophobic amino acid residues. It also catalyzes the cleavage of amides of individual amino acids and all other carboxylic acid derivatives, in addition to that of polymeric amides. A number of kinetic studies of this enzyme have been performed on the overall reaction rates (k_{cat}/K_M) or the steady-state kinetic parameters (k_{cat}, K_M).[1,2] However, the parameters by themselves are not sufficient to represent the microscopic rate or equilibrium constant for the elementary reactions of this enzyme. In order to obtain an insight into the mechanism of an α-CHT catalysis, the thermodynamic parameters[3] of individual reaction rate processes, as shown by Eq. 1, are necessary, especially through pre-steady-state kinetic measurements.

$$E + S \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_{acyl}} ES' + P_1 \xrightarrow{k_{deacyl}} E + P_2. \quad (1)$$

Here E, S, ES, ES′, P_1, and P_2 denote an enzyme, a substrate, an enzyme-substrate complex, an acyl-enzyme, a first leaving group, and a second leaving group, respectively, and K_s, k_{acyl}, and k_{deacyl} represent the dissociation constant of the ES complex, the acylation rate constant, and the deacylation rate constant, respectively.

Activation volumes or reaction volumes, evaluated from the pressure dependence of chemical reactions, are useful parameters for determining the differences in the reaction mechanisms.[4] From recent studies of trypsin,[5] carboxypeptidase A,[6] thermolysin,[7] and α-CHT[8] catalysis, it has been shown that these parameters also provide important information on enzyme reactions.[9]

Regarding the studies concerning the reaction mechanism of an enzyme under high pressure, the effects on pressure inactivation must be removed from the pressure effect on the reaction rate, as an enzyme protein becomes inactivated by a high-pressure treatment. Miyagawa and Suzuki[10] indicated that there is no loss of the activity of α-CHT in a pH 7.8 Tris buffer solution up to 3 kbar from measurements of enzyme activity after a pressure treatment. It has been clarified that α-CHT undergoes a reversible pressure inactivation from the rate of the hydrolysis of phenyl esters catalyzed by α-CHT up to 3 kbar.[8a] Therefore, it is necessary to check the true active enzyme concentration ($[E]_a$) directly under high pressure in order to study the reaction mechanism. These $[E]_a$ values can be stoichiometrically determined[11] by means of the pre-steady-state kinetics in the hydrolysis of *p*-nitrophenyl ester catalyzed by α-CHT.

In the present work, first the $[E]_a$ values at each pressure up to 2.4 kbar at 25 °C were determined from the measurements of the pre-steady-state in a hydrolysis reaction of *p*-nitrophenyl pivalate (pNPT) catalyzed by α-CHT. After the removal of the effect on the pressure inactivation of the enzyme, the reaction mechanism of α-CHT is discussed in terms of the volume change during substrate binding and the activation volumes of the acylation and deacylation processes.

Experimental

Materials. α-Chymotrypsin (α-CHT)(3× crystallized, Sigma Chemical Co.) was used without further purification. *p*-Nitrophenyl pivalate (pNPT) was recrystallized five times from hexane, mp 94.5—95.2 °C (lit,[12] 94—95 °C). The demineralized water was distilled. Acetonitrile was distilled over diphosphorus pentaoxide before use.

Apparatus and Procedure. The method for measuring the rate of hydrolysis reactions under high pressure has been described in a previous report.[13] The rate was measured at pH 7.8 in a 0.005 M† Tris-HCl buffer solution up to 2.4 kbar at 25±0.5 °C. The pressure dependence on the pH value was negligible, since the volume change of the dissociation process was -1 cm^3 mol^{-1}.[14] The rate of the hydrolysis of the pre-steady-state was determined by a change in the optical density (400 nm). This was attributable to the λ_{max} of the *p*-nitrophenolate ion formed as a product, monitored by means of a Hitachi 340 type

† 1 M=1 mol dm^{-3}.

spectrophotometer. It takes 45 seconds (on the average) for a compression up to a certain experimental pressure after mixing an ester and α-CHT solutions in a high-pressure cell.

Stock solutions of the enzyme (9.0×10^{-4} M) were prepared daily in a 0.01 M sodium acetate buffer. Each enzyme solution that was stored below 4 °C during use, was stable for at least 48 h.[11] Total substrate concentrations ($[S]_o$ were varied from 7.5×10^{-6} to 2.5×10^{-5} M, and the total enzyme concentration ($[E]_o$) was 4.5×10^{-6} M.

Results

The equilibrium and rate constants (K_s, K_M, k_{acyl}, and k_{deacyl}), determined by assuming a pre-steady-state, and $[E]_a$ values were analyzed by the following modified Bender's method.[11] Under the conditions of $[E]_o \ll [S]_o$, and $k_{acyl} \gg k_{deacyl}$ for a *p*-nitrophenyl ester substrate,[11] a typical plot of P_1 formation at each pressure as a function of time (t), obtained by spectrophotometry, is shown in Fig. 1. It can be represented by an equation of the form:

$$P_1 = At + B(1-e^{-Ct}) \tag{2}$$

$$A = \frac{k_{deacyl}[E]_a[S]_o}{[S]_o+K_M} + k_s([S_o]-[E_o]) \tag{3}$$

$$B = \frac{[E]_a[S]_o^2}{([S]_o+K_M)^2} \tag{4}$$

$$C = \frac{k_{acyl}[S]_o}{[S]_o+K_s} \tag{5}$$

$$K_M = K_s k_{deacyl}/k_{acyl}. \tag{6}$$

Here k_s denotes the rate constant of the spontaneous hydrolysis of the ester. The K_s and k_{acyl} are obtained by the least-squares method from Lineweaver-Burk plots (Eq. 7, Fig. 2) of the obtained C from the curvature of the initial "burst" of P_1 (Fig. 1).

$$\frac{1}{C} = \frac{K_s}{k_{acyl}}\cdot\frac{1}{[S]_o} + \frac{1}{k_{acyl}}. \tag{7}$$

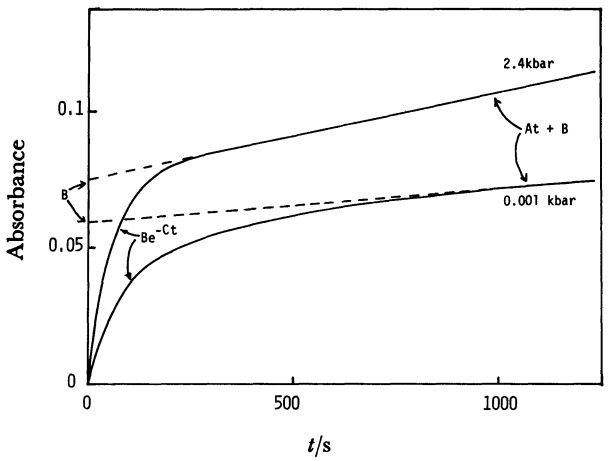

Fig. 1. The α-CHT-catalyzed hydrolysis of pNPT at 25 °C and two pressures, pH 7.8 (0.005 M Tris). Each ester concentration is 2.5×10^{-5} M.

K_M and k_{deacyl} are obtained by the same method (Eq. 8, Fig. 3) of the obtained A from a part of the straight line in Fig. 1.

$$\frac{1}{D} = \frac{1}{A-k_s([S]_o-[E]_o)} = \frac{K_M}{k_{deacyl}[E]_a}\cdot\frac{1}{[S]_o} + \frac{1}{k_{deacyl}[E]_a}. \tag{8}$$

On the other hand, B can be obtained from the intercept by extrapolating the above straight line (Fig. 1). As K_M values, being independent of $[E]_a$,

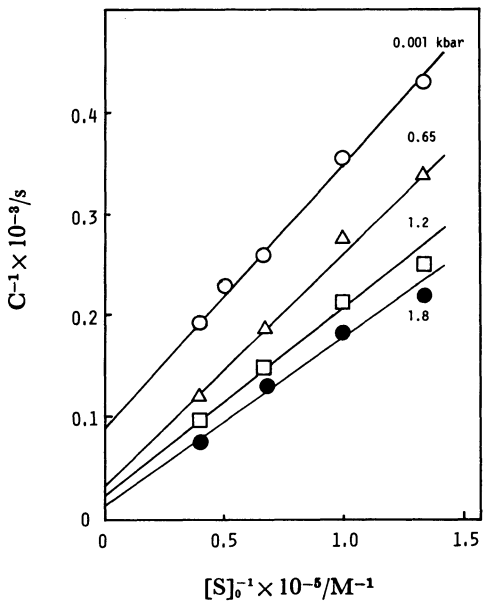

Fig. 2. Lineweaver-Burk plots for the hydrolysis of pNPT in the acylation process, pH 7.8 (0.005 M Tris).

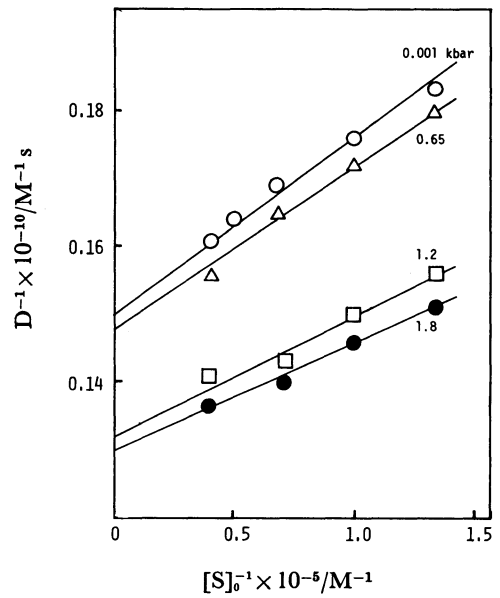

Fig. 3. Lineweaver-Burk plots for the hydrolysis of pNPT in the deacylation process, pH 7.8 (0.005 M Tris).

Table 1. $[E]_a$ and E_p at Various Pressures and 25 °C[a)]

	P/kbar				
	0.001	0.65	1.2	1.8	2.4
$10^6[E]_a$/mol kg^{-1} [b)]	3.84	3.71	3.71	3.77	3.92
E_p[c)]/%	85	82	82	84	87

a) pH 7.8, 0.005 M Tris. b) ±0.1. c) ±2.

Table 2. Kinetic Parameters of the Hydrolysis of pNPT Catalyzed by α-CHT at Various Pressures and 25 °C[a)]

P/kbar	$10^4 k_{deacyl}$/s^{-1}	$10^2 k_{acyl}$/s^{-1}	$10^6 K_M$/M	$10^5 K_s$/M
0.001	1.33	1.19	1.89	3.37
0.65	1.35	2.33	1.57	5.19
1.2	1.51	4.05	1.40	7.61
1.8	1.54	6.54	1.23	10.3

a) pH 7.8, 0.005 M Tris. ±0.05.

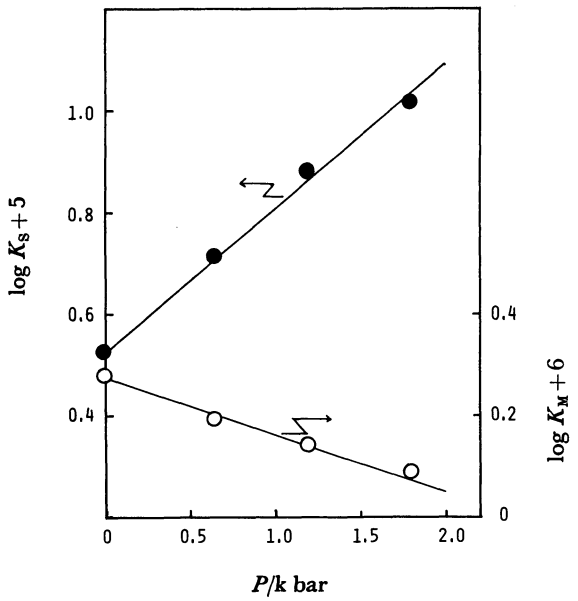

Fig. 4. Log K_S and log K_M vs. pressure at 25 °C.

(1.23×10^{-6} M—1.89×10^{-6} M) at each pressure are smaller than an $[S]_0$ value of 2.5×10^{-5} M. The B value corresponds to $[E]_a$ in Eq. 4. The values of $[E]_a$ and the enzyme purity (E_p) (calculated from the ratio of $[E]_a$ to the $[E]_0$ at each pressure) are summarized in Table 1. These values tell us that there is no loss in activity of α-CHT within ±5% by a compression of up to 2.4 kbar. The rate constants, corrected by the $[E]_a$ and the values of K_M and K_s at each pressure, are shown in Table 2.

From the linear relations of the logarithms of K_s, K_M, k_{acyl}, and k_{deacyl} vs. the pressure established for pNPT in Figs. 4 and 5, the volume changes (ΔV_K, and ΔV_{K_M}) and the activation volumes ($\Delta V^{\ddagger}_{acyl}$ and $\Delta V^{\ddagger}_{deacyl}$) accompanying each process are summarized in Table 3.

Table 3. Volume Changes and Activation Volumes for α-CHT Catalysis at 25 °C[a)]

cm^3 mol^{-1}			
ΔV_{K_s}	ΔV_{K_M}	$\Delta V^{\ddagger}_{acyl}$	$\Delta V^{\ddagger}_{deacyl}$
−14±1	6±1	−24±1	−2±1

a) pH 7.8, 0.005 M Tris.

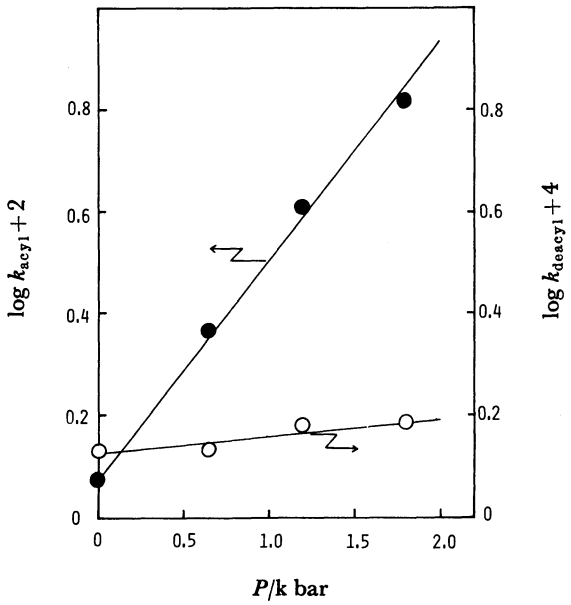

Fig. 5. Log k_{acyl} and log k_{deacyl} vs. pressure at 25 °C.

Discussion

The established overall enzyme reaction of α-CHT with a tetrahedral intermediate (ET, ET′) in the acylation or deacylation step is given by Eq. 9.

$$E + S \underset{K_s}{\rightleftharpoons} ES \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ET \xrightarrow{k_2} \underset{+\ P_1}{ES'} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ET' \xrightarrow{k_4} E + P_2 \quad (9)$$

(k_{acyl} over the first arrow; k_{deacyl} over the second arrow)

As the k_1 step is rate limiting in the acylation process of a p-nitrophenyl ester substrate,[15)] the observed k_{acyl} is given by Eq. 10.

$$k_{acyl} = k_1. \quad (10)$$

While, in a deacylation step, the rate constant is given by Eq. 11,

$$k_{deacyl} = K_3 k_4, \quad (11)$$

where $K_3 = k_3/k_{-3}$.

Substrate Binding. The volume change accompanying the formation of a Michaelis complex ($-\Delta V_K$) is 14 cm^3 mol^{-1}. This value is discussed in

terms of the weak intermolecular interactions of noncovalent bonds (ΔV_{inter}) and of the conformational change of the enzyme itself (ΔV_{conf}) for the incorporation of pNPT in a binding site.

$$-\Delta V_{K_s} = \Delta V_{inter} + \Delta V_{conf}. \quad (12)$$

The driving force of the incorporation of pNPT into the binding site is mainly the hydrophobic interaction between pNPT and α-CHT. The size of the binding site is approximately 1.0—1.2 nm×0.55—0.65 nm×0.33—0.45 nm determined by the X-ray study of Steiz et al.[16] The van der Waals radius of the pivaloyl group over 0.5 nm[17] is too large to incorporate it into a binding site of this enzyme at a depth of 0.33—0.45 nm. This pocket corresponds to the radius of the isopropyl group which removes the methyl group from the pivaloyl group. Therefore, we consider that a hydrophobic interaction between the binding site of α-CHT and the isopropyl group can be formed. The volume change accompanying the transfer of an isopropyl group from the water medium (the process of the formation of hydrophobic interaction) is 3.03 $cm^3 mol^{-1}$. This was determined from the difference in the corresponding volume change (between 5.47 $cm^3 mol^{-1}$ for isobutyl alcohol and 2.44 $cm^3 mol^{-1}$ for methyl alcohol). These were determined from the density data of an aqueous alcohol solution.[18]

Therefore, the value of ΔV_{conf} is 11 $cm^3 mol^{-1}$, accompanying the incorporation of a nonspecific substrate like pNPT into α-CHT. On the other hand, in our previous paper[8b] it was reported that ΔV_{conf} is negligibly small during the incorporation of a specific substrate as *N*-Ac-L-Trp-amide (ATA). The difference in ΔV_{conf} between nearly zero for ATA and about 11 $cm^3 mol^{-1}$ for pNPT obtained in this work may be considered to be the specific (ATA)-nonspecific (pNPT) substrate with a bulky pivaloyl group.

Acylation Process. Taniguchi and Suzuki[8a] reported that the $\Delta V^{\neq}_{acyl}$ for the hydrolysis of 2-valeryloxy- and 2-heptanoyloxy-benzoic acids catalyzed by α-CHT are −20 and −21 $cm^3 mol^{-1}$, respectively. A $\Delta V^{\neq}_{acyl}$ of −24 $cm^3 mol^{-1}$, found in our work, agrees with the above data. From Eq. 10, $\Delta V^{\neq}_{acyl}$ corresponds to the volume change for the process from ES to ET. The k_1 process is characterized by a large decrease in volume in contrast to a volume increase of 15 $cm^3 mol^{-1}$ for the k_2 process[8b] in acylation. According to the single-proton-transfer (SPT) mechanism[19] (Fig. 6), the following four interactions may be mainly formed at the transition state in a k_1 process.

(1) The formation of a covalent bond between the oxygen atom of Ser-195 and the carbonyl carbon atom of the substrate, (2) the charge concentration at the carbonyl oxygen atom of the substrate and at the nitrogen atom of the imidazole ring of His-57, the formation of two hydrogen bonds (H-bonds) (3) between the oxygen atom of Ser-195 and nitrogen atom of imidazole ring of His-57, and (4) between the nitrogen atom of above ring and the oxygen atom of Asp-102, take place simultaneously. It is reported that the volume changes accompanying the formation of each interaction for model systems are −10 $cm^3 mol^{-1}$ for covalent bond,[20] −5 $cm^3 mol^{-1}$ for H-bonding,[8b] and −5.5 $cm^3 mol^{-1}$ for charge concentration,[8b] respectively. Accordingly, the large negative volume change of −24 $cm^3 mol^{-1}$ is considered to correspond to the sum of the volume decreases for interactions (1)—(4).

Deacylation Process. The $\Delta V^{\neq}_{deacyl}$ (−2 $cm^3 mol^{-1}$) is consistent with the data of Lockyer et al.[21] (−2 $cm^3 mol^{-1}$). It is reported[21] that the values of $\Delta V^{\neq}_{deacyl}$ for other *p*-nitrophenyl esters are −6—−4 for acetate and −3 $cm^3 mol^{-1}$ for isobutylate. From an average volume change of −4 $cm^3 mol^{-1}$, the slightly negative volume decrease takes place in the deacylation process. From Eq. 11, the $\Delta V^{\neq}_{deacyl}$ is given by Eq. 13.

$$\Delta V^{\neq}_{deacyl} = \Delta V_{K_3} + \Delta V^{\neq}_{4}. \quad (13)$$

According to the SPT mechanism, we showed the initial state (ES′) of the deacylation process, ET′, and the expected transition state in Fig. 7. Interactions of (1)—(8) may be possible to form between ES′ and the transition state through the ET′. Because a volume change of −4 $cm^3 mol^{-1}$ is small, in spite of the

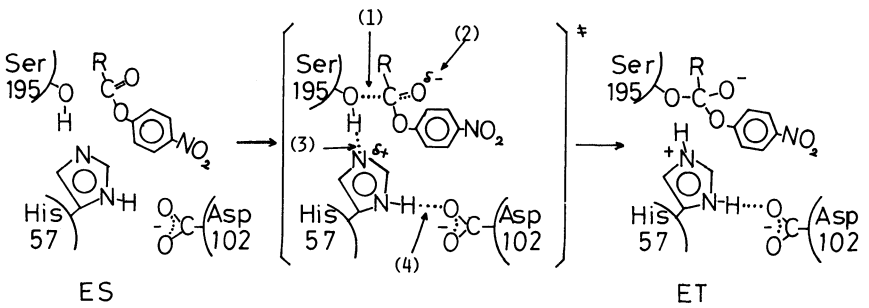

Fig. 6. Schematic drawing of the reaction mechanism of α-CHT on the process of ET formation.

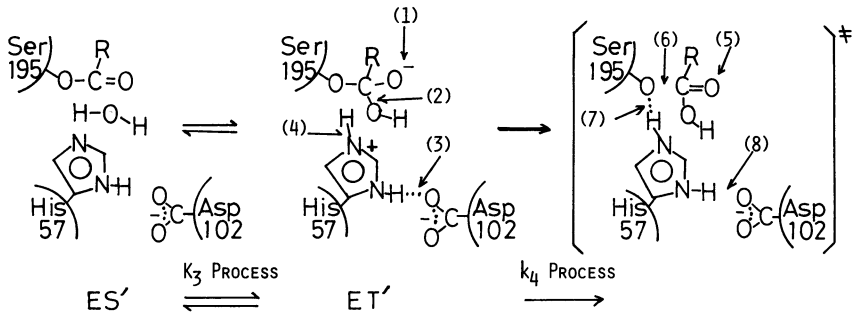

Fig. 7. Schematic drawing of the reaction mechanism of α-CHT on the process of deacylation.

formation of many interactions, the volume changes for some interactions are expected to cancell each other. Those interactions are considered to be (1)—(8) interactions, except (7) in Fig. 7, for the following reason.

At first, there was no contribution from the volume change during (1) hydration and (5) dehydration at the carbonyl oxygen atom for a reverse reaction, and (3) the formation and (8) the cleavage of one H-bond between the nitrogen atom of imidazole ring of His-57 and the oxygen atom of Asp-102. Also, similar volume changes may be expected to (2) [the formation of a covalent bond between the oxygen atom of H_2O and the carbonyl carbon atom of the substrate], and (6) [the cleavage of a covalent bond of ET′ for the formation/cleavage of C–O covalent bond]. The volume change for (4) [a proton transfer between the oxygen atom of H_2O and the nitrogen atom of imidazole of His-57, accompanying a cleavage/formation of a H-bond], may be cancelled out. Accordingly, the volume change for (7) [the formation of a H-bond between the oxygen atom of Ser-195 and nitrogen atom of imidazole ring of His-57] is considered to correspond to the experimental value of $-4\ cm^3\ mol^{-1}$.

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