Kinetic Anomalies in Chymotryptic Hydrolyses of *p*-Nitrophenyl Acetate and *N*-Benzoyl-L-alanine Methyl Ester

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Kinetic and thermodynamic parameters were evaluated for the acylation and the deacylation steps in the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin at pH 7.8 and at temperatures between 15 and 35 °C by the use of stopped-flow and ordinary ultraviolet spectrophotometers. In contrast to the temperature dependencies of k_2 and K_s reported in the literature (P. A. Adams and E. R. Swart, *Biochem. J.*, 161, 83 (1977)), no kinetic anomaly was observed in either of the steps, but reasonable straight lines were obtained in both Arrhenius and van't Hoff plots. On the other hand, in the chymotryptic hydrolysis of *N*-benzoyl-L-alanine methyl ester a sharp kinetic anomaly was found. The discrepancy in the case of *p*-nitrophenyl acetate is discussed in connection with a possible conformational change of the enzyme, an alteration of the rate-limiting step or differences in the experimental procedures. The cause of the anomaly observed in the case of *N*-benzoyl-L-alanine methyl ester is also discussed in detail.

Keywords α-chymotrypsin; hydrolysis; kinetic anomaly; *p*-nitrophenyl acetate; *N*-benzoyl-L-alanine methyl ester; rate-limiting step; conformation change

The hydrolysis of a non-specific substrate by α-chymotrypsin has been reasonably explained[1] by the following two-intermediate mechanism (Eq. 1):

$$E+S \underset{}{\overset{K_s}{\rightleftharpoons}} (ES) \xrightarrow{k_2} (ES)' \xrightarrow{k_3} E+P_2 \qquad (1)$$
$$\qquad\qquad\qquad\qquad\quad + P_1$$

E:	enzyme	P_1, P_2:	product
S:	substrate	K_s:	dissociation constant of (ES)
(ES):	Michaelis complex	k_2:	acylation rate constant
(ES)′:	acyl enzyme	k_3:	deacylation rate constant

Although a number of kinetic studies have been reported on the chymotryptic hydrolysis of non-specific substrates, they were mostly restricted to analyses of the deacylation step (k_3) because of the extremely high rates of the acylation step (k_2) and the pre-equilibrium step (K_s) even in non-specific substrate systems. Kinetic anomalies have often been reported, *i.e.*, Arrhenius plots of k_3 or van't Hoff plots of the apparent Michaelis constant (K_m^{app}) in several substrate systems showed a sharp intersection of two straight lines[2] at *ca.* 25 °C or a smoothed curve with a bending point[3] at around 25 °C. Many investigators have tried to clarify the cause of these anomalies.

Such anomalies, when observed, have often been attributed to a conformational change of either the enzyme or the acyl enzyme at a certain temperature, where the Arrhenius or van't Hoff plots break. Some grounds for this argument have been provided by several reports. Rajender *et al.*[4] reported that α-chymotrypsin has a transition at *ca.* 25 °C, where the enzyme is equilibrated between its two active substates. Kaplan and Laidler[2] suggested a reversible conformational change between active and inactive enzymes. Wang *et al.*[3] claimed that the anomalies observed in the hydrolysis of acyl-α-chymotrypsin were caused by the existence of the equilibrium between two types of acyl enzyme with different reactivities rather than by a conformational change of the enzyme itself. Baggott and Klapper[5] proposed that the enzyme might possess more than one site for hydrolyzing some kinds of substrate within the region of the active site. On the other hand, Wilson and Cabib[6] concluded that the anomalies observed in a series of reactions with acetylcholine esterase, which was believed to hydrolyze its substrate by a mechanism similar to that of α-chymotrypsin, were due to an alteration of the rate-limiting step in the consecutive reaction steps.

In order to examine the above-mentioned arguments in more detail, it is better to study the anomalies exhibited not only in the deacylation step but also in the pre-equilibrium and the acylation steps. The latter two steps might be more directly affected by a structural change of the enzyme, if it occurs, than the former step. Along this line, Adams and Swart[7] reported the temperature dependencies of k_2 and K_s in the chymotryptic hydrolyses of *p*-nitrophenyl esters. Their results exhibit sharp kinetic anomalies in both Arrhenius plots of k_2 and van't Hoff plots of K_s at 21—22 °C. In particular, the latter plots show a clear intersection of two straight lines, changing their gradients from negative below 21 °C to positive above 22 °C. This indicates that the sign of thermodynamic parameters for the equilibrium between (E+S) and (ES) changes suddenly at that temperature, which implies possible structural change of E, (ES) or both at 21—22 °C, in line with the report of Rajender *et al.*[4] Even in the chymotryptic hydrolyses of non-specific substrates, the temperature dependency of the reaction still remains in dispute.

In this work, the reported kinetic anomalies were examined in careful and repeated experiments on the individual reaction steps of chymotryptic hydrolysis of *p*-nitrophenyl acetate and *N*-benzoyl-L-alanine methyl ester. We found no anomaly in any step of the hydrolysis of *p*-nitrophenyl acetate. However, for the hydrolysis of *N*-benzoy-L-alanine methyl ester, a clear kinetic anomaly in the Arrhenius plots of the overall rate constant was observed. Reaction mechanisms are proposed which might account for these observations.

Experimental

Theory Under saturated conditions ($[S_0] \gg [E_0]$), as employed by Adams and Swart,[7] the concentration of product $[P_1]$ is related to time as follows:

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

where A is the rate of steady-state reaction (deacylation in this case), B the amount of burst and C the apparent first-order rate constant of acylation.

$$A=\frac{k_{cat}[E_0][S_0]}{K_m^{app}+[S_0]} \tag{3}$$

$$k_{cat}=\frac{k_2k_3}{k_2+k_3}\fallingdotseq k_3 \quad (\because k_2\gg k_3)$$

$$K_m^{app}=\frac{k_3}{k_2+k_3}\cdot K_s$$

$$B=[E_0]\left(\frac{\frac{k_2}{k_2+k_3}}{1+\frac{K_m^{app}}{[S_0]}}\right)^2\fallingdotseq[E_0]\left(1+\frac{K_m^{app}}{[S_0]}\right)^{-2} \tag{4}$$

$$C=\frac{k_2[S_0]}{K_s+[S_0]} \tag{5}$$

where K_m^{app} is the apparent Michaelis constant which characterizes the reaction steps before deacylation. K_m^{app} and k_3 ($\fallingdotseq k_{cat}$) can be obtained from the observed rate parameter A, while K_s and k_2 could be evaluated from the observation of the pre-steady-state reaction by using Eq. 5 as follows.

A typical reaction trace observed in chymotryptic hydrolysis of *p*-nitrophenyl acetate by use of the stopped-flow apparatus is shown in Fig. 1. The difference between the curve observed during the pre-steady-state region and the straight line extrapolated from the steady-state reaction region is indicated as D. The evaluation of rate parameters B and C was performed with adaptation of Eq. 2 as a non-linear model function by the method of non-linear least-squares regression by use of the program "SALS".[8]

Enzyme α-Chymotrypsin was purchased as three-times-recrystallized freeze-dried powder from Sigma Chemical Co. and was used without further purification. Active enzyme concentration was determined from the deacylation experiment at 25 °C by application of the rearranged formula[9] based on Eq. 4 as follows;

$$\frac{1}{\sqrt{B}}=\frac{1}{\sqrt{[E_0]}}+\frac{K_m^{app}}{\sqrt{[E_0]}}\cdot\frac{1}{[S_0]} \tag{6}$$

whence $[E_0]$ was determined as an intercept by plotting $1/\sqrt{B}$ against $1/[S_0]$. The purity of the enzyme used was thus determined to be 61% by weight. Stock solutions of the enzyme were made up daily and stored at 4 °C. For the reaction with *p*-nitrophenyl acetate (ultraviolet (UV) measurement), a phosphate buffer (0.033 mol dm^{-3}, pH 7.8) was used. For the reaction with *N*-benzoyl-L-alanine methyl ester (titration in a pH-stat), deionized, distilled water free from carbon dioxide, containing 0.1 mol dm^{-3} NaCl, was used without employing any buffer. Concentrations of the enzyme were adjusted to 3.5×10^{-7} mol dm^{-3} for the steady-state measurement and 3.5×10^{-5} mol dm^{-3} for the pre-steady-state measurement with *p*-nitrophenyl acetate, and 3.6×10^{-6} mol dm^{-3} for the overall hydrolysis rate measurement with *N*-benzoyl-L-alanine methyl ester.

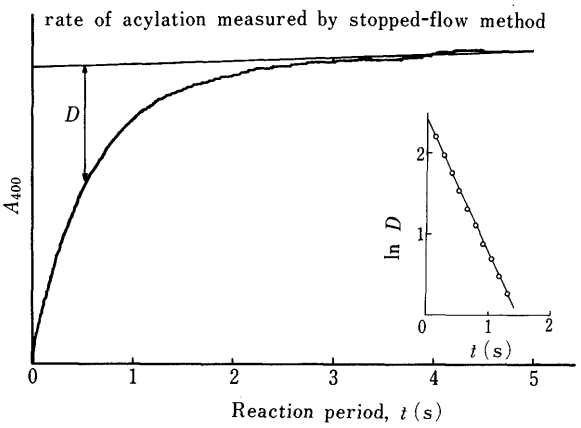

Fig. 1. A Typical Reaction Trace Observed for Hydrolysis of *p*-Nitrophenyl Acetate by α-Chymotrypsin at pH 7.8 and 25 °C in 0.033 mol dm^{-3} Phosphate Buffer Containing 4% Acetonitrile

This shows the resolution of the rapid acylation of the enzyme from the steady-state deacylation stage. $D=[P_1]_{ext}-[P_1]=(At+B)-[P_1]=B\exp(-Ct)$.

Substrate Commercially available *p*-nitrophenyl acetate was purified by three recrystallizations from a hexane–chloroform (1 : 1) solution (mp 79.0—80.0 °C). The stock substrate solution was prepared with addition of acetonitrile. The concentration of acetonitrile was 4% (v/v) in a reaction solution.

N-Benzoyl-L-alanine methyl ester was prepared by the ordinary method of esterification and benzoylation of L-alanine followed by recrystallization from hexane (mp 57.5—58.5 °C), and dissolved into de-ionized, distilled water at use.

Extinction Coefficient of *p*-Nitrophenolate Ion The molar extinction coefficient of *p*-nitrophenolate ion was measured under conditions of complete hydrolysis of *p*-nitrophenyl acetate in a 0.01 mol dm^{-3} NaOH solution as $\varepsilon_{400}=18{,}000$, which was consistent with the observation of Kézdy and Bender.[10] 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 and 4% acetonitrile.

temperature (°C)	15.0	20.0	25.0	30.0	35.0	40.0
ε_{400}	15300	15600	15900	16200	16400	16600

Measurement of Chymotryptic Hydrolysis of *p*-Nitrophenyl Acetate Kinetic measurement of the chymotryptic hydrolysis of *p*-nitrophenyl acetate was performed in two ways.

(1) Steady-State Reaction (Deacylation) Measurements: The release of *p*-nitrophenolate ion was followed by measurement of the increase in absorbance at 400 nm by use of a Shimadzu UV-210A spectrophotometer. The hydrolysis of *p*-nitrophenyl acetate proceeds in the buffer solution independently of the enzymatic reaction (the autolysis of substrate). Thus, the autolysis was started by the addition of 148 μl of the stock substrate solution to 3.45 ml of the temperature-equilibrated buffer solution in the reaction cuvette. After about 10 min the enzymatic reaction was initiated by the addition of 100 μl of the stock enzyme solution to the cuvette, followed by immediate stirring of the mixed solution. The net rate of the enzymatic steady-state reaction was obtained by subtracting the autolysis rate from the measured rate.

(2) Pre-Steady-State Reaction (Acylation) Measurements: The rapid release of *p*-nitrophenolate ion was followed by measurement of the increase in absorbance at 400 nm by using a stopped-flow spectrophotometric apparatus (Union Giken, RA-1100). After the buffer solution had temperature-equilibrated in both reservoirs in the apparatus, 0.4 ml of the stock enzyme solution (1 mmol dm^{-3}) was added to one reservoir containing 11 ml of buffer solution, and an appropriate volume of the stock substrate solution to the other, followed by prompt stirring of the solution in each reservoir. Since the temperature equilibrium was immediately attained, the reaction could be initiated without a significant effect of autolysis of α-chymotrypsin. The reaction was initiated by the rapid and simultaneous injections of equal volumes of each solution from the individual reservoirs. Each experiment thus started was traced by measurement of the increase in absorbance at 400 nm until the steady-state reaction was attained.

Measurement of Chymotryptic Hydrolysis of *N*-Benzoyl-L-alanine Methyl Ester Steady-state chymotryptic hydrolysis of *N*-benzoyl-L-alanine methyl ester was traced by use of a pH-stat (Radiometer, Titrator TTT2b, Autoburette ABU12b, Titrigraph SBR3, glass electrode G202, calomel electrode K401). The stock substrate solution (2.6 ml) and 1 mol dm^{-3} NaCl solution (0.3 ml) were mixed in the reactor and temperature-equilibrated. The pH of the mixed solution was adjusted to 8.0 by adding a small amount of the NaOH solution prepared for titration and then the titrator was started. After a few minutes the reaction was initiated by the addition of 100 μl of the stock enzyme solution (0.15 mmol dm^{-3}) to the reactor. The concentrations of NaOH solution used for titration were between 0.01 and 0.03 mol dm^{-3}. During the reaction, the surface of the reacting solution was protected by the flow of nitrogen gas from being exposed to the air. The rate of steady-state reaction was obtained from the gradient of the consumption of NaOH against time.

Results and Discussion

Kinetic Analysis of Deacylation in Chymotryptic Hydrolysis of *p*-Nitrophenyl Acetate Rearrangement of Eq. 3

allows us to analyze the steady-state reaction (deacylation) of the enzymatic hydrolysis of *p*-nitrophenyl acetate.

$$\frac{[S_0]}{A}=\frac{K_m^{app}}{k_3[E_0]}+\frac{[S_0]}{k_3[E_0]} \quad (7)$$

Figure 2 shows such a relationship. A plot of $[S_0]/A$ against $[S_0]$ gives a straight line of slope $1/k_3[E_0]$ and an intercept $K_m^{app}/k_3[E_0]$, as illustrated by Eq. 7. Figure 3 shows Arrhenius plots of k_3 and van't Hoff plots of K_m^{app} obtained above. Both plots could be reasonably approximated with straight lines. The deacylation step of acetyl chymotrypsin has been studied by Marshall and Chen,[11] Adams and Swart,[7] Bender *et al.*,[12] and Martinek *et al.*[13] All of these investigators have obtained straight lines in Arrhenius plots of k_3. The numerical values of k_3 obtained in this work are in good agreement with those in the literature.

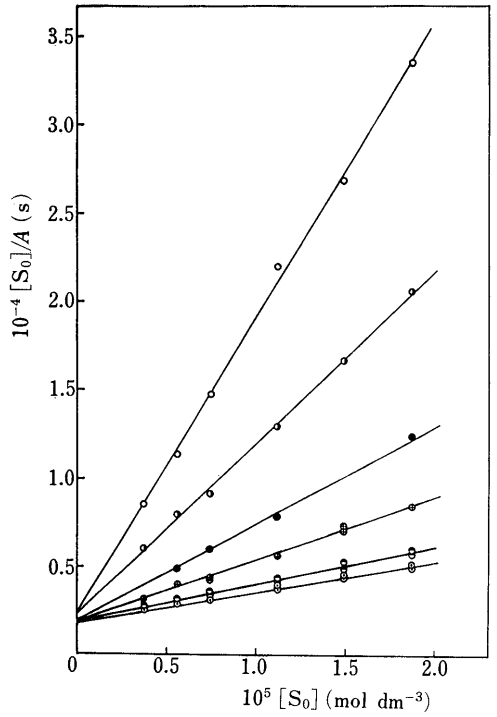

Fig. 2. Hanes–Woolf Plots for the Deacylation Step of the Hydrolysis of *p*-Nitrophenyl Acetate by α-Chymotrypsin at pH 7.8

$[E_0]=3.5\times10^{-7}$ mol dm^{-3}. Temperatures are (°C): ○, 15.0, ◑ 20.0, ● 25.0, ⊕ 30.0, ◓ 35.2, ⊙ 40.0.

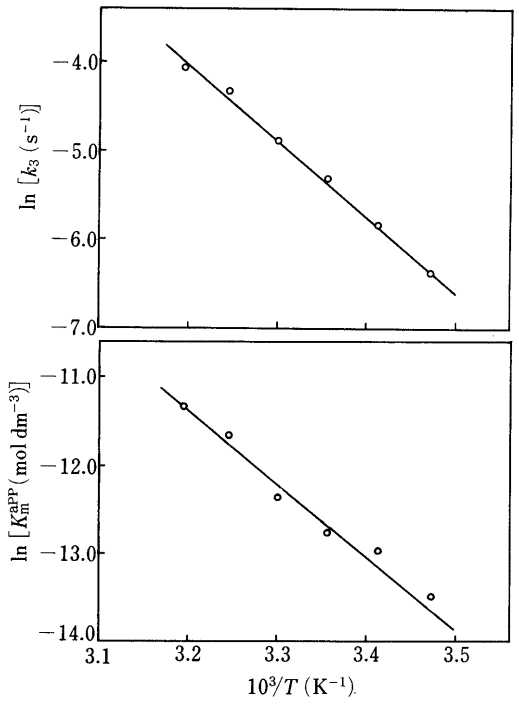

Fig. 3. Arrhenius Plots of k_3 and van't Hoff Plots of K_m^{app} for the Hydrolysis of *p*-Nitrophenyl Acetate by α-Chymotrypsin at pH 7.8

Kinetic Analysis of Pre-steady-State Steps in Chymotryptic Hydrolysis of *p*-Nitrophenyl Acetate The following Eq. 8 rearranged from Eq. 5 allows us to determine both the acylation rate constant k_2 and the pre-acylation equilibrium constant K_s.

$$\frac{[S_0]}{C}=\frac{K_s}{k_2}+\frac{[S_0]}{k_2} \quad (8)$$

The values of $[S_0]/C$ are plotted against substrate concentration in Fig. 4. Figure 5 shows the Arrhenius plots of k_2 and the van't Hoff plots of K_s, from which reasonable linear approximations are obtained. These results are in striking contrast to those of Adams and Swart[7] although the present experimental conditions were not so different from theirs. They reported that the temperature dependencies of k_2 and K_s showed sharp anomalies at 21—22°C.

Then, setting the reaction conditions just the same as those of Adams and Swart,[7] *i.e.*, the same pH value and concentrations of the enzyme and acetonitrile, by use of the stopped-flow method, we conducted further experiments at four critical temperatures, *i.e.*, 15, 20, 25 and 35 °C. Figure 6 shows plots of $[S_0]/C$ against $[S_0]$ obtained in our re-examination. The results are also shown in Fig. 7, in which

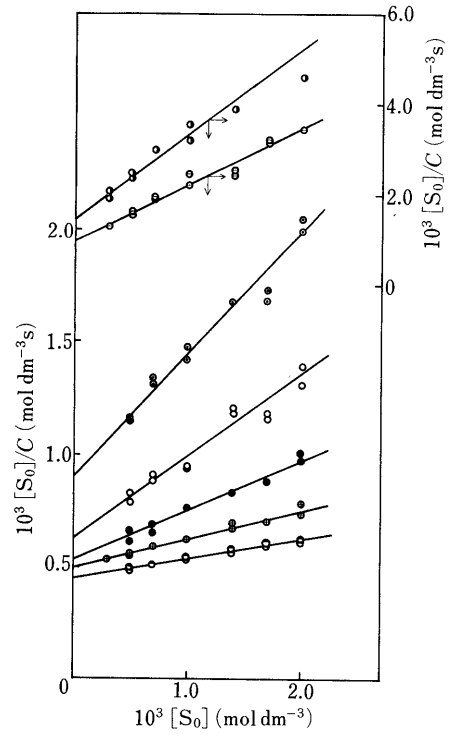

Fig. 4. Evaluation of k_2 and K_s for the Acylation and the Initial Equilibrium Step of the Hydrolysis of *p*-Nitrophenyl Acetate by α-Chymotrypsin at pH 7.8

$[E_0]=3.5\times10^{-5}$ mol dm^{-3}. Temperatures are (°C): ◑ 5.7, ⊖ 10.3, ⊙ 14.9, ○ 19.9, ● 24.9, ⊕ 29.9, ◓ 34.8.

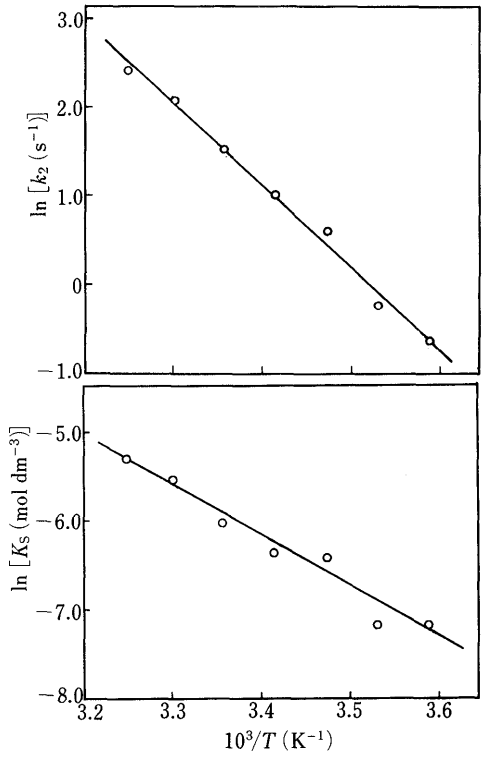

Fig. 5. Arrhenius Plots of k_2 and van't Hoff Plots for the Hydrolysis of *p*-Nitrophenyl Acetate by α-Chymotrypsin at pH 7.8

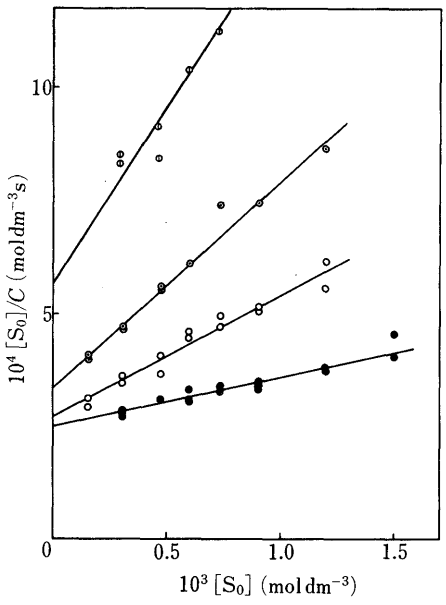

Fig. 6. Evaluation of k_2 and K_s from Our Reexamination of the Results of Adams and Swart[7] at pH 7.4 in 0.04 mol dm^{-3} Phosphate Buffer Containing 1.0% Acetonitrile

$[E_0] = 1.8 \times 10^{-6}$ mol dm^{-3}. Temperature are (C); ⓘ 15.0, ⊙ 20.0, ○ 25.0, ● 35.0.

the broken lines correspond to the results of Adams and Swart.[7] Although the numerical values of k_2 at 25 °C are almost equal to each other, the values of k_2 at 35 °C and temperature dependencies are quite different. It should be noted that there was one distinct difference between two experimental procedures. According to Adams and Swart,[7] 1.6 ml of the enzyme solution was injected into a temperature-equilibrated substrate solution after the enzyme solution had first been incubated and temperature-equilibrated in a syringe. During the incubation the enzyme might be deactivated to some extent, because the autolysis of α-chymotrypsin (cannibalistic reaction) could proceed.[14] Consequently, there is a possibility that the k_2-values obtained by Adams and Swart[7] above 25 °C could be underestimated. Since their k_3-values at the deacylation step were probably determined under saturated conditions by another steady-state measurement, those parameters did not exhibit any anomaly. Table I summarizes the numerical values of K_s, k_2 and k_3 at several temperatures studied in this work. Table II lists all the kinetic and thermodynamic parameters obtained in the present work.

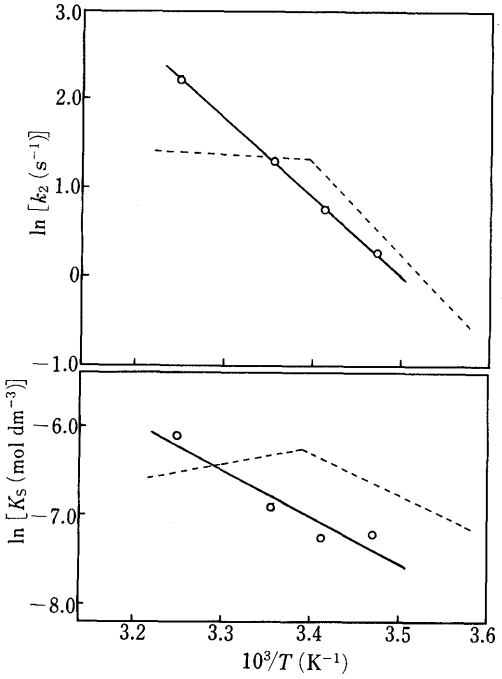

Fig. 7. Arrhenius Plots for k_2 and van't Hoff Plots for K_s from Our Reexamination of the Results of Adams and Swart[7]

The solid lines show the present results and the broken lines show the results of Adams and Swart.[7]

TABLE I. Rate and Dissociation Constants Obtained for Chymotryptic Hydrolysis of *p*-Nitrophenyl Acetate and *N*-Benzoyl-L-alanine Methyl Ester

p-Nitrophenyl acetate (pH 7.8)					*N*-Benzoyl-L-alanine methyl ester (pH 8.0)	
Temp. /°C	k_3 /10^{-3} s^{-1}	Temp. /°C	k_2/s^{-1}	K_s/10^{-3} mol dm^{-3}	Temp. /°C	k_{cat} /10^{-1} s^{-1}
		5.7	0.531	0.775		
		10.3	0.796	0.770	10.0	0.349
15.0	1.71	14.9	1.83	1.64		
20.0	2.94	19.9	2.74	1.73	20.0	0.994
25.0	4.98	24.9	4.55	2.41	25.0	1.78
30.0	7.60	29.9	7.93	3.94	30.0	2.63
35.2	13.2	34.8	11.2	5.01	35.0	3.69
40.0	17.2					

Kinetic Anomaly in Chymotryptic Hydrolysis of *N*-Benzoyl-L-alanine Methyl Ester *N*-Benzoyl-L-alanine methyl ester, which is regarded as a more specific substrate than *p*-nitrophenyl acetate, was employed as another substrate for

Table II. Thermodynamic and Kinetic Parameters for the Hydrolysis of *p*-Nitrophenyl Acetate and *N*-Benzoyl-L-alanine Methyl Ester by α-Chymotrypsin

	p-Nitrophenyl acetate		*N*-Benzoyl-L-alanine methyl ester	
	Formation of ES complex (K_s^{-1})	$K_m^{app\,-1}$	$K_m^{app\,-1}$ High temp.	Low temp.
ΔH (kJ mol^{-1})	−49.0	−69.5	0.0	−5.2
ΔS (JK^{-1} mol^{-1})	−115	−128	39.0	21.5
ΔG (kJ mol^{-1})	−14.7	−31.4	−11.6	−11.6
	Formation of acyl enzyme (k_2)	Hydrolysis of acyl enzyme (k_3)	Formation of products (k_{cat}) High temp.	Low temp.
$\Delta H^{\neq}$ (kJ mol^{-1})	74.8	68.7	53.9	81.0
$\Delta S^{\neq}$ (JK^{-1} mol^{-1})	18.8	−58.7	−78.5	12.5
$\Delta G^{\neq}$ (kJ mol^{-1})	69.2	86.2	77.3	77.3

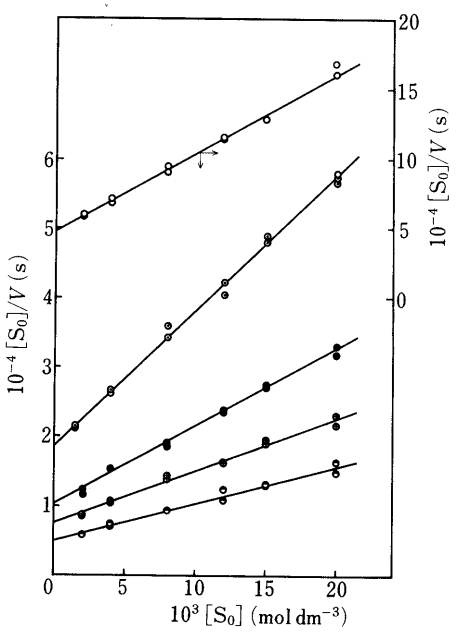

Fig. 8. Hanes–Woolf Plots for the Deacylation Step of the Hydrolysis of *N*-Benzoyl-L-alanine Methyl Ester by α-Chymotrypsin at pH 8.0 in 0.1 mol dm^{-3} NaCl Aq. Solution

Temperatures are (°C): ○ 10.0, ⊙ 20.0, ● 25.0, ⊕ 30.0, ◓ 35.0.

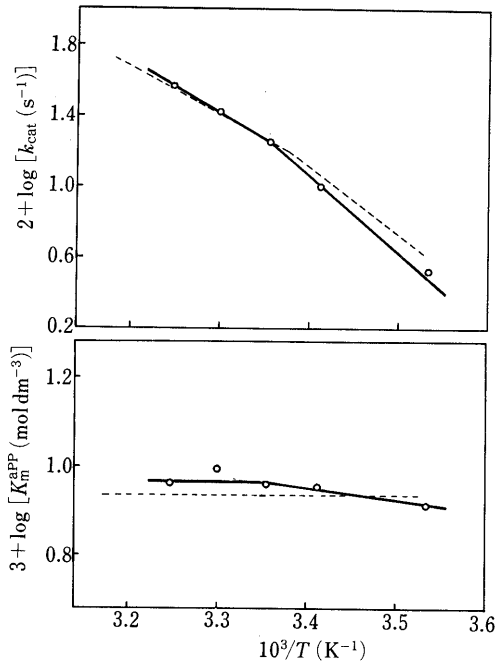

Fig. 9. Arrhenius Plots for k_{cat} and van't Hoff Plots for K_m^{app} for the Hydrolysis of *N*-Benzoyl-L-alanine Methyl Ester at pH 8.0

The solid lines show the results of this work and the broken lines show the results of Kaplan and Laidler.[2]

Table III. Typical Kinetic and Thermodynamic Anomalies Observed in Chymotryptic Hydrolysis in the Literature

Substrate	Rate or equilibrium constant for which the anomaly is observed	Temperature of the anomaly (°C)	Reference
N-Benzoyl-D- and L-alanine methyl ester	k_3	25	(2)
N-Acetyl-L-tryptophan ethyl ester	k_2/K_s	25	(4)
p-Nitrophenyl acetate	k_2, K_s	21 22	(7)
p-Nitrophenyl propionate	K_s/k_2	25	(7)
p-Nitrophenyl butyrate	K_s/k_2	29	(7)
p-Nitrophenyl trimethyl-acetate	k_2, K_s	26 28	(7)
p-Nitrophenyl 2-furoate	k_3		(3)
p-Nitrophenyl 2-(5-*n*-propyl)furoate	k_3	Smoothed curve with inflection points	(3)
p-Nitrophenyl 2-(5-ethyl)furoate	k_3		(5)

chymotryptic hydrolysis. Kaplan and Laidler[2] investigated temperature dependency in the hydrolysis of this substrate by α-chymotrypsin and found that the Arrhenius plots of steady-state rate constant, postulated to be k_3 by them instead of k_{cat} in this work, showed a sharp anomaly at 25 °C. The results of the present re-investigation are shown in Fig. 8 (Hanes–Woolf plots) and in Fig. 9 (Arrhenius and van't Hoff plots); data obtained by Kaplan and Laidler[2] are indicated by broken lines in Fig. 9. Both results are parallel. The present Arrhenius plots of k_{cat} also show a sharp kinetic anomaly at 25 °C with a value of activation enthalpy almost equal to that in the literature.[2]

Discussion on the Anomalies in Temperature Dependency

Table III summarizes the reported kinetic and/or thermodynamic constants which did show non-linear temperature dependency in chymotryptic hydrolysis. The three k_3's at the bottom of the table are for deacylation of *p*-nitrophenyl 2-(5-alkyl)furoate and correspond to a smoothed curve with bending points over relatively wide ranges of temperature. In the other cases, there are sharp breaks in the temperature dependencies. Various explanations for these kinetic anomalies have been proposed. One is a conformation change of the enzyme. This argument is supported by the data of Rajender *et al.*[4] and Kim and Lumry.[15] They concluded that at pH 8 α-chymotrypsin

changes its conformation at around 25 °C from one active substate (at lower temperatures) to another (at higher temperatures) with the enthalpy and entropy changes of *ca.* 200 kJ mol^{-1} and 680 J K^{-1} mol^{-1}, respectively. Recently, based on measurement of the dynamic heat capacity of α-chymotrypsin solution, Imaizumi *et al.*[16] suggested that a slight conformational change of the enzyme begins at *ca.* 27 °C and is completed at *ca.* 47 °C at pH 5.2. The results also imply that the transition temperatures would shift to lower values at pH above 5.2.

In the present experiments with *p*-nitrophenyl acetate, the temperature dependencies of k_3, K_m^{app}, k_2 and K_s could be reasonably approximated by straight lines. Therefore, it may be concluded that even if the enzyme transconforms between two active substates, the extent of the conformational change at around 25 °C is not large enough for the kinetic and thermodynamic parameters to be significantly affected and that no phenomenological reflection of such a change is able to be observed with *p*-nitrophenyl acetate as the substrate.

On the other hand, the sharp kinetic anomaly observed with *N*-benzoyl-L-alanine methyl ester may indicate that such a conformation change of the enzyme does occur and affects the kinetic results, presumably because this is a more specific substrate than *p*-nitrophenyl acetate.

However, the possibility should also be considered that the reaction involves two different intermediates, (ES) and (ES′), in the acylation and the deacylation steps, respectively, each step having its individual specific temperature dependency. That is, there may be a change of the rate-limiting step in the consecutive reaction scheme. A suggestive case is that of acetylcholine esterase,[6] where the kinetic anomalies could reasonably be attributed to a change of the rate-limiting step. Kaplan and Laidler[2] concluded that the rate-limiting step of chymotryptic hydrolysis of *N*-benzoyl-L-alanine methyl ester was deacylation from the pH dependence exhibited by this substrate at 20 °C, so that they approximated k_{cat} to be equal to k_3. However, the individual rate constants of acylation and deacylation were not obtained at any temperature. Ambiguity remains because of their approximation over the whole temperature range employed by them.

Another possibility to explain the anomalies is different modes of chymotryptic attack on the substrate, with two different deacylation steps; however, this can be excluded because it was confirmed that no chymotryptic reaction took place with *N*-benzoyl-L-alanine.

At the present stage a reasonable explanation is that if there exists no significant numerical difference between the rate constants for the acylation and the deacylation steps in Eq. 1, Arrhenius plots of k_{cat}, which is a function of k_2 and k_3, can exhibit a bending curve and be approximated by two straight lines with an apparent breaking point.

Kinetic measurement of the acylation step by use of *N*-CBZ-L-alanine *p*-nitrophenyl ester is under way to clarify further the cause of the anomaly observed in the present study.

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