This assumes that Zn–OH has a fractionation factor of ϕ = 1.0. Now if we estimate the Zn–OH_2 fractionation factor by linear interpolation between $\phi_{H_3O^+}$ (pK_a = 1.74) and ϕ_{H_2O} (pK_a = 15.74), then the fractionation factor for Zn–OH_2 ($pK_a \simeq 8$–9) would be predicted to be ϕ = 0.85. For this equilibrium then $K_{H_2O}/K_{D_2O} = (0.85)^2/(0.69)^3 = 2.2$, and $\Delta pK_a = 0.3$. It can be seen that the estimated SIE for ionization of Zn–OH_2 is smaller than for imidazole. However, because of error in this estimate and in the measured SIE for k_{+4}, we are not prepared to state that either imidazole or Zn–OH_2 is acting as the ionizable acid–base catalyst in the second rate process observed during aldehyde reduction. In eq 3 we have shown only one of many possibilities for the ultimate fate of proton removed from alcohol during alcohol oxidation.

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Kinetic and Thermodynamic Study of the Specificity in the Elementary Steps of α-Chymotrypsin-Catalyzed Hydrolysis Reaction

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Abstract: Pre-steady-state kinetic processes of α-chymotrypsin-catalyzed hydrolysis were studied at several temperatures for *N*-(2-furyl)acryloyl derivatives of Tyr, Phe, and Leu methyl esters, and the kinetic and thermodynamic parameters of the individual elementary steps were determined. For a wide range of pH, the rate profile of the hydrolyses of these esters, under the condition of enzyme in excess of the substrate, confirmed the generally accepted three-step mechanism and did not require the postulation of the existence of additional intermediates. The spectra of the two intermediates (enzyme–substrate complex and acyl-enzyme) expected for the three-step mechanism were quantitatively measured. From the pH dependences of three kinetic parameters (enzyme–substrate dissociation constant, acylation rate constant, and deacylation rate constant), their pH-independent limiting values were obtained, showing varying specificity depending on the particular elementary step. The temperature dependences of the reaction parameters indicated that the specificity in the deacylation step is controlled by the entropic term as far as the specific substrates are concerned. This was not so in the case of nonspecific substrates. The acylation step for specific substrates exhibited less negative or positive $\Delta S^{\ddagger}$ values, and the enzyme–substrate dissociation constant was rather insensitive to the change in temperature.

α-Chymotrypsin is a well-known endoproteinase which hydrolyzes the peptide bonds at the C end of hydrophobic amino acid residues. A number of studies on its specificity have been performed on the overall reaction rates (k_{cat}/K_m) or on the steady-state kinetic parameters (k_{cat} and K_m).[1,2] However, these parameters do not always represent the microscopic rate or equilibrium constant for the elementary reactions of this enzyme. In order to obtain an insight into the mechanism of the α-chymotrypsin catalysis, studies on the specificity[3,4] and thermodynamic parameters[5] of the individual rate processes of the reaction, as shown by eq 1, are necessary, especially by means of pre-steady-state kinetic measurements:

$$E + S \overset{K_s}{\rightleftarrows} E \cdot S \overset{k_2}{\rightarrow} ES' \overset{k_3}{\rightarrow} E + P_2 \quad (1)$$
$$+ P_1$$

(E, S, E·S, ES′, P_1, and P_2 denote enzyme, substrate, enzyme–substrate complex, acyl-enzyme, first leaving group, and second leaving group (acid), respectively, and K_s, k_2, and k_3 are the dissociation constant of E·S complex, acylation rate constant, and deacylation rate constant, respectively.) However, detailed kinetic and thermodynamic studies of the individual steps have been restricted to the deacylation step of the acyl-enzyme[6-10] or to the binding of substrate analogues.[11-14]

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Those for the actual substrate-binding step and for the acylation step have been rarely reported.[15-17] Even in a number of reported thermodynamic results for the deacylation step, serious discrepancies occur concerning their conclusions on the origin of the specificity-determining factor, whether it is enthalpic or entropic. These discrepancies occur mainly because measurements have been made of the catalytic rates ($k_{cat.}$), not of the deacylation rates (k_3).[10]

In our previous report, we showed the advantages of employing a chromophoric specific substrate,[18] *N*-(2-furyl)-acryloyl-L-tryptophan methyl ester, the use of which had been originally proposed by Bernhard et al.,[19] for quantitative evaluation of most of the individual kinetic parameters involved in the catalytic reaction, especially under conditions where the enzyme concentration was much higher than that of the substrate. In this paper we applied this chromophoric substituent to other amino acid esters, namely methyl esters of Tyr, Phe, and Leu, and compared the results with the previous ones. The dependence of the reaction parameters of the individual steps on the nature of the side-chain groups of the amino acid esters and the temperature dependences of all of the rate parameters were studied. We believe that the present study is the first such investigation for the specific substrates on the basis of pre-steady-state kinetics.

Materials and Methods

Three-times crystallized α-chymotrypsin (bovine pancreas) was purchased from Worthington Biochemical Co. (lots CDI 34D698, 34S888, and 36S771). The preparation and activity determination of the enzyme solutions were previously described.[18] *N*-(2-Furyl)-acryloyl-L-tryptophan methyl ester (Fa-L-TrpOMe) was from Cyclo Chemicals. Fa-L-TyrOMe, Fa-L-PheOMe, and Fa-L-LeuOMe were prepared from the corresponding L-amino acid methyl ester hydrochlorides and β-(2-furyl)acryloyl chloride, according to the method described in the literature,[20] with a slight modification. Anal. Fa-L-TyrOMe ($C_{17}H_{18}N_1O_5$) C, H, N. Fa-L-PheOMe ($C_{17}H_{17}N_1O_4$) C, H, N. Fa-L-LeuOMe ($C_{14}H_{19}N_1O_4$) C, H, N. Melting points: Fa-L-TyrOMe, 152-153 °C (lit. 152-153 °C[21]); Fa-L-PheOMe, 101-102 °C (lit. 100-101 °C[22]); Fa-L-LeuOMe, 107-108 °C. *N*-Acetyl-L-tryptophan *p*-nitrophenyl ester (Ac-L-TrpONp) and Ac-L-TrpOMe were obtained from Cyclo Chemicals Co. and Aldrich Chemicals Co., respectively. Indoleacryloylimidazole (IAI) and furylacryloylimidazole (FAI) were prepared according to the method described in the literature.[19] Anal. IAI ($C_{14}H_{11}N_3O_1$) C, H, N. FAI ($C_{10}H_8N_2O_2$) C, H, N. Melting points: IAI, 190 °C (lit. 190 °C); FAI, 109-111 °C (lit. 111-114 °C). Cinnamoylimidazole (CI) was purchased from Sigma Chemicals Co., and recyrstallized from cyclohexane. Buffers were prepared from guaranteed grade reagents. Deionized and distilled water produced by an auto-still was used for preparation of aqueous solutions.

A stopped-flow spectrophotometer (Union RA1100) and a UV-visible spectrophotometer (Union SM401) were used to measure the reaction rates. A detailed description of these apparatuses was given in the previous paper.[18] The dead time of the stopped-flow apparatus was determined by measurements on the reaction between poly(4-vinyl)-*N*-(2,4-dinitrophenyl)pyridinium chloride and OH^-,[23] which involved a colored intermediate and the colorless initial and final states at around 580 nm with favorable rate constants for the measurement of the dead time.

For the measurement of the deacylation rate (k_3), conditions of enzyme concentration in large excess to the substrate were employed. The acylation rates (k_2) were measured under either excess enzyme ($[E]_0 \gg [S]_0$) or under excess substrate ($[S]_0 \gg [E]_0$). In both cases, k_2 and K_s' were obtained by plotting the data in the Eadie form. For Fa-L-TyrOMe and Fa-L-PheOMe, the $[E]_0 \gg [S]_0$ condition was favored, whereas $[S]_0 \gg [E]_0$ was mainly applied for Fa-L-LeuOMe, because of the difference in K_s' values of these esters.

Kinetic processes were followed at the wavelength around which the maximum difference spectrum was observed between the substrate and the acyl enzyme (330 nm for Fa derivatives of L-amino acid methyl esters), between the substrate and the product acid anion (305 nm for Ac-L-TrpOMe), between the acyl enzyme and the product acid (350 nm for IAI, 300 nm for CI, and 320 nm for FAI), or between the substrate and the product alcohol (348 nm for Ac-L-TrpONp at lower pH).

Temperature was controlled by circulating the thermostated water (Lauda K2R). Thermodynamic parameters were calculated by plotting the data in the form of $\ln(k/T)$ vs. $1/RT$, and the standard deviations were calculated by the statistical method described by Youden.[24]

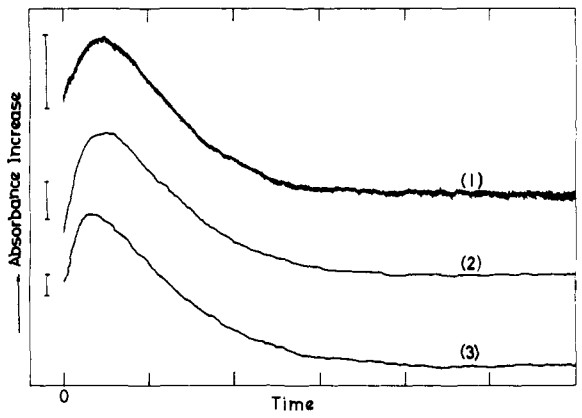

Figure 1. Reaction traces of α-chymotryptic hydrolysis of Fa-L-TyrOMe obtained by an ordinary spectrophotometer (1) and by a stopped-flow spectrophotometer (2, 3). (1) pH 2.3, citrate, $[S]_0 = 6.6 \times 10^{-6}$ M, $[E]_0 = 1.0 \times 10^{-4}$ M. Abscissa scale, 400 s/division. (2) pH 3.5, citrate, $[S]_0 = 6.5 \times 10^{-6}$ M, $[E]_0 = 1.0 \times 10^{-4}$ M. 2 s/division. (3) pH 5.9, phosphate, $[S]_0 = 9.6 \times 10^{-6}$ M, $[E]_0 = 8.5 \times 10^{-5}$ M. 100 ms/division. The bars in the left side denote ordinate scale, OD change of 0.005. 25 °C, μ = 0.25.

Results and Discussion

Reaction Traces and the Intermediates. α-Chymotrypsin-catalyzed hydrolysis of Fa-L-TyrOMe under the condition of $[E]_0 \gg [S]_0$ was followed at 330 nm as shown in Figure 1. Each reaction trace consisted of two phases, a fast increase in the absorbance followed by a slow decrease. Similar results were obtained for other methyl esters of amino acids in the wavelength range of 320-350 nm. As discussed in the previous paper[18] and by Bernhard,[25] these reaction traces are explained by the three-step mechanism including two intermediates, namely the E·S complex and acyl-enzyme (ES') (eq 1). It was shown, however, for one of the present substrates[21] that at pH 2.25 the reaction traces showed a characteristic variation depending on the measured wavelength, and that an additional intermediate, such as oxazolinone,[26,27] was likely to be involved. Our results show no indication of an additional intermediate down to pH 2.30, irrespective of the wavelength (320-350 nm). The optical density of the final state of each trace in Figure 1 was accounted for in terms of the sum of those due to the free enzyme and the product acid. Therefore, the contribution from the enzyme-product complex ($E \cdot P_2$) seemed very small, even with the undissociated product acid at low pH. The reported traces of previous authors[21,26] can only be explained with an extremely low dissociation constant and an extremely high extinction coefficient of $E \cdot P_2$ complex, which are very unlikely.

Assuming the three-step mechanism, the spectral changes like those shown in Figure 1 were analyzed, and the difference in the molar extinction coefficients of the intermediates was calculated at pH 6. For this calculation, the kinetic parameters obtained below were used, according to the procedure explained in the Appendix. This pH value was selected because of the moderate acylation rates at this pH, which enabled us to calculate the spectrum. Spectra of the two intermediates for the Fa-L-TyrOMe hydrolysis are shown in Figure 2, which also includes the wavelength dependence of the two measured quantities, *I* and *II* (*I*, the extrapolation of the slow decrease

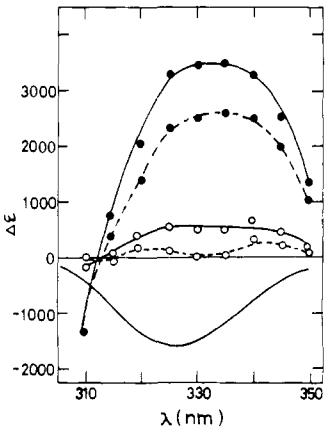

Figure 2. Difference spectra of the intermediates for α-chymotryptic hydrolysis of Fa-L-TyrOMe. 25 °C, μ = 0.25. Free substrate (S) is taken as the standard: (●—) *I*; (○—) *II*; (●---) ES′; (○--) E·S; (—) P_2 (anion). Undissociated product acid (P_2H) is equal to S. For definitions of *I* and *II*, see text.

Table I. Extinction Coefficients of the Intermediates and the Products with Reference to Those of the Free Substrates[a]

substrate	$\Delta\epsilon_{E\cdot S}$ (330 nm)	$\Delta\epsilon_{ES'}$ (330 nm)	$\Delta\epsilon_{P_2^-}$ (325 nm)
Fa-L-TyrOMe	+50	+2500	−1540
Fa-L-PheOMe	+200	+2450	−1630
Fa-L-TrpOMe	+200	+3300	−1815

[a] $\Delta\epsilon$ in M^{-1} cm^{-1}, pH 6.0, 25 °C, μ = 0.25.

Table II. pH Dependences of the Reaction Parameters of Binding and Acylation Steps[a]

substrate	pH	condition[b]	K_s', 10^{-5} M	k_2, s^{-1}	k_2/K_s', 10^5 M^{-1} s^{-1}
Fa-L-Tyr-OMe	4.22	e	3.3 ± 1.5	0.93 ± 0.18	0.22 ± 0.10
	4.94	e	1.8 ± 0.9	5.43 ± 0.68	1.8 ± 1.4
	5.52	e	4.0 ± 0.9	28.4 ± 3.7	5.5 ± 1.3
	5.98	e	2.9 ± 0.6	98.5 ± 10	31 ± 9
	6.30	e	1.6 ± 0.7	147 ± 17	58 ± 40
	6.71	e	3.1 ± 0.7	308 ± 31	91 ± 27
Fa-L-Phe-OMe	6.00	e	2.5 ± 1.2	28.3 ± 4.6	7.4 ± 4.6
	6.22	e	4.2 ± 0.5	41.9 ± 2.5	9.8 ± 1.1
	6.46	e	3.0 ± 0.9	53.6 ± 8.3	22 ± 11
	6.62	e	3.1 ± 1.0	90 ± 13	28 ± 10
	6.93	s	3.1 ± 0.6	102 ± 7	33 ± 9
	7.02	e	1.6 ± 0.2	105 ± 4	66 ± 14
	7.42	e	3.1 ± 0.3	170 ± 6	55 ± 7
	8.90	e	4.0 ± 2	203 ± 23	50 ± 10
Fa-L-Leu-OMe	5.86	s	11 ± 2	1.25 ± 0.14	0.11 ± 0.03
	6.10	s	17 ± 2	1.82 ± 0.14	0.11 ± 0.01
	6.38	s	18 ± 6	3.0 ± 0.6	0.13 ± 0.06
	6.46	s	14 ± 2	4.6 ± 0.3	0.33 ± 0.06
	6.57	e	9 ± 3	5.0 ± 1.0	0.48 ± 0.16
	6.86	s	9 ± 1	4.1 ± 0.2	0.44 ± 0.07
	6.89	s	4 ± 1	6.1 ± 0.5	1.4 ± 0.6
	7.55	s	9 ± 1	9.0 ± 0.4	0.95 ± 0.25
	7.72	s	4 ± 1	7.82 ± 0.35	1.7 ± 0.6
	8.57	e	≥20	...	0.96 ± 0.05
	9.00	e	≥20	...	0.97 ± 0.05

[a] 25 °C, μ = 0.25. [b] e: $[E]_0 \gg [S]_0$. s: $[S]_0 \gg [E]_0$.

in absorbance to time zero, *II*, optical density change within the dead time of the stopped-flow apparatus).

The $\Delta\epsilon$ values at the wavelengths where the difference spectra showed maxima for Fa-L-TyrOMe, Fa-L-PheOMe, and Fa-L-TrpOMe are shown in Table I. For all three substrates, the acylated enzymes had difference extinction coefficients of about +3000 M^{-1} cm^{-1} against the free substrates. A spectrophotometric study on the acyl-enzyme of Fa-L-Trp-α-chymotrypsin was reported by Miller.[20] In that case, the acyl-enzyme was obtained by mixing the enzyme with the free acid at low pH (2.37). Although the $\Delta\epsilon$ values obtained there were somewhat larger than ours (the main cause of the differences can be attributed to the rather higher estimated value of K_p by Miller), the shape of the spectrum given there is consistent with the present result. This consistency in the spectra of the intermediates, independently obtained from both ends of the reaction (S + E and P + E), provides further support for the three-step mechanism.

Though absorbance increases within the dead time of the stopped-flow apparatus were clearly observed, the intrinsic changes due to the formation of the noncovalently attached substrates (E·S) seemed very small or within experimental error. Accordingly, an intended study of the difference spectra and T-jump kinetics of the binding process of amide (Fa-L-$TrpNH_2$) or the D enantiomer (Fa-D-TrpOMe) to the enzyme was unsuccessful. Though Bernhard and Lau described a quantitative blue shift of the spectrum of the substrate (Fa-L-TrpOMe) below 300 nm bound to the enzyme,[25] from the spectral change within the dead time of the stopped-flow apparatus, the strong absorbance of the enzyme at wavelengths lower than 310 nm under our experimental conditions prevented the precise determination of the spectrum in that region.

pH Dependence of the Kinetic Parameters and the Specificity. From the reaction curves obtained under conditions of $[E]_0 \gg [S]_0$ such as in Figure 1, three kinetic parameters are obtained.[18] The apparent dissociation constant of the E·S complex (K_s') and the acylation rate constant (k_2) are obtained from the enzyme-concentration dependence of the fast phase of the reaction in which the absorbance increases, taking into account the slower phase in which the absorbance decreases. The deacylation rate constant (k_3) is directly obtained as the inverse of the relaxation time of the slow decreasing phase. Under the condition of $[S]_0 \gg [E]_0$, only K_s' and k_2 were obtained. In this case k_3 values obtained under the $[E]_0 \gg [S]_0$ condition were used to calculate k_2 from the $[S]_0$ dependence of the apparent acylation rate constant ($= [k_2[S]_0/(K_s' + [S]_0)] + k_3$). The pH dependences of these kinetic parameters at 25 °C for Fa-L-TyrOMe, Fa-L-PheOMe, and Fa-L-LeuOMe are shown in Table II (K_s', k_2) and in Figure 3 (k_3). The limiting values at high pH and the pK_a values were obtained by Eadie analogue rearrangement of the data ($k \times [H^+]$ vs. k) and are listed in Table III. In the case of the k_2 value for Fa-L-TyrOMe, the very high acylation rate for this substrate limited the pH range of the measurements, and therefore the limiting value was obtained from the lower half of the sigmoid. The k_2/K_s' value was known from the abscissa intercept of the Eadie plot of the acylation rate. The limiting parameters and pK_a values for k_2/K_s' are also listed in Table III. The higher pK_a value of the group in the free enzyme ($pK_{a_2} \simeq 9$)[3,28,29] was not evaluated from k_2/K_s'–pH profiles because of the difficulties in measuring the acylation rates in the higher pH region. $(k_2/K_s')_{lim}$ was calculated by assuming $pK_{a_2} \simeq 9$. In Table III, the reaction parameters for Fa-L-TrpOMe, obtained in the previous paper,[18] are compared. For the acid–base equilibria of the enzyme, the enzyme–substrate complex, and the acyl-enzyme, the four esters gave similar values, except for a slightly lower pK_a for k_2 of Fa-L-LeuOMe. Furthermore, the protonation equilibrium at the active site seems insensitive to the presence of the noncovalently attached substrate or to

Table III. Limiting Parameters and Acid-Dissociation Constants for Elementary Steps of α-Chymotrypsin-Catalyzed Hydrolyses of Four Specific Substrates[a]

substrate	deacylation		acylation		binding	second-order rate	
	pK_a	$k_{3\,(lim)}$, s^{-1}	pK_a	$k_{2\,(lim)}$, s^{-1}	K_s'[b], 10^{-5} M	pK_{al}	$k_2/K_{s(lim)}$, $10^5\ M^{-1}\ s^{-1}$
Fa-L-TyrOMe	6.85	110	(6.85)	770	2.8	...	(300)
Fa-L-PheOMe	6.85	35	6.85	200	3.2	7.0	90
Fa-L-LeuOMe	6.85	1.5	6.60	8.5	11	7.1	2
Fa-L-TrpOMe[c]	6.85	29	6.85	360	0.8	6.9	600

[a] 25 °C, μ = 0.25. The limiting and pK_a values were calculated according to the following equations. For k_2 and k_3, $k = k_{(lim)}/(1 + [H^+]/K_a)$; for k_2/K_s', $k_2/k'_{s(app)} = k_2/K'_{s(lim)}/(1 + [H^+]/K_{al})/(1 + K_{a2}/[H^+])$. The standard deviations of pK_as are 0.05 (for deacylation and acylation) or 0.1 (for second-order rate), and those for the rate parameters are 5% (for k_2 and k_3) or 10% (for K_s' and k_2/K_s'). [b] Average values for the whole pH range for Tyr and Phe and average values in the neutral pH region for Leu and Trp. [c] From ref 18.

the covalently linked acyl group, in the case of these specific substrates.

The pH dependence of K_s' is interesting. Tyr and Phe derivatives showed no significant trend below pH 9, while Fa-L-LeuOMe showed K_s' increasing with decreasing pH, which resembles the case of Fa-L-TrpOMe.[18] In the latter case, the pH-dependent K_s' was ascribed to the existence of another dissociative group, whose pK_a is 6.0 in the free enzyme and 5.1 in the E-S complex, locating in such a position that it cannot influence the binding of Tyr or Phe derivative. Though a nonproductive binding mode[30] was excluded in the case of the Trp derivative on the basis of several reasons,[18] such a binding mode is to be considered for the case of the Leu derivative. Introduction of the Fa group caused the most pronounced decrease in K_s' for the Leu derivative among the four esters, as seen by comparing the K_s' values in Table III with the reported K_s', K_m, or K_i values of the corresponding acetyl derivative,[31-36] and a slightly lower pK_a value for k_2 was observed for the Leu derivative. When we compare the structure of the β-aryl group of Leu with the *N*-acyl group, furylacryloyl, the latter being more bulky than the former, the chance of the occurrence of nonproductive binding seems high. Reversed order of the *N*-acyl group and β-aryl group may occur, in which the scissile ester bond will be located apart from the enzyme surface. Alternatively, a binding of the bulky *N*-acyl group into the tosyl hole (*ar*) with the β-aryl and ester part bound in the *n* and *am* sites,[37,38] respectively, may be considered. In either case the nonproductive binding of Fa-L-LeuOMe can influence the dissociative group, which interacts with Fa-L-TrpOMe, not with Fa-L-TyrOMe or with Fa-L-PheOMe.[18]

As seen from Table III, the order of the limiting kinetic parameters for Tyr, Trp, Phe, and Leu derivatives depends on the reaction step. Though this type of information on the specificity shown by the enzyme in the individual steps can be obtained by comparing the previously reported pre-steady-[3,39] and steady-[31-36,40,41] state parameters for various kinds of substrates, those reference values were obtained under different conditions, for different chemical species, often by measurement at single pH, and hence might contain unknown factors. The present article reports a set of the intrinsic (pH-independent) parameters for every elementary step for a series of substrates. Binding affinity of the substrate varies as Trp > Tyr ≃ Phe ≫ Leu, and the acylation rate is in the order of Tyr > Trp > Phe ≫ Leu. This order in k_2 is slightly different from that for $k_{cat.}$ of amide substrates[33-35] (Tyr > Phe > Trp), though the $k_{cat.}$ values for the amide substrates are known to be practically equal to the acylation rate constants. This may be related to the requirement of the secondary interaction for Phe substrates. As Bizzozero et al. reported,[4] Phe substrates exert other interactions than provided by the β-aryl group. In amide substrates, the amide NH can provide a secondary interaction with Phe-41 of the enzyme.[42] However, another explanation based on the difference in the detailed mechanisms

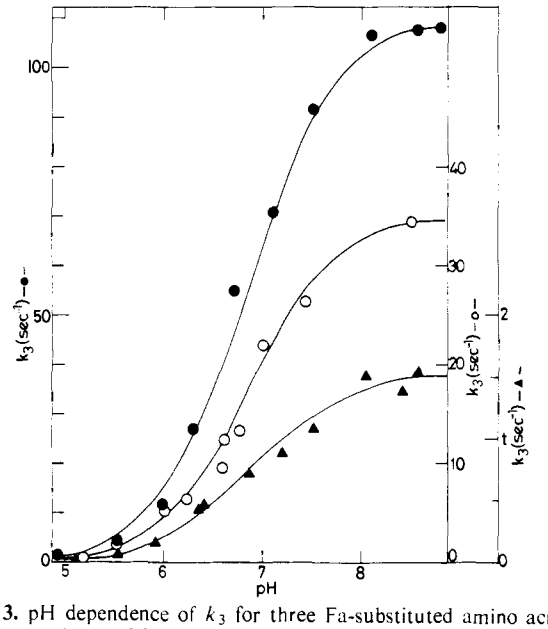

Figure 3. pH dependence of k_3 for three Fa-substituted amino acid-α-chymotrypsins. 25 °C, μ = 0.25. Citrate, phosphate, and borate: (●) Fa-L-Tyr-; (○) Fa-L-Phe-; (▲) Fa-L-Leu-.

of the acylation for ester and amide substrates[43,44] may be proposed. The deacylation rate constants follow the order Tyr > Phe > Trp ≫ Leu. It is usually assumed that k_3 is equal to $k_{cat.}$ for ester substrates. The present result, however, clearly shows that this is not rigorously justified for methyl esters of specific amino acids.[40] The k_2/k_3 values are 7.0 for Tyr, 5.7 for Phe, 7.1 for Leu, and 12.4 for Trp, and then these give the $k_{cat.}$ values of 87.5% of k_3 for Tyr, 85% for Phe, 88% for Leu, and 93% for Trp. When the relative specificity in k_3 is considered in a qualitative fashion, examination of the $k_{cat.}$ and k_3 values results in similar conclusions: Trp:Tyr:Phe:Leu = 1:3:1-2:0.01 (or less). This is not the case when a quantitative discussion on the k_3 values is to be made, as in the case of the temperature dependence of k_3. The assumption of $k_{cat.} = k_3$ can only be true in the cases of highly reactive leaving groups such as *p*-nitrophenolate;[41] e.g., in the case of Ac-L-TrpONp, $k_{cat.}$ is more than 99% of k_3.[45]

As a result, the overall reactivity of the substrates, k_2/K_s', varies in the order Trp > Tyr > Phe ≫ Leu, which is the same as that for $k_{cat.}/K_m$.[36] It was noted that the very high value of k_2/K_s' for Fa-L-TrpOMe resulted in a k_2 value higher than k_1.[18] Such a reversion in the rate-limiting step in the acylation reaction occurs only when k_2/K_s' values are higher than half of k_1, the maximum value of which was estimated to be 6-8 × $10^7\ M^{-1}\ s^{-1}$.[18] This is not the case for the three Fa-L-amino acid methyl esters studied here.

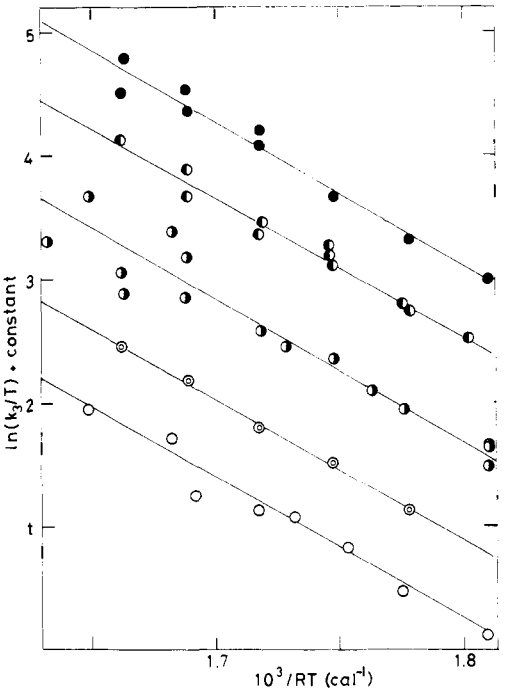

Figure 4. Temperature dependences of k_3 for five specific amino acid-α-chymotrypsins. pH 8.90, borate. μ = 0.25: (●) Fa-L-Tyr-; (◐) Fa-L-Trp-; (◑) Fa-L-Phe-; (◎) Fa-L-Leu; (○) Ac-L-Trp-.

Temperature Dependence of the Reaction Parameters. The three reaction parameters were measured at different temperatures. In view of the previously reported discontinuities of the temperature dependences for this enzyme,[10,17,46,47] the highest temperature was set at 40 °C for k_3 and 25 °C for k_2. The deacylation step (k_3) for five specific amino acid α-chymotrypsins (four Fa derivatives and the Ac-L-Trp-) was studied at pH 8.9 (Figure 4), so that the temperature dependence of the pK_a at the active site (around 6.9 at 25 °C) will have no effect on the result (see below). The absence of other groups of higher pK_a was supported by the pH dependence of k_3 at 25 °C and by the work of other authors[15,16,48] (at least up to pH 9 at 25 °C). Since the protonation at the active site is exothermic, the pK_a value increases with decreasing temperature, and therefore the pH of the reaction solution should be set as high as possible. It should be noted that the ordinate scale in Figure 4 is relative and does not show the magnitudes of the rates. All of the plots gave straight lines in the temperature range used. Calculated thermodynamic parameters are listed in Table IV, which also contains the results for nonspecific substrates, β-substituted acryloyl-α-chymotrypsins. The $\Delta H^{\ddagger}$ values for the five specific substrates are practically equal, while the $\Delta H^{\ddagger}$ values for three nonspecific substrates are considerably higher and they are not equal with each other. Among the previous discussions on the relation of specificity of the deacylation reaction with thermodynamic parameters,[6-10,48] a serious discrepancy was found as to which factor, enthalpic or entropic, is more important. Bender et al.[6] reported that the kinetic specificity was entropy controlled. Other authors reported for fatty derivatives[7-9] compensatory changes between activation entropies and enthalpies, the latter controlling the specificity. There have been some other reports that also showed compensatory changes, but also that the specificity is controlled by $\Delta S^{\ddagger}$ or by both.[10,49] As mentioned before, one of the main causes of these discrepancies is the fact that the previous authors measured $k_{cat.}$, not k_3. With regard to this point, the study by Baggot and Klapper,[10] for *p*-nitrophenyl esters of 2-(5-*n*-alkyl)furoic acids under the $[E]_0 > [S]_0$ condition, was the sole exception. They indicated the compensatory change of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. Similar to their result, the nonspecific substrates presently studied showed a compensatory change.

Table IV. Thermodynamic Parameters of Deacylation Step for Fa-L-amino acid- and β-Arylacryloyl-α-chymotrypsin[a]

acyl group	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, kcal/mol
Fa-L-Trp-	11.3 ± 0.6	−13.6 ± 1.9
Fa-L-Tyr-	11.6 ± 0.8	−11.3 ± 2.8
Fa-L-Phe-	11.7 ± 1.1	−12.4 ± 3.6
Fa-L-Leu-	11.3 ± 0.3	−22.6 ± 1.1
Ac-L-Trp-	11.4 ± 1.1	−13.7 ± 3.9
indoleacryloyl-	14.1 ± 0.4	−23.9 ± 1.5
cinnamoyl-	14.8 ± 0.5	−17.7 ± 1.6
furylacryloyl-	19.4 ± 0.8	−5.8 ± 2.6

[a] μ = 0.25. pH 8.9, borate buffer.

Bender et al.[1,6] proposed that specificity was entropy controlled for both specific and nonspecific substrates, and discussed this in terms of the freedom of the internal rotation of the bonds. The present study, however, seemed to limit their explanation to the specific substrates. The interpretation of differences in the specificity in terms of the rotational freedom of the acyl-enzyme may be applicable only to the amino acid derivatives. In such cases the hydrogen bond between NH of the acyl group and Ser-214[50] will cause a considerable restriction of freedom and the differences in the activation entropies of deacylation simply reflect the facility of freezing the residual rotational freedom of the acyl-enzyme, which is determined by the interaction of the β-aryl group with the tosyl hole of the enzyme. On the contrary, for substrates without an α-NH, such as those derived from aliphatic acids or acryloyl derivatives, much more freedom should be frozen in the activation process and other energetic terms, e.g., hydration and interaction of the alkyl chain with other sites of the enzyme than the tosyl hole, can affect the specificity. As a result, a compensatory change of activation parameters is observed, instead of the simple relation seen for the amino acid derivatives.

The temperature dependences of k_2 and K_s' for Fa-L-PheOMe are shown in Table V. The study of Fa-L-PheOMe was undertaken because it allowed the quantitative determination of the temperature dependence of the acylation step, because of the substrate's suitable reaction rates and binding affinity. Additionally, the *p*-nitrophenyl ester of *N*-acetyl-L-tryptophan[45] was studied for comparison. The acylation kinetics were measured at pH values near 6, because it is difficult to obtain reliable thermodynamic parameters from measurements at higher pH. For both substrates the linearity of the three points in the Arrhenius plot was good, and the apparent thermodynamic parameters were obtained. To determine the intrinsic changes in energies, corrections due to the enthalpy of the dissociation at the active site (ΔH_{diss}) were made,[9] according to eq 2 and 3:

$$\Delta G^{\ddagger}_{app} = RT \ln (1 + [H^+]/K_a) + \Delta G^{\ddagger}_{int} \quad (2)$$

$$\Delta H^{\ddagger}_{app} = \Delta H_{diss}/(1 + K_a/[H^+]) + \Delta H^{\ddagger}_{int} \quad (3)$$

The reaction was measured at the same pH value at different temperatures. The subscripts app and int denote the apparent values and the intrinsic values, respectively. In the calculation of the intrinsic parameters, the ΔH_{diss} value was taken from ref 16 (ΔH_{diss} = 5.0 kcal/mol). Though this value was not obtained from a pre-steady-state kinetic measurement, it is the single reported value of ΔH_{diss} for the acylation step, and we believe that it does not differ much from the true value, judging from the ΔH values of free histidine and free aspartic acid and from ΔH_{diss} for the deacylation step.[9,16,51] Ac-L-TrpONp

Table V. Temperature Dependence of k_2 and K_s'[a]

substrate	pH	temp	k_2, s^{-1}	$\Delta H^{\ddagger}_2$,[b] kcal/mol	$\Delta S^{\ddagger}_2$,[b] eu	K_s', 10^{-5} M
Fa-L-PheOMe	5.96	8.8	6.1 ± 0.8	14.3 ± 0.6	−4	3.5 ± 1.6
		16.6	13.2 ± 0.8			4.4 ± 0.7
		25.0	26.1 ± 4.2			2.5 ± 1.2
Ac-L-TrpONp	5.92	10.1	252 ± 13	16.2 ± 0.4	+10	9 ± 1
		17.1	535 ± 23			14 ± 1
		25.0	1120 ± 180			15 ± 3

[a] μ = 0.25, 0.1 M, phosphate buffer. [b] Corrected for ΔH value of the active-site protonation.

showed a larger activation enthalpy than Fa-L-PheOMe, and the difference in k_2 values of these two substrates is due to the entropic term. In any case, these activation enthalpies are fairly large (even larger than those for the k_3 process of specific substrates, Table IV), and the activation entropies are less negative or positive. A positive activation entropy was reported for the acylation of α-chymotrypsin by a *p*-nitrophenyl acetate (at pH 7.4, 279–293 K),[17] and these positive $\Delta S^{\ddagger}$ values might be characterized to the ester substrates with a good leaving group, such as *p*-nitrophenol.

Though it is well known that the esterolytic reactions with dissociative mechanisms exhibit less negative or positive $\Delta S^{\ddagger}$ values,[52] the conformational entropies of both the enzyme and the substrate should be considered in enzymatic reactions. The $\Delta S^{\ddagger}$ values observed here indicated that the substrate and the enzyme conformations were already properly arranged in the state of the E·S complex for the successive chemical reaction and therefore large entropy freezing is not needed. For the acylation process, a relaxation of the packed tight conformation of the E·S complex toward the loose acyl-enzyme was claimed.[53] It may be said that at least a considerable part of this relaxation of the protein conformation is realized already in the transition state of the acylation step, which is reflected in the positive or zero activation entropy.

Since the measured K_s' values contained rather large deviations, only a qualitative discussion can be made on the binding step; K_s' is less sensitive to the temperature change and ΔH is almost zero. In contrast, fairly negative ΔH and ΔS values were reported for the binding of substrate analogues or inhibitors to the active enzymes.[11,14] There exist in the literature[12,47] pH dependences of ΔH for substrate analogue binding in the direction of more negative values with increasing pH, and the present ΔH value obtained at pH ~6 may be explained in part by this pH dependence of ΔH. On the other hand, for the binding of the substrate analogue to the chemically inactivated enzymes,[14] no "induced-fit" conformation change was observed, and ΔH for binding was close to zero, which is similar to the present temperature insensitivity of K_s'.

In the hydrolysis of a specific substrate, a part of the binding energy is utilized for the successive chemical reaction by destabilizing the substrate.[54] Our result indicates that the "induced-fit" conformation change for the binding of a specific substrate to the active enzyme is not so large. The entropy loss due to the conformational change of the enzyme and the substrate may be of the same order as or smaller than the positive contribution from the desolvation of both the enzyme and the substrate. Aside from the energetic optimization, the time which is required to complete the conformation change[55] seems likely to be an important factor for the difference in the extents of the conformational changes upon binding between the cases of specific substrates and those of pseudosubstrates, since the hydrolytic reactions of the specific substrates may occur more rapidly than the rates of such conformational changes.[53]

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Appendix

In a single turnover experiment ($[E]_0 \gg [S]_0$), the optical density of the total system of:

$$E + S \underset{}{\overset{K_s}{\rightleftarrows}} E \cdot S \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 + P_1 \qquad \text{(A-1)}$$

follows

$$OD(t) = \epsilon_E[E]_0 + \epsilon_S[S] + \epsilon_{ES}[E \cdot S] + \epsilon_{ES'}[ES'] + \epsilon_{P2}[P_2] \qquad \text{(A-2)}$$

where ϵ_is are the respective molecular extinction coefficients and the possible $\epsilon_{EP_2}[EP_2]$ term is eliminated because of its insignificance. Optical absorbance of the enzyme molecule is assumed constant during the reaction and P_1 is considered colorless.

When the optical density is described as a difference from the final state of the single turnover reaction, where all of the substrates are to change into the products, the relative optical density ($\Delta OD(t)$) is given as follows:

$$\Delta OD(t) = \epsilon_E[E]_0 + \epsilon_S[S] + \epsilon_{E\cdot S}[E \cdot S] + \epsilon_{ES'}[ES'] + \epsilon_2[P_2] - (\epsilon_E[E]_0 + \epsilon_{P_2}[S]_0) \qquad \text{(A-3)}$$

$$= (\epsilon_S - \epsilon_{P_2})[S] + (\epsilon_{E\cdot S} - \epsilon_{P_2})[E \cdot S] + (\epsilon_{ES'} - \epsilon_{P_2})[ES'] \qquad \text{(A-4)}$$

$$= \Delta\epsilon_s[S] + \Delta\epsilon_{E\cdot S}[E \cdot S] + \Delta\epsilon_{ES'}[ES'] \qquad \text{(A-5)}$$

$\Delta\epsilon_i$s denote $\epsilon_i - \epsilon_{P_2}$.

As was shown in ref 18, the concentration of each molecular species at time t is characterized by two relaxation times (τ_1 and τ_2):

$$\tau_1^{-1} = k_2[E]_0/([E]_0 + K_s') \qquad \text{(A-6)}$$

$$\tau_2^{-1} = k_3 \qquad \text{(A-7)}$$

With the reaction parameters and initial concentrations, eq A-5 is rewritten as:

$$\Delta OD(t) = \Delta\epsilon_S \frac{K_s[S]_0}{[E]_0 + K_s} \exp(-t/\tau_1) + \Delta\epsilon_{E\cdot S} \frac{[E]_0[S]_0}{[E]_0 + K_s} \exp(-t/\tau_1) - \Delta\epsilon_{ES'} \frac{k_2[E]_0[S]_0}{k_2[E]_0 - k_3([E]_0 + K_s)} \times \{\exp(-t/\tau_1) - \exp(-t/\tau_2)\} \qquad \text{(A-8)}$$

$$= \left\{\frac{\Delta\epsilon_s K_s + \Delta\epsilon_{ES}[E]_0}{[E]_0 + K_s} - \frac{\Delta\epsilon_{ES'}k_2[E]_0}{k_2[E]_0 - k_3([E]_0 + K_s)}\right\} \times [S]_0 \exp(-t/\tau_1) + \frac{\Delta\epsilon_{ES'}k_2[E]_0[S]_0}{k_2[E]_0 - k_3([E]_0 + K_s)} \exp(-t/\tau_2) \qquad \text{(A-9)}$$

Under practical conditions two quantities indicated in Figure 5, *I* and *II*, are measured: *I*, extrapolation of τ_2 process to time zero:

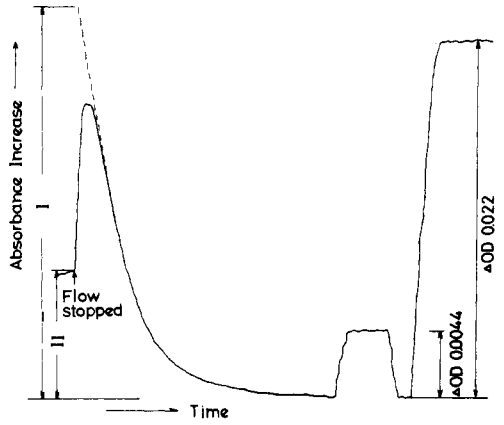

Figure 5.

$$I = \frac{\Delta\epsilon_{ES'} k_2[E]_0[S]_0}{k_2[E]_0 - k_3([E]_0 + K_s)} \qquad \text{(A-10)}$$

II, OD(t) at t equals the dead time of the apparatus (in the present case, 3 ms). The ΔOD value was known from the calibrations by a 1 or 5% cut of the light intensity (shown in left side), which correspond to a ΔOD of 0.0044 or 0.022, respectively, for a 1-cm cell. $\Delta\epsilon_{ES'}$ is obtained from I by eq A-10, using the experimentally obtained values of $\Delta\epsilon_S$, k_2, k_3, and K_s. At pH 6, k_2 is not so large and K_s' is practically equal to K_s. Therefore, the errors due to the preequilibrium hypothesis for the binding step[18] can be ignored. From the $\Delta\epsilon_{ES'}$ value, $\Delta\epsilon_{E,S}$ is calculated according to eq A-9.

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