Use of the Specificity Constant of α-Chymotrypsin[1]

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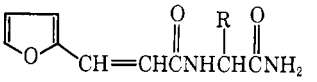
Abstract: The turnover constant, k_{cat}, the Michaelis–Menten constant, K_m, and the specificity constant, k_{cat}/K_m, were calculated for the α-chymotrypsin-catalyzed hydrolysis of the N-3-(2-furyl)acryloylamides of L-tryptophan, L-phenylalanine, L-α-aminoheptanoic acid, and L-leucine. No correlation was observed between the structure (hydrocarbon side-chain character) of these amino acid derivatives and either k_{cat} or K_m, but an excellent correlation was found between the structure and the specificity constant, k_{cat}/K_m. The relative specificities $(k_{cat_1}/K_{m_1})/(k_{cat_2}/K_{m_2})$ between any of the above amides was the same (within experimental error) as those calculated previously between the analogous N-acetylamino acid methyl esters. Therefore, the proposal that the specificity constant is the most accurate reflection (of all kinetic constants) of the α-chymotrypsin specificity for a substrate is confirmed. From this proposal (1) an interpretation is established for the binding specificity of a substrate to the enzyme, and (2) the specificity constant and relative specificity can be used to predict the behavior of α-chymotrypsin toward any given substrate which is a derivative of an α-L-amino acid.

The enzyme α-chymotrypsin selectively catalyzes the hydrolysis of ester and peptide bonds (the C–X bond in Figure 4) in which the reactive carbonyl group belongs to the L-amino acids tryptophan, tyrosine, phenylalanine, and, to a lesser extent, leucine. The kinetic pathway of this enzyme has been elucidated for the nonspecific substrates *p*-nitrophenyl acetate,[3] esters of *trans*-cinnamic acid,[4] and recently more dramatically for the specific substrate N-3-(2-furyl)acryloyl-L-tryptophan methyl ester.[5] The α-chymotrypsin-catalyzed hydrolysis of an ester (or other) substrate is a three-step process (eq 1) in which are formed (1) ES, an enzyme–substrate complex, (2) ES′, the acyl enzyme, and P_1 the alcohol, and (3) E, the enzyme, and P_2, the carboxylic acid. The steps following the reversible complex formation have been characterized.[6,7] Since the enzyme-catalyzed process is now known at the level of the individual steps, it is possible to probe meaningfully the striking property of enzyme specificity.[7-9] In other words, one may now begin to understand why some substrates, particularly those amino acid derivative with the larger hydrocarbon side chain,[9] are enzymatically hydrolyzed much faster than others, and why amino acid derivatives of the L configuration are generally hydrolyzed much faster than those of the D configuration.[10] Each of the three steps in the mechanism of eq1 has its own specificity. Most of the data in the literature has been determined in terms of k_{cat} $(k_2k_3/(k_2 + k_3))$, the turnover constant, and K_m $(K_sk_3/(k_2 + k_3))$, the apparent binding constant, which are not necessarily related to the individual rate constants in a simple manner. It has been proposed[7] that of all kinetic parameters k_{cat}/K_m most accurately reflects the specificity of substrates to α-chymotrypsin. In order to test this hypothesis it seemed desirable to obtain data and calculate the specificity constants not for a series of compounds in which k_{cat} and K_m may be complex functions (see above) as in ester substrates,[8b] but for a series in which k_{cat} and K_m measure simple constants (k_2 and K_s, respectively). Amides meet this criterion.[11]

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



Therefore, the N-3-(2-furyl)acryloylamides of L-tryptophan, L-phenylalanine, L-α-aminoheptanoic acid, and L-leucine were selected for examination because these particular amides possess a chromophore[12] ideal for monitoring the hydrolytic reaction of the primary amide bond. The data from these experiments are analyzed in terms of the specificity constant, k_{cat}/K_m.

Experimental Section

Materials. 3-(2-Furyl)acryloyl Chloride. 3-(2-Furyl)acrylic acid (10.3 g, 0.0725 mole), Aldrich Chemical Co., mp 139.5–141.5° (lit.[13] mp 140–142°), and 15 ml (0.21 mole) of thionyl chloride, Distillation Products, Inc., were mixed and allowed to stand at 25° for 1.5 hr in a flask protected by a calcium chloride tube, as described earlier.[14] Distillation of the resultant brown mixture at 134° (3.5 mm) afforded 10.2 g (88%) of a light yellow oil which crystallized upon cooling, mp 32–34°. This material was used immediately since partial decomposition (discoloration) occurred rapidly. However, redistillation of the discolored mixture yielded colorless chloride.

N^{α}-3-(2-Furyl)acryloyl-L-tryptophanamide (Cyclo Chemical Co., Lot K5502-2) was crystallized from ethyl acetate–hexane, mp 176–178°, $[\alpha]^{25}D$ −60.5 ± 6.0° (*c* 3, ethanol), uv max (3.2% acetonitrile–pH 8 Tris) 305 mμ (ε 27,600). *Anal.* Calcd for $C_{18}H_{17}N_3O_4$: C, 66.86; H, 5.30; N, 13.00. Found: C, 66.81; H, 5.31; N, 13.01.

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(2) National Institutes of Health Postdoctoral Fellow, 1966–1968.

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Table I. The k_{cat} and K_m Values for the α-Chymotrypsin-Catalyzed Hydrolysis of N-3-(2-Furyl)acryloyl-L-amino Acid Amides at pH 8.05 ± 0.05, 25°, 0.1 *M* Tris

Amino acid	$k_{cat} \times 10^2$ sec^{-1}	$K_m \times 10^3$ *M*	k_{cat}/K_m, M^{-1} sec^{-1}	$\frac{k_{cat}/K_m}{k_{cat_{Phe}}/K_{m_{Phe}}}$
Tryptophan	6.9 ± 0.4	1.4 ± 0.4	49 ± 4	5.2
Phenylalanine	4.22 ± 1.2	4.5 ± 1.5	9.4 ± 2.0	1
α-Aminoheptanoic acid	0.105 ± 0.015	0.90 ± 0.20	1.18 ± 0.08	0.12
Leucine			0.19 ± 0.02	0.02

The hydrolysis product was identified as N-3-(2-furyl)acryloyl-L-tryptophan by comparison of the uv spectrum, uv max (3.2% acetonitrile–pH 8 Tris) 302 mμ (ε 28,100), with the uv spectrum of the authentic acid,[15] uv max (3.2% acetonitrile–pH 8 Tris) 302 mμ (ε 25,320). This is distinguishable from 3-(2-furyl)acrylic acid, uv max (3.2% acetonitrile–pH 8 Tris) 292 mμ (ε 25,000).

The N-3-(2-furyl)acryloylamino acid amides described below were prepared according to the following method. Two mmoles of L-amino acid amide hydrochloride (recrystallized from acetone–water) and 5.0 mmoles of triethylamine (Matheson Coleman and Bell Co., bp 88.5–89.0°) were mixed in 10 ml of benzene. A solution of 2.1 mmoles of 3-(2-furyl)acryloyl chloride in 10 ml of benzene was added to this mixture which was gently swirled. After 1 hr 25 ml of water and 25 ml of ethyl acetate were added and the organic phase was washed with 15 ml of 3 *N* hydrochloric acid, dried (magnesium sulfate), and treated with charcoal. Evaporation at reduced pressure and addition of hexane to the residue induced crystallization. After two recrystallizations (ethyl acetate–hexane) the desired N-3-(2-furyl)acryloylamino acid amide was obtained in 20–40% yield.

N-3-(2-Furyl)acryloyl-α-phenylalaninamide was prepared from L-phenylalaninamide hydrochloride [Cyclo Chemical Co., Lot K5150, $[\alpha]^{25}D$ +19.9° (*c* 2, water), lit.[16] $[\alpha]^{25}D$ +20.7° (*c* 1, water)] and had mp 224–227°, $[\alpha]^{25}D$ −73.5 ± 7.3° (*c* 1, acetonitrile), uv max (3.2% acetonitrile–pH 8 Tris) 305 mμ (ε 25,800). *Anal.* Calcd for $C_{16}H_{16}N_2O_3$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.51; H, 5.85; N, 9.67.

The hydrolysis product was identified as N-3-(2-furyl)acryloyl-L-phenylalanine by comparison of the uv spectrum, uv max (3.2% acetonitrile–pH 8 Tris) 302.5 mμ (ε 27,000), with the uv spectrum of the authentic acid,[15] uv max (3.2% acetonitrile–pH 8 Tris) 302.5 mμ (ε 26,600).

N-3-(2-Furyl)-L-α-aminoheptanamide was prepared from L-α-aminoheptanamide hydrochloride [Cyclo Chemical Co. Lot K5460, $[\alpha]^{25}D$ +9.4 ± 0.6° (*c* 3, water). *Anal.* Calcd for $C_7H_{17}N_2OCl$: C, 46.53; H, 9.48; N, 15.50. Found: C, 46.74; H, 9.42; N, 15.26] and had mp 179–181°, $[\alpha]^{25}D$ +24.2 ± 6.7° (*c* 1, acetonitrile), uv max (3.2% acetonitrile–pH 8 Tris) 304 mμ (ε 26,000). *Anal.* Calcd for $C_{14}H_{20}N_2O_3$: C, 63.62; H, 7.62; N, 10.54. Found: C, 63.75; H, 7.64; N, 10.39.

The hydrolysis product was assumed to be N-3-(2-furyl)acryloyl-L-α-aminoheptanoic acid on the basis of the uv spectrum, uv max (3.2% acetonitrile–pH 8 Tris) 302.5 mμ (ε 26,100).

N-3-(2-Furyl)acryloyl-L-leucinamide was prepared from L-leucinamide hydrochloride [Cyclo Chemical Co., Lot K4934, $[\alpha]^{25}D$ +10.8° (*c* 2, water), lit.[16] $[\alpha]^{25}D$ 10.0° (*c* 1, water)] and had mp 176–178°, $[\alpha]^{25}D$ +18.3 ± 3.6° (*c* 1.5, acetonitrile), uv max (3.2% acetonitrile–pH 8 Tris) 305 mμ (ε 27,200). *Anal.* Calcd $C_{13}H_{18}N_2O_3$: C, 62.38; H, 7.25; N, 11.19. Found: C, 62.36; H, 7.30; N, 10.85.

The hydrolysis product was identified as N-3-(2-furyl)acryloyl-L-leucine by comparison of the uv spectrum, uv max (3.2% acetonitrile–pH 8 Tris) 302.5 mμ (ε 28,400), with the uv spectrum of the authentic acid (obtained by alkaline hydrolysis of the amide), mp 160–162°, uv max (pH 8 Tris) 302.5 mμ (ε 27,800), mass spectrum *m/e* 251 (3.2%). *Anal.* Calcd for $C_{13}H_{17}NO_4$: C, 62.14; H, 6.82; N, 5.57. Found: C, 61.60; H, 6.83; N, 6.01.

α-Chymotrypsin. Worthington α-chymotrypsin (three times crystallized, salt free), Lots CDI-6KB and CDI-7CD, was used without further purification. Enzyme solutions were routinely passed through 0.22-μ Millipore filters to guarantee 100% optically clear solution. The operational normality of the enzyme solution was determined[17] by titration with N-*trans*-cinnamoylimidazole according to method A.[17] Stock substrate solutions were made from Eastman Spectra Grade acetonitrile which had been distilled from phosphorus pentoxide. All water used was glass distilled in a Corning AG-2 still after passing through an Amberlite MB 3 resin and a cationic exchanger. Tris(hydroxymethyl)aminomethane buffer (0.1 *M*) was prepared from Trizma Base (Sigma Chemical Co.). Acetate buffer was prepared[18] from Mallinckrodt reagent grade sodium acetate trihydrate. All pH values were measured on a Radiometer 4C pH meter. At the end of each kinetic run the pH of the solution was measured.

Kinetic Measurements. Kinetics of hydrolysis were determined using a Cary 14 PM recording spectrophotometer (0–0.1 and 0–1.0 absorbance slide wires) equipped with a thermostated cell compartment. The hydrolyses of the N-furylacryloyl-L-amino acid amides were conducted at 25° and pH 8.05 ± 0.05, the pH maximum of k_{cat}/K_m,[19] and were observed against a buffer blank as follows (amino acid, wavelength in mμ, Δε observed): tryptophan, 340, 740 ± 20; phenylalanine, 344, 490 ± 20; α-aminoheptanoic acid, 340, 720 ± 20; leucine 340, 765 ± 30. In all cases $\epsilon/\Delta\epsilon$ = 5.0 ± 0.5.

A typical experiment on the Cary spectrophotometer is described for the hydrolysis of N-3-(2-furyl)acryloyl-L-phenylalaninamide as follows. Three milliliters of 0.1 *M* Tris buffer was equilibrated at 25.0 ± 0.1° in the cuvet in the sample compartment of the spectrophotometer. An acetonitrile stock solution of the amide (75 μl, 5.1×10^{-2} *M*) and 25 μl of acetonitrile were added and recording commenced. After approximately 1 min, 50 μl of enzyme stock solution (1.14×10^{-3} *M*) was added and recording commenced after 20 sec. The absorbance data were converted into initial rate data by using the difference in molar absorptivities of the ester and acid. The change in molar absorptivity was determined by making one reaction go to completion.

Results

The kinetics of the hydrolysis reaction were analyzed[20] in terms of eq 1, which, under turnover conditions, is identical with the Michaelis–Menten[21] equation where P_1 represents ammonia, and P_2, the N-3-(2-furyl)acryloylamino acid. The general equation for velocity is

$$v = \frac{k_{cat}[E][S]}{K_m + [S]} \quad (2)$$

where

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \quad (3)$$

and

$$K_m = K_s \frac{k_3}{k_2 + k_3} \quad (4)$$

For amides, acylation is the rate-limiting step,[11] *i.e.*, $k_3 \gg k_2$, so that eq 3 and 4 reduce to $k_{cat} = k_2$ and $K_m = K_s$, respectively, even if nonproductive binding does occur (see Discussion).

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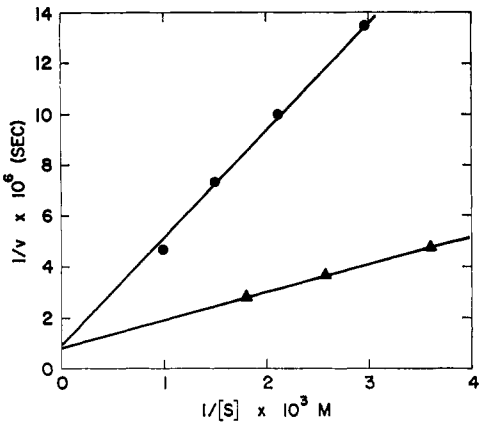

Figure 1. The reaction of α-chymotrypsin with (1) N-3-(2-furyl)-acryloyl-L-tryptophanamide (▲), [E] = 1.81 × 10⁻⁵ *M*, [S] = 2.77–5.55 × 10⁻⁴ *M*; (2) *N*-3-(2-furyl)acryloyl-L-phenylalaninamide (●), [E] = 1.98 × 10⁻⁵ *M*, [S] = 4.0–12.1 × 10⁻⁴ *M*; pH 8.05 ± 0.05, 25°, 3.2% v/v acetonitrile–water.

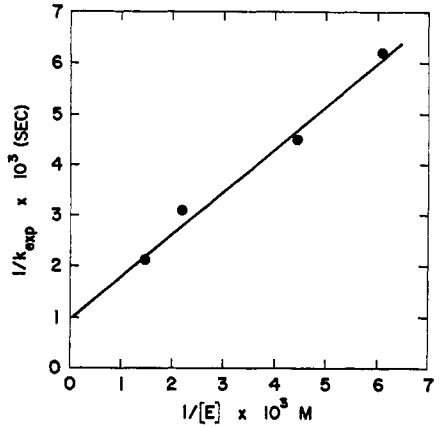

Figure 2. The reaction of α-chymotrypsin with N-3-(2-furyl)-acryloyl-L-heptanamide, [S] = 8.75 × 10⁻⁵ *M*, [E] = 1.1–6.7 × 10⁻⁴ *M*, pH 8.05 ± 0.05, 25°, 3.2% v/v acetonitrile–water.

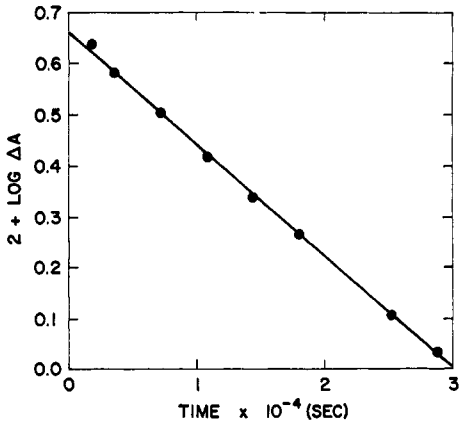

Figure 3. The first-order reaction of α-chymotrypsin (3.38 × 10⁻⁴ *M*) with N-3-(2-furyl)acryloyl-L-leucinamide (6.26 × 10⁻⁵ *M*) at pH 8.05 ± 0.05, 25°, 3.2% v/v acetonitrile–water.

The experiments with the amides of tryptophan and phenylalanine were conducted with [S] ≫ [E]. The data, shown in Figure 1, for a single Lineweaver–Burk plot was obtained from the linear steady-state rates of several reactions using different initial substrate conditions. These rates were measured for a fraction of the total extent of reaction, thus $v = \Delta abs/(\Delta\epsilon \times \Delta t)$ where Δabs is the change in optical density observed, Δε is the calculated change in molar absorptivity and Δ*t* is the time of observation of the absorbance change. The total change in molar absorptivity was determined by hydrolyzing each amide completely. The long extrapolation of the lines in Figure 1 introduce considerable uncertainty in the kinetic parameters (see Table I). The enzymic hydrolysis of N-3-(2-furyl)-acryloyl-L-α-aminoheptanamide was sufficiently slow so that the experiments were conducted under the condition [E] ≫ [S], K_m ≫ [S]. The data were calculated from the first-order rate constants, k_{exp}, of several reactions using different initial enzyme concentrations. These reactions were measured for a large fraction of the reaction. The value for the infinite absorbance was calculated from a Kézdy plot[22] of the absorbances observed. In all reactions, good first-order plots were obtained. The analysis of data obtained under similar conditions has been described earlier.[23] By plotting $1/k_{exp}$ *vs.* 1/[E], shown in Figure 2, k_2 and K_s/k_2 were calculated from the intercept and slope, respectively.

The enzymic hydrolysis of N-3-(2-furyl)acryloyl-L-leucinamide was observed at [E] ≫ [S] and K_m ≫ [S] (and pH 8) for several hours. Under these conditions it was necessary to determine the amount of enzyme undergoing denaturation. At pH 8 k_d, the first-order rate constant for denaturation, is equal to 1.83 × 10⁻⁵ sec⁻¹.[19] The enzyme concentration in the leucinamide experiment was calculated to decrease by not more than 15%, and an excellent first-order plot (Figure 3) could be drawn for three half-lives. Using the scheme of eq 1, the velocity equation (eq 2) reduces to

$$v = k_{cat}[E][S]/K_m$$

The observed first-order rate constant is equal to $k_{cat}[E]/K_m$. The k_{cat}/K_m value was calculated to be 0.19 ± 0.02 M^{-1} sec⁻¹ by using the average enzyme concentration over the period of observation.

The kinetic constants k_{cat}, K_m, and $k_{cat}K/_m$ calculated from the plots shown in Figures 1–3 are presented in Table I. In this table, the value of K_m for the phenylalanine compound is about ten times larger than the value for the tryptophan compound. This trend is also found in four derivatives[24] of N-acetyl-L-phenylalanine compared to the same derivatives of N-acetyl-L-tryptophan (amide, ethyl ester, methyl ester, *p*-nitrophenyl ester). The values of k_{cat} of N-acetyl-L-tryptophanamide (4.37 ± 0.90 × 10⁻² sec⁻¹) and N-acetyl-L-phenylalaninamide (4.6 × 10⁻² sec⁻¹) are quite close to the values found for the corresponding N-furylacryloyl analogs.[24] This means that the phenylalanine derivatives bind better with the enzyme than the tryptophan derivatives. The α-aminoheptanoic acid residue has been used previously only once, as the N-acetyl methyl ester.[25] There, as in this work, its specificity lay between the phenylalanine and leucine derivatives in the series. We have not attempted to

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Table II. Relative k_{cat}/K_m Values for the Chymotrypsin-Catalyzed Hydrolysis of N-Acetylamino Acid Methyl Esters

Amino acid[a]	k_{cat}, sec^{-1}	$K_m \times 10^5$ M	$k_{cat}/K_m \times 10^{-4}$ M^{-1} sec^{-1}	$\frac{k_{cat}/K_m}{k_{cat_{Phe}}/K_{m_{Phe}}}$	Ref
			Set 1		
Try[b]	27.7	9.5	29.2	7.6	24
Phe[b]	57.5	150	3.8	1	24
			Set 2		
Phe[c]	48.2	180	2.7	1	*e*, *f*
Leu[c]	3.6	290	0.12	0.05	*e*
			Set 3		
Try[c]	49	11.7	42	10	8a, *g*
Phe[c]	52.5	125	4.2	1	8a, 25
Hep[c]	13.4	164	0.81	0.20	8a, 25
Leu[c]	4.6	290	0.16	0.04	8a, *h*
			Set 4		
Try[b,d]	30.5	0.2	1500	4.7	24
Phe[b,d]	77	2.4	320	1	24

[a] Try = tryptophan, Phe = phenylalanine, Hep = α-aminoheptanoic acid, Leu = leucine. [b] pH 7.0, 25°, 3.17% v/v acetonitrile–water. [c] pH 7.9, 25°, aqueous solution 0.10 M sodium chloride. [d] *p*-Nitrophenyl ester. [e] G. Hein and C. Niemann, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1341 (1961). [f] M. L. Bender and W. L. Glasson, *J. Am. Chem. Soc.*, **82**, 3336 (1960). [g] J. R. Knowles, *Biochem. J.*, **95**, 180 (1965). [h] G. E. Hein, J. B. Jones, and C. Niemann, *Biochem. Biophys. Acta*, **65**, 353 (1962).

measure nonproductive binding. However, we have shown (see Discussion) that this phenomenon does not affect the calculations on which we base our hypothesis.

From the experimental point of view, enzyme specificity can be analyzed as (1) "relative binding specificity," the ratio of the binding constant, K_m, of a substrate to the binding constant of a reference substrate, and as (2) "relative kinetic specificity," the ratio of the turnover constant, k_{cat}, of a given substrate to that of a reference substrate under identical conditions. Then the variation of the specificity constant, defined as k_{cat}/K_m,[7] reflects the way a structural change in the hydrocarbon side chain attached to the asymmetric carbon atom of the particular L-amino acid amide affects the binding and/or reactivity. The other three groups, N-3-(2-furyl)acryloylamino, carboxamide, and hydrogen atom, are constant throughout the series. The ratio between specificity constants was calculated from the α-chymotrypsin-catalyzed hydrolysis of ester substrates (see Table II). A comparison of specificity constant ratios from ester substrate data is presented in Table II while that from amide data is presented in Table I. The correspondence of the values obtained from N-acetylamino acid methyl esters and N-3-(2-furyl)acryloylamino acid amides is indeed good. Reproducibility of specificity constant ratios within a factor of two from different laboratories is considered acceptable because of inherent difficulties in handling very low enzyme concentrations and extreme sensitivity of enzymatic reactions toward minute changes of reaction medium such as pH, temperature, and ionic strength.

Discussion[26]

It is possible, as has been suggested[27] earlier, that a substrate may bind to the enzyme in such a way that subsequent catalytic steps cannot occur. This process is called nonproductive binding. The only way for a substrate thus bound to react with the enzyme is to dissociate and then to reassociate with the enzyme in the productive mode. From such a scheme (eq 5

$$\begin{array}{ccccccc} E + S & \overset{K_s}{\rightleftharpoons} & ES & \xrightarrow{k_2} & ES' & \xrightarrow{k_3} & E + P_2 \\ \searrow\!\!\!\!\!\!\uparrow K_i & & & & + & & \\ ES_X & & & & P_1 & & \end{array} \qquad (5)$$

where ES_x is the nonproductive enzyme–substrate complex), one may derive an expression for the reaction velocity (see eq 3), k_{cat} and K_m.[7] Even in this more

$$k_{cat} = k_2/(1 + k_2/k_3 + K_s/K_i) \qquad (6)$$

$$K_m = K_s/(1 + k_2/k_3 + K_s/K_i) \qquad (7)$$

complex system, k_{cat}/K_m is still equal to k_2/k_s, a ratio of constants on the productive pathway of catalysis. Thus the specificity constant applies to amides (k_2 rate limiting), aryl and certain alkyl esters (k_3 rate limiting), and other carboxylic derivatives.

We have already defined relative binding specificity and relative kinetic specificity (see Results) between two substrates of the enzyme α-chymotrypsin. For every substrate which is hydrolyzed by α-chymotrypsin in a measurable way, the constant, k_{cat}/K_m can be calculated. This constant may then be compared with the constant calculated from the enzymic hydrolysis (under identical conditions) of another (reference) substrate. One can then tabulate and assess the specificity of one substrate (or any substrate) relative to a reference substrate, based on the physical process of binding and the chemical processes of acylation and deacylation. This is done in Tables I and II.

Enzyme specificity has been analyzed[7] by determining the individual effects on the α-chymotrypsin constant k_{cat}/K_m of the groups R_1, R_2, and X (see Figure 4) attached to the asymmetric carbon atom of an amino acid derivative. The influence of the X group on k_{cat}/K_m has been shown not to involve binding of X to the enzyme but rather to involve the electronic nature of X,[28] whereas the influence of the R_1 group on k_{cat}/K_m

(26) The definition and some implications of the specificity constant are discussed in ref 7.

(27) H. T. Huang and C. Niemann, *J. Am. Chem. Soc*, 74, 4634, 5963 (1952); G. E. Hein and C. Niemann, *ibid.*, 84, 4487 (1962); T. H. Applewhite, R. B. Martin, and C. Niemann, *ibid.*, 80, 1457 (1958); J. A. Thoma and D. E. Koshland, *ibid.*, 82, 3329 (1960); E. A. Zeller, G. Ramachander, G. A. Fleisher, T. Ishimaru, and V. Zeller, *Biochem. J.*, 95, 262 (1965); E. A. Zeller, *Biochem. Z.*, 339, 13 (1963); J. A. Thoma, *Biochemistry*, 5, 1365 (1965); K. R. Hanson, *ibid.*, 1, 723 (1962); T. E. Barman and H. Gutfreund, Proceedings of the International Colloquium on Rapid Mixing Sampling Techniques, Academic Press, New York, N. Y., 1964, pp 339–343.

(28) See ref 7, Table III.

(over a range of several hundred in magnitude of k_{cat}) has been shown to be due to binding to the enzyme and independent of X and R_2.[29] Similarly the influence of the R_2 group on k_{cat}/K_m has been shown to be due to enzyme binding and independent of X and R_1.[30] The specificity of R_2 has been expressed in free energy terms by

$$\log \frac{(k_{cat}/K_m)_{R_2R_1X}}{(k_{cat}/K_m)_{R_2OR_1X}} = S_{R_2} \qquad (8)$$

where the specificity factor S_{R_2} is dependent exclusively on the nature of R_2. It is these data that we have confirmed by studying the series of amides shown in Table I. For the N-acetyl-L-amino acid methyl esters k_{cat}/K_m varies from 0.16×10^4 to 42.0×10^4 M^{-1} sec^{-1}, while in the N-3-(2-furyl)acryloyl-L-amino acid amides k_{cat}/K_m varies from 0.019 to 49 M^{-1} sec^{-1}. For the two series of derivatives of tryptophan, phenylalanine, α-aminoheptanoic acid, and leucine presented in Tables I and II, the ranges of reactivity are quite different. No correlation between substrate structure (hydrocarbon side-chain character) and either the values of k_{cat} and values of K_m was apparent in the data. However, the encouraging observation was: the relative specificities as defined by k_{cat}/K_m are the same within experimental error in both tables. Increasing the hydrophobic nature of either the substrate R_1 or R_2 group decreases the free energy of activation of the over-all enzymic hydrolysis. Since the R_1 and R_2 terms contribute independently to the specificity constant, two independent binding areas must exist in the α-chymotrypsin molecule. Thus we have confirmed the earlier proposed hypothesis[7] for the specificity of α-chymotrypsin based on a catalytic center and two independent binding sites, a hydrophobic site (for the R_2 group) and a site (for the R_1 group) involving both hydrogen bonding and hydrophobic bonding.[31] A scheme whereby the binding of a substrate may take place independently at each site, but where only one complex, ES, is reactive, is shown in eq 9. If enzyme–substrate complex formation is much faster than the kinetic steps, the steady-state assumption for ES′ yields the rate expression of eq 10. If nonproductive binding occurs, a similar

$$\begin{array}{ccccccc} E + S & \overset{K_1}{\rightleftharpoons} & a & & & & \\ \uparrow\downarrow K_2 & & \uparrow\downarrow K_3 & & & & \\ b & \overset{K_4}{\rightleftharpoons} & ES & \xrightarrow{k_2} & ES' & \xrightarrow{k_3} & E + P_2 \\ & & & & + & & \\ & & & & P_1 & & \end{array} \qquad (9)$$

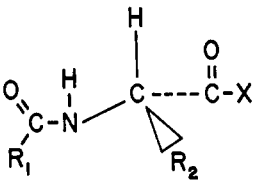

Figure 4. An L-amino acid derivative.

$$v = k[E][S]/D(S + 1/D) \qquad (10)$$

$$D = 1/K_1 + 1/K_2 + (1/K_1K_3)(1 + k_2/k_3)$$

but more complex expression is obtained.[7] With or without nonproductive binding, $k_{cat}/K_m = k_2/K_1K_3 = k_2/K_2K_4$, a ratio of constants on the productive pathway of catalysis.

The two-point attachment of the substrate to the enzyme is a necessary and sufficient condition for explaining the stereospecificity of α-chymotrypsin because the configuration of the substrate is also known. The designation to the substrate of L or D configuration fixes the relative position of the proton and the reactive carbonyl group on the asymmetric carbon atom. It is quite reasonable to regard the enzyme surface as an asymmetric environment. The active serine residue may therefore react with the bound substrate only when the reactive carbonyl group is in one of the two possible orientations, that imposed by the L configuration of the substrate (Figure 4).

In conclusion, the data presented have confirmed the hypothesis that the specificity of α-chymotrypsin-catalyzed reactions is independent of the nature of the leaving group and of the group acylating the α-amino nitrogen group. From this behavior we have proposed an interpretation for binding prerequisite to enzymic hydrolysis. This interpretation accounts for selectivity of enzyme–substrate interaction on the basis of substrate configuration and hydrocarbon side-chain character. Most important, since this hypothesis for specificity has been substantiated, the specificity constant (and relative specificity) can now be used with confidence to predict the behavior of α-chymotrypsin toward any given substrate.

(29) See ref 7, Table IV.
(30) See ref 7, Table VI.
(31) For the hypothesis of four independent binding sites see G. E. Hein and C. Niemann, *J. Am. Chem. Soc.*, **84**, 4495 (1962), and ref 26. For the application of this theory to the binding of amides see C. L. Hamilton, C. Niemann, and G. R. Hammond, *Proc. Natl. Acad. Sci. U. S.*, **55**, 664 (1966).