Mechanism of Chymotrypsin. Structure, Reactivity, and Nonproductive Binding Relationships†

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ABSTRACT: It is shown that anilide substrates of chymotrypsin may bind in a nonproductive mode, with the anilide ring in the substrate binding pocket. Acetyl- and trimethylammonium-substituted *N*-acetyl-L-tyrosineanilides bind predominantly productively. *Hydrophobic substituents such as -Cl and -NO_2 groups cause nonproductive binding to be 50–100 times stronger than productive.* The tight binding of these hydrophobic substrates is not due to the accumulation of a covalent intermediate. Similarly, the lowered k_{cat} values are not due to electronic effects on chemical reactivity but are a consequence of the nonproductive binding. At pH 7.8 the dissociation constants of *N*-acetyl-L-tyrosine-4-chloroanilide, *N*-acetylglycine-4-chloroanilide, and formyl-4-chloroanilide are 0.78, 0.98, and 0.40 mM, respectively. The binding is almost completely associated with that of the 4-chloroanilide portion. The dissociation constants of a series of substituted formanilides follow π, the hydrophobicity constant. The binding of substituted *N*-acetyltyrosineanilides can be separated into the productive and nonproductive binding components. The latter also follows π for the formanilide ring. Earlier structure-reactivity studies on *N*-acetyl-L-tyrosineanilide hydrolysis used substituents that had correlated π and σ^- values. Artifactual results were obtained which are due to variable nonproductive binding causing compensating changes in k_{cat} and K_M. A more extensive series of substrates in this study using substituents in which π and σ^- are not correlated shows that there is no simple relationship between σ^- and observed values of k_{cat} and K_M. There is now no experimental evidence for pre-transition-state protonation. Direct evidence is presented against the accumulation of a tetrahedral intermediate in the anilide hydrolysis. It is shown that when the competition between productive and nonproductive binding is pH dependent, the pH dependence of k_{cat} is controlled by a pK_a different from that of the catalytic group in the productive complex.

The chymotrypsin-catalyzed hydrolysis of specific anilide substrates is of some mechanistic and historical importance. Substituents in the aniline ring cause inductive effects which alter chemical reactivity but, it would be thought, cause only small steric effects. The hydrolysis of a series of substituted anilides of *N*-acetyl-L-tyrosine and *N*-acetyl-L-tryptophan has led to three theories of the mechanism of action of chymotrypsin based on the experimental linear free-energy relationships.

It was found that for a limited series of substituted anilides both k_{cat}[1] and K_M are decreased by electron withdrawal and that the apparent pK_a for the enzyme–substrate complex is in some cases significantly lowered (Inagami *et al.*, 1969). These authors suggested the possible contribution of an acidic group in the catalysis. Wang and coworkers (Wang and Parker, 1968; Parker and Wang, 1969) have formulated a general theory of enzyme catalysis by "directed proton transfers" based on the variation of k_{cat} with aniline basic strength. Caplow (1969) and Lucas and Caplow (1972), on the basis of the variation of k_{cat}, K_M, and the pK_a of k_{cat} with substituents, postulated the accumulation of a tetrahedral intermediate in the reaction—the enzyme stabilizing the otherwise unstable intermediate.

It occurred to us that the structure–reactivity relationships involved are not due to chemical reactivity changes but reflect substituent effects on the competition between productive and nonproductive modes of binding. The anilide portion may bind also in the hydrophobic pocket and will compete with the tryptophan and tyrosine aromatic rings. The variations in k_{cat} and K_M may then be due to the substituent effects on the hydrophobic nature of the aniline ring (see Figure 1).

The purpose of this study is to test this hypothesis and clarify the mechanistic situation.

Experimental Section

Materials

Three times crystallized, salt-free, lyophilized α-chymotrypsin (lot CDI 8LK) was obtained from Worthington. Protein concentrations were determined spectrophotometrically in 10^{-3} M HCl at 280 nm using a molar extinction coefficient of 5×10^4. Active-site titrations were performed at pH 2.4 using the Cbz-L-tyrosine *p*-nitrophenyl ester method (Kézdy *et al.*, 1964). The α-chymotrypsin contained 94% of the theoretical active sites.

Preparation of the Substrates and Inhibitors. FORMANILIDES. Acetic anhydride (14 ml) was added to 42 ml of 98% formic acid. After 5 min, the aniline was added to the stirred mixture which was left overnight at 0°. The formic and acetic acids were removed under reduced pressure at 50°. The compounds were chromatographed where necessary and crystallized to give the following products (melting point, crystallization solvent): 4-nitro, 196–197° (lit. 194–195°), water; 4-chloro, 101–102° (lit. 102°), water; 4-methoxy, 78–79° (lit. 81°) ether–petroleum ether (60–80°); 4-methyl, 50–51° (lit. 53°) ether–petroleum ether, chromatographed on

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‡ J. F. is a member of Fonds National de la Recherche Scientifique (Belgium).

[1] Abbreviations used in this paper are: PMSF, phenylmethanesulfonyl fluoride; methylchymotrypsin, α-chymotrypsin methylated on the imidazole of His-57 (Nakagawa and Bender, 1970); k_{cat} and K_M, the Michaelis–Menten parameters; K_I and K_S, dissociation constants for enzyme–inhibitor and enzyme–substrate complexes, respectively.

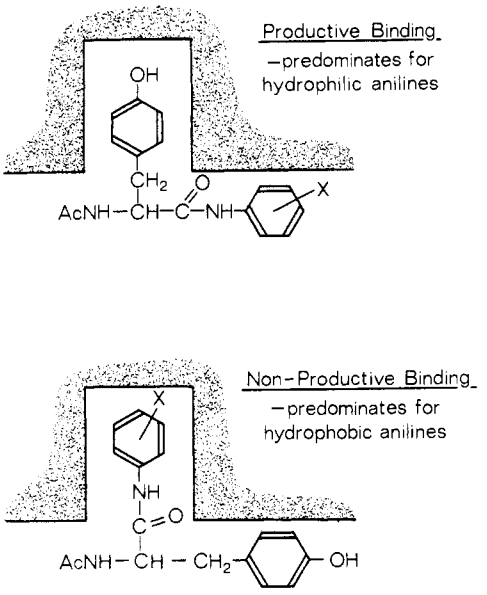


FIGURE 1: Illustration of productive and nonproductive binding for chymotrypsin and anilide substrates.

silica gel 20% acetone–petroleum ether; 3-chloro, 55–57° (lit. 58°), ether–petroleum ether, chromatographed on silica gel 20% acetone–petroleum ether; 4-cyano, 188–190° (lit. 189–190°), water; 4-acetyl, 104–105°, 20% ethanol–water; 3-methoxy, 55–57° (lit. 57°) ether–petroleum ether. (Literature melting points are from the Dictionary of Organic Compounds, 1966.)

ACETYLTYROSINEANILIDES were prepared by the azide coupling procedure as described by Inagami *et al.* (1969). Further purification was accomplished by chromatography on silica gel in 12% ethanol–chloroform. The melting points and yields were: 4-chloro, 229–231° (lit. 224–225°, 228–230°, 227–228°; Inagami *et al.*, 1969; Lucas and Caplow, 1971; Parker and Wang, 1968), ethyl acetate, 60%; 4-methoxy, 217–218° (lit. 218–219°, 220–221°; Lucas and Caplow, 1972; Inagami *et al.*, 1969), crystallized from ethyl acetate–ethanol–water, or 205–206° (lit. 205–206°); Parker and Wang, 1968), crystallized from acetone–petroleum ether, 60%; 4-acetyl, 245–247°, crystallized from acetone–petroleum ether, 20%; *p*-*N*-dimethylamino, 212–214°, crystallized from ethyl acetate, 70%. The *N*-acetyl-L-tyrosine-4-trimethylammoniumanilide iodide salt was prepared by methylation of *N*-acetyl-L-tyrosine-4-dimethylaminoanilide which was prepared as above. The dimethylaminoanilide (400 mg) was dissolved in 5 ml of dimethylformamide and 400 mg of iodomethane was added. After 1 hr 15 ml of acetone was added and the product slowly crystallized (mp 215–217°).

The anilides were all chromatographically homogeneous and had satisfactory nuclear magnetic resonance (nmr) spectra and elemental analyses. Optical purity was in all cases greater than 98% as shown by the amount of aniline released on chymotryptic hydrolysis monitored either spectrophotometrically or titrimetrically.

The acetylglycine-4-chloroanilide was synthesized by coupling acetylglycine and 4-chloroaniline with dicyclohexylcarbodiimide in dimethylformamide. The product crystallized from the solution by the addition of ethyl acetate and cooling. It was recrystallized from methanol, mp 226–227°. The *N*-formylphenylalanine semicarbazide has been described previously by Fersht and Requena (1971).

Methylchymotrypsin was prepared by the method of Henderson (1971) and was purified by affinity chromatography (Cuatrecasas *et al.*, 1968) and lyophilized. It was titrated before use with acetyl-L-tryptophan *p*-nitrophenyl ester at pH 7.8 after reinhibition by PMSF and found to contain 72% of the theoretical number of active sites. The dimethylformamide was obtained from B.D.H. dried over molecular sieves and distilled under vacuum. The *n*-octanol was obtained from B.D.H. and used without further purification.

Methods

The hydrolysis of the anilides at 25° was monitored under the experimental conditions given in Table I using a Gilford 2400 spectrophotometer or a Radiometer autotitrator. pH measurements were recorded on a Radiometer pH 26c meter at the same temperature. The stopped-flow device was as previously described (Fersht, 1972). The binding constants for the formanilides were measured by the competitive inhibition of the α-chymotrypsin-catalyzed hydrolysis of 5 mM *N*-formyl-L-phenylalanine semicarbazide at pH 7.75 (Tris), $\mu = 0.1$ in 5% dimethylformamide solutions at at least three inhibitor concentrations, using the equation

$$\frac{V - V_I}{V_I} = \frac{[I]}{K_I}\left(1 + \frac{[S]_0}{K_S}\right)^{-1}$$

where V and V_I are the rates in the absence and in the presence of inhibitor (I), respectively. The kinetics were followed by the automatic assay for semicarbazide previously described (Fersht and Requena, 1971). The values of k_{cat} and K_M for the substrate in the presence of 5% dimethylformamide were found to be 0.0325 sec^{-1} and 7.7 mM, respectively. (The comparison of these values with the values found by Fersht and Requena (1971), $k_{cat} = 0.0288$ sec^{-1} and $K_M = 2.28$ mM, gives the correction factors for the presence of 5% dimethylformamide used in Table VI.)

The binding constants of formanilide and acetylglycine-4-chloroanilide were found from the inhibition of the hydrolysis of *N*-acetyl-L-tyrosine-4-chloroanilide under the same conditions.

The binding of *N*-acetyl-L-tyrosine-4-chloroanilide to methylchymotrypsin was determined from the competitive inhibition of the acylation of the enzyme by acetyl-L-tryptophan *p*-nitrophenyl ester under pseudo-first-order conditions ($= [S] \ll K_S$). If k is the rate constant in the absence of inhibitor I and k_I in its presence then

$$k = k_{cat}\frac{[S]}{K_S}$$

$$k_I = \frac{k_{cat}[S]}{K_S\left(1 + \frac{[I]}{K_I}\right)}$$

so that

$$\frac{1}{k_I} = \frac{1}{k}\left(1 + \frac{[I]}{K_I}\right)$$

The *partition coefficients of the formanilides* between water and *n*-octanol were measured by shaking 5 ml of an aqueous solution of the formanilide ($\sim 10^{-2}$ M) with 1 or 5 ml of *n*-octanol equilibrated at 25°. Each layer (100 μl) was then diluted

TABLE I: Experimental Conditions for the Hydrolysis of *N*-Acetyl-L-tyrosineanilides by α-Chymotrypsin at 25°, pH 7.77, μ = 0.1, 5% Dimethylformamide.

Anilide	[Substrate] (M)	[Enzyme] (μM)	No. of Substrate Concns	[Substrate][a] for First-Order Kinetics (M)	λ (Δε)
4-$N(CH_3)_2$	5.1×10^{-4}–5.1×10^{-3}	9.3	7	10^{-3}	305[b]
4-CH_3O				3.3×10^{-4}	300
4-Cl	2×10^{-4}–1.5×10^{-3}	28	6		300 (1.04×10^3)
4-$N^+(CH_3)_3$	10^{-3}–3×10^{-2}	8.55	7	10^{-4}	300 (6.00×10^2)
4-CH_3CO	10^{-2}–10^{-3}	9.5	9	5×10^{-4}	340 (5.88×10^3)

[a] Reaction followed under pseudo-first-order conditions where [S] $\ll K_M$ using 10^{-5} M enzyme. [b] For the first-order kinetics. pH-Stat used for Michaelis–Menten plots.

into 3 ml of ethanol and the anilide concentration was assayed spectrophotometrically.

Stopped-Flow Search for Intermediates. (a) PROFLAVIN DISPLACEMENT. One syringe of the mixer contained 8×10^{-5} M α-chymotrypsin and 5×10^{-5} M proflavin in a μ = 0.1 pH 7.77 Tris buffer; the other syringe contained 2×10^{-3} M *N*-acetyl-L-tyrosine-4-chloroanilide, 10% dimethylformamide, and 5×10^{-5} M proflavin in the same buffer. There were no detectable relaxations at 465 nm. pH–jump experiments were performed by preincubating the enzyme and substrate in a dilute pH 6.0 buffer and proflavin and mixing in the stopped-flow spectrophotometer with the pH 7.77 Tris and proflavin. Again there were no relaxations.

(b) DIRECT SEARCH. One syringe of the mixer contained 1 mM or 2×10^{-4} M α-chymotrypsin in the pH 7.77 Tris buffer. The other syringe contained 5 mM *N*-acetyltyrosine-4-acetylanilide in 10% dimethylformamide and pH 7.77 Tris. The absorbance at 340 was monitored. The rate of increase in absorbance was the same as found for the steady-state studies. The initial burst (see Figure 4) was found from the initial absorbance on mixing the above solutions and repeating using blanks omitting either substrate or enzyme. The "burst" was found to be <0.015 using 1 mM enzyme and <0.005 using 2×10^{-4} M. Under these conditions the enzyme is 14.3% saturated with substrate.

Results

The experimental data and conditions are given in Tables I–IV and in Figures 2–5. The values of k_{cat} are corrected for active-site content (94%). Except for the more soluble 4-trimethylammoniumanilide and the 4-chloroanilide where K_M is low, the poor solubility of the substrates prevented measurements at concentrations higher or even equal to the K_M. This makes the separation of k_{cat} and K_M somewhat imprecise. Some values of k_{cat}/K_M were checked by monitoring under pseudo-first-order conditions where [S] $\ll K_M$. There is excellent agreement between the values from this study and those from other workers where duplicated except for the binding of *N*-acetyl-L-tyrosine-4-chloroanilide to methylchymotrypsin. The solubility is low, about 1 mM, but supersaturated solutions of up to 2 mM are sufficiently metastable to be used. The procedure of following the inhibition of the acylation of the enzyme by acetyl-L-tryptophan *p*-nitrophenyl ester under pseudo-first-order conditions is very sensitive, the rate constants being independent of enzyme concentration, and the value of 4.75 mM is considered reliable. The value of 11 mM (Lucas and Caplow, 1972) was determined for an enzyme of undetermined active-site content.

The anilide ring binds well to chymotrypsin. The inhibition

TABLE II: Inhibition Constants for Formanilides and α-Chymotrypsin and Partition of Formanilides between *n*-Octanol and Water.

Formanilide	σ^-	Partition[a] *n*-Octanol: Water, *P*	π (log P/P_0)	K_I[b] (mM)
4-Me	−0.17	40.5	0.48	3.0
4-MeO	−0.11[c]	10.8	−0.08	8.8
—	0	13.2 (P_0)	0.00	4.0
3-MeO	0.11	17.7	0.13	6.8
4-Cl	0.23	108.3	0.91	0.40
3-Cl	0.37	80.0	0.78	0.80
4-CN	1.0	11.9	−0.04	12.6
4-CH_3CO	1.0	8.76	−0.18	19.0
4-NO_2	1.27	27.0	0.31	3.8
(Diformylhydrazine				>2 M)

[a] 25°. [b] pH 7.75, μ = 0.1, Tris buffer, 5% dimethylformamide, 25°. [c] van Bekkum *et al.* (1959). −0.268 should not be used for anilines.

TABLE III: Dissociation Constants of Chloroanilides and Chymotrypsins.[a]

4-Chloroanilide	Dissociation Constant (mM) α-Chymotrypsin	*N*-Methyl(His 57)-α-chymotrypsin
N-Acetyl-L-tyrosine	0.78	4.75 ± 0.10
N-Acetylglycine	0.98	0.96
Formyl	0.40	0.51
N-Acetyl-L-tryptophan	0.57[b]	
	0.77[c]	

[a] 25°, 5% dimethylformamide, pH 7.8. [b] Caplow (1969), pH 7.64, 10% dimethylformamide. [c] Caplow (1969), pH 8.18.

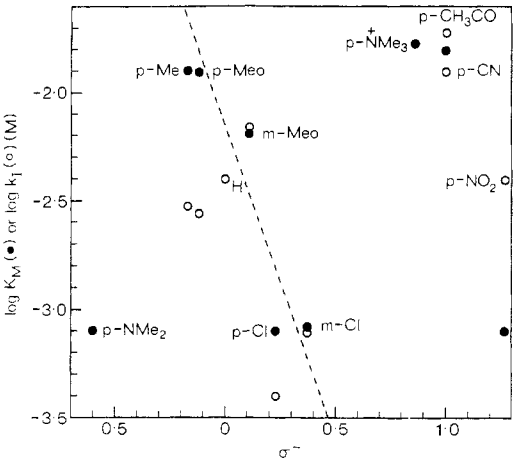


FIGURE 2: Plots of log K_M against σ^- for *N*-acetyl-L-tyrosineanilide hydrolysis (●) and log K_I against σ^- for competitive inhibition by formanilides (○).

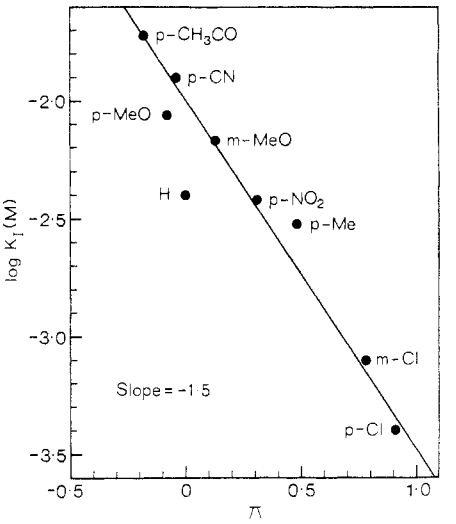


FIGURE 3: Plot of log K_I for formanilide inhibition of α-chymotrypsin against π, the hydrophobicity constant.

constants for substituted formanilides given in Table II parallel the hydrophobic nature of the substituent. The hydrophobic nature may be quantified by the use of π, the log of the partition constant of the compound between water and *n*-octanol (Fujita et al., 1964). There is a linear free-energy relationship between log K_I and π (see Figure 2). This correlation has been noted for several series of inhibitors (Martinek *et al.*, 1970).

The *N*-acetylglycine moiety binds very poorly to chymotrypsin, K_S for *N*-Ac-Gly-OMe is 3380 mM and for *N*-Ac-Gly-OEt it is 823 mM (Berezin *et al.*, 1971). Yet, as seen in Table III, *N*-acetylglycine-4-chloroanilide binds tightly with K_I = 0.98 mM, a value very close to that of the K_M for *N*-acetyl-L-tyrosine-4-chloroanilide. *The binding of all 4-chloroanilides in Table III may be accounted for mainly by the binding of the anilide ring in the hydrophobic pocket.*

TABLE IV: Summary of Kinetic Data for the α-Chymotrypsin-Catalyzed Hydrolysis of *N*-Acetyl-L-tyrosineanilides.[a]

Anilide	k_{cat} (sec^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} sec^{-1}) *b*	*c*	Ref
4-NMe_2	0.254	8	31.7	29.5	*d*
4-Me	0.10	13	7.7		
4-MeO	0.29	12	17.4	18.9	*e*
	0.230	13	17.7		*f*
3-MeO	0.052	6.6	7.9		*e*
4-Cl	0.0143	0.785	19.0		*d*
	0.0137	0.67	20.4		*e*
	0.0202	0.81	25.4		*f*
3-Cl	0.0111	0.82	13.4		*e*
4-$N^+(Me)_2$	1.27	16.8	75.5	73	*d*
4-CH_3CO	1.41	15.5	91.0	91.5	*d*
4-NO_2	0.0505	0.7	72		*e*
	0.06	0.9	66		*g*

[a] 25°, pH 7.8, 5% dimethylformamide. [b] From the ratio of k_{cat} and K_M. [c] From pseudo-first-order kinetics in this study. [d] This study. [e] Inagami *et al.* (1969). [f] Lucas and Caplow (1972). [g] Faller (1971).

The data may be analyzed in terms of the model in Figure 1.

Examination of the values of K_M in Table IV shows a parallel with the Π values of the anilide substituent rather than σ^-, the inductive effect: the hydrophobic Cl and NO_2 groups lower K_M while the hydrophilic N^+ME_3, CH_3CO, and, to a lesser extent, MeO groups involve high K_M values, the binding being predominantly productive. Acetyltryptophan derivatives generally bind better than acetyltyrosine derivatives and it would be expected from the above that the ratio of productive to nonproductive binding in the tryptophan anilides will be higher than for the tyrosine derivatives. The ratio of K_M values at pH 8.2 for acetyl-L-tryptophan-4-methoxyanilide:4-chloroanilide is 2.5:1 (Caplow, 1969) and that for the tyrosine derivatives at pH 7.8 is 16:1.

In the Table IV possible values of dissociation constants for nonproductive binding (K_{NP}) are calculated from values of K_S, the dissociation constant for productive binding, and from the observed K_M from the relationship $1/K_M = 1/K_S + 1/K_{NP}$, assuming plausible values of K_S by extrapolation from known results. Separation of the values for K_{NP} and plotting against π for formanilides (Figure 4) gives a similar correlation to the plot for the formanilides themselves (Figure 3).

In Figure 6 values of k_{cat} for the productive binding mode are given, calculated for K_S = 55 mM in the equation

$$k_{cat}(\text{productive mode}) = k_{cat}(\text{obsd})\left(\frac{K_{NP} + K_S}{K_{NP}}\right)$$

There is little correlation between the values for K_S for the productive modes and σ^-. The compensating changes in the observed values of k_{cat} and K_M are due to competition between productive and nonproductive binding.

Direct Evidence against Accumulation of a Tetrahedral Intermediate. The experiments with proflavin, a probe for the accumulation of intermediates (Bernhard *et al.*, 1966; Brandt *et al.*, 1967; Fersht and Requena, 1971), indicate that no build up of a tetrahedral, or any other, intermediate occurs in the stopped-flow time range. It has been shown (Robinson, 1970; Kallen and Jencks, 1966) that the tetrahedral adduct of an anilide has a similar spectrum to that of the parent aniline. A direct spectrophotometric search for an intermediate was

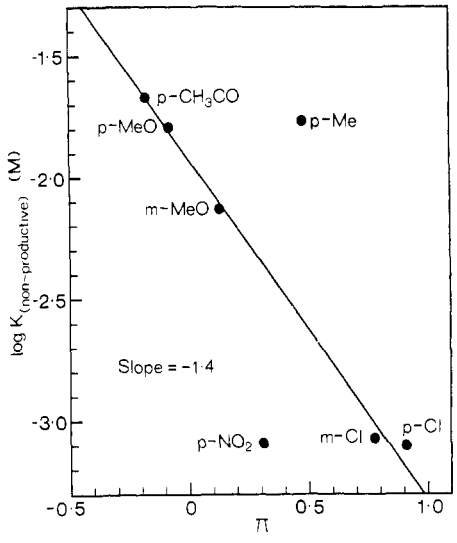
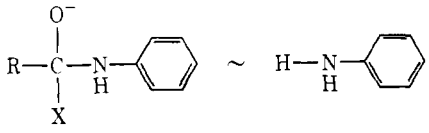


FIGURE 4: Plot of log $K_{nonproductive}$ for *N*-acetyl-L-tyrosineanilide hydrolysis against π for the formanilides. K_{NP} is calculated for K_S = 55 mM. Using K_S = 20 mM a similar plot is obtained but the slope increases to −1.6.



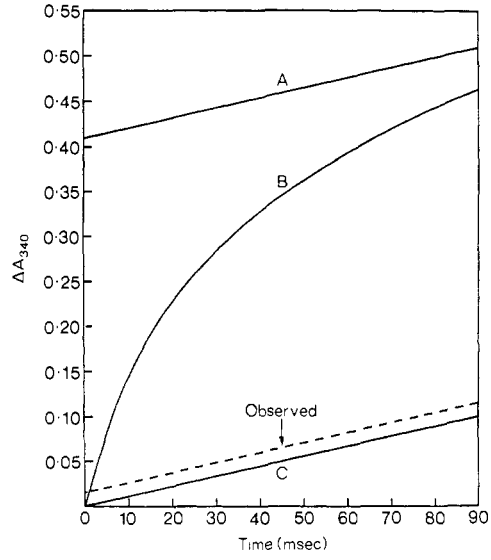


FIGURE 5: Observed result and theoretical predictions for the detection of a tetrahedral intermediate. *N*-Acetyl-L-tyrosine-4-acetylanilide and α-chymotrypsin were mixed in the stopped-flow spectrophotometer to give final concentrations of 5×10^{-4} and 2.5×10^{-3} M, respectively, at pH 7.8. If a tetrahedral intermediate accumulates within the 4-msec dead time, curve A is expected; a burst followed by a steady-state production of aniline. A slower accumulation would give B. No accumulation would give C. The observed result is a burst of <3.6% of that expected for complete accumulation.

made by mixing *N*-acetyl-L-tyrosine-4-acetylanilide with chymotrypsin in the stopped-flow spectrophotometer and monitoring at the λ_{max} of the 4-acetylaniline. A rapid accumulation of a tetrahedral intermediate before the time resolution of stopped-flow spectrophotometry would give an initial burst followed by a steady-state production of 4-acetylaniline. A slow accumulation would give an observable exponential transient. No accumulation of an intermediate would give just the steady-state production of aniline, as shown in Figure 6. The latter was observed; less than 4% of the enzyme–substrate complex can exist as a tetrahedral intermediate. Under the experimental conditions 14.3% of the enzyme is bound with substrate. The conversion of this fraction of 5×10^{-4} M enzyme to anilide tetrahedral intermediate would give a burst of 0.42 absorbance unit, using a $\Delta\epsilon_{340}$ of 5.88×10^3 (see Table I). The observed burst was <0.015.

Values of k_{cat} and K_M for the α-chymotrypsin hydrolysis of *N*-acetyl-L-tyrosinamides have been collated from the literature and added to the data of this study for comparative purposes. The constants for the hydrazides were extrapolated from the corresponding *N*-formylphenylalanine derivatives using a factor of ten from the known ratios of k_{cat}/K_M for Ac-Tyr:Ac-Phe and formyl-Tyr:acetyl-Tyr. Dimethyform-

TABLE V: Calculated Values for K_S(Nonproductive) from Assumed Values K_S(Productive) and K_M for *N*-Acetyl-L-tyrosineanilides.

Anilide	K_M (mM)	K_S(Nonproductive) (mM) Calcd for K_S(Productive) = (mM) 16	20	50	56
4-N^+Me_3	16.8		105	25	24
4-CH_3CO	15.5	500	69	22	21
4-Me	13	69	37	17.5	17
4-MeO	12.5	58	33	16.7	16.1
4-NMe_2	8				
3-MeO	6.6	11.2	9.9	7.6	7.5
3-Cl	0.82	0.86	0.86	0.83	0.83
4-NO_2	0.8	0.84	0.83	0.81	0.81
4-Cl	0.78	0.82	0.81	0.79	0.79

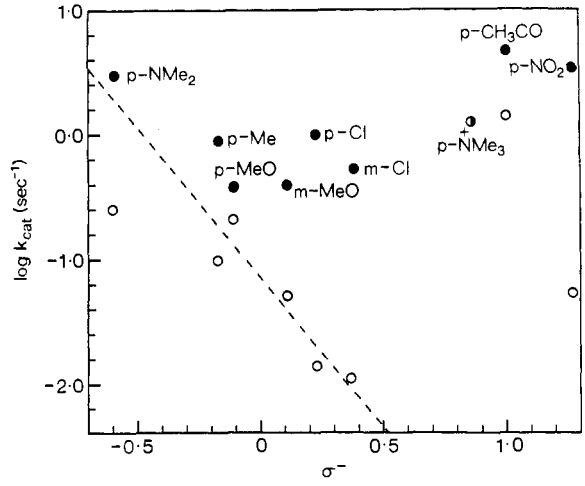


FIGURE 6: Plots of log k_{cat} against σ^-. The open circles are the observed values and the closed circles (vertically above) are calculated for the productive binding mode. The dashed line is the artifact when a limited series of substituents is used.

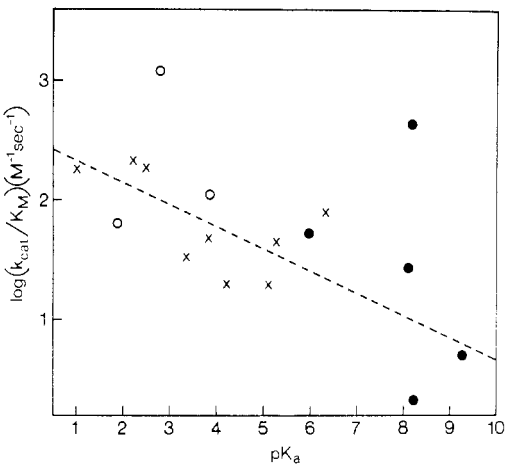


FIGURE 7: Plot of log (k_{cat}/K_M) for the α-chymotrypsin hydrolysis of *N*-acetyl-L-tyrosinamides against the pK_a of the leaving group amine (see Table VI): ×, anilides; ●, primary amines; ○, extrapolated hydrazide data.

amide (5%) generally increases K_M by 3.3-fold and k_{cat} by 30% (this study and Inagami *et al.*, 1969). The anilide data were altered by these numbers. The results are given in Table VI. The K_M value for the *N*-acetyl-L-tyrosine-4-trimethylammoniumanilide which should have no nonproductive binding is significantly low. There is probably some binding to a leaving group site.

Discussion

Competition between productive and nonproductive binding modes (Figure 1) is suggested from the fact that: (a) the binding of all the 4-chloroanilides to chymotrypsin in Table II is similar and is therefore attributable mainly to that of the anilide ring; (b) K_M varies as π, the hydrophobicity constant, rather than σ^-; furthermore, (c) K_M may be divided into productive and nonproductive binding components and a plot of K_{NP} against $\pi_{formanilide}$ is similar to that of $K_{I formanilide}$ *vs.* $\pi_{formanilide}$; and (d) in acetyltryptophan derivatives there is a smaller variation in K_M due to the greater hydrophobic character of the indole ring than the phenol in tyrosine.

Substrates with hydrophobic aniline rings bind predominantly in the nonproductive mode. The lowered k_{cat} values reflect the small contribution of productive building. Hydrophilic substituents such as the trimethylammonium and methoxy groups have little nonproductive binding. The k_{cat} and K_M values are correspondingly high.

The observed variations of k_{cat} and K_M are due to compensatory changes: nonproductive binding lowers K_M and k_{cat}. As Brot and Bender (1969) have emphasized, the specificity constant k_{cat}/K_M, which is always equal to k_2/K_S, is the only reliable parameter in such cases for structure–reactivity relationships.

The studies of Inagami *et al.* (1969), Parker and Wang (1968), Caplow (1969), and Lucas and Caplow (1972) are unfortunate in that for the substituents used, Me–, MeO–, Cl, and $-NO_2$, σ^- and π change in a parallel manner. Using a more extensive series of compounds for which π and σ are not correlated it is seen that there is no simple relationship between the Michaelis–Menten parameters and σ^-.

This undermines the experimental basis of the three aforementioned theories of chymotrypsin action and reinforces earlier evidence against them (Fersht and Requena, 1971; Fersht, 1971, 1972; Lucas and Caplow, 1972).

General Problem of Distinguishing between Nonproductive Binding, Strain, and Induced Fit. A single series of measurements of k_{cat} and K_M for a series of substrates cannot in general distinguish between the above possibilities (see Jencks, 1969, for example). Chymotrypsin is a simple case. There are two hydrophobic sites (Hansch and Coats, 1970). The hydrophobic binding pocket is strongly sensitive to π. The leaving group site is weakly hydrophobic, the binding varying as 0.25π (Hansch and Coats, 1970). By using "portions" of the substrate and measuring the sensitivity of the binding to substituent variation it is now shown that the compensatory changes in k_{cat} and K_M for the anilide hydrolysis are due to nonproductive binding effects. In the absence of this information it could possibly have been argued, for example, that these results were due to strain, binding energy being used to lower activation energy.

In general, for proteolytic or esterolytic enzymes that involve an acyl enzyme it is expected that nonproductive binding should be manifested in the acylation step. The covalent bond in the acyl enzyme eliminates most nonproductive modes.

In enzymes with an extended active site such as elastase (Thompson and Blout, 1970) and papain (Lowe and Yuthavong, 1971) the situation is much more complex. It might be possible to measure the sensitivity of each subsite to hydrophobic binding by judicious modification of substrates and inhibitors. In the absence of such information it is difficult to distinguish between strain, nonproductive binding, and induced fit.

The demonstration in this study that the substitution of a chlorine in the aniline ring causes nonproductive binding to be 50–100 times greater than productive shows the danger in using substrates with extraneous hydrophobic regions such as aniline, phenol, or benzoyl portions.

Reaction Mechanism. NATURE OF THE RATE-DETERMINING STEP. There is no simple relationship between k_{cat}/K_M for amide hydrolysis and the pK_a of the leaving group amine (see Table VI and Figure 7) except that the Brønsted β is low as found by Inward and Jencks (1965) for amines and furoylchymotrypsin. A low β value is intermediate between those found for rate-determining attack and rate-determining breakdown of a tetrahedral intermediate (Fersht, 1971).

The $^{14}N/^{15}N$ kinetic isotope experiments of O'Leary and Kluetz (1970) suggest rate-determining breakdown of a tetrahedral intermediate with α-chymotrypsin and *N*-acetyltryptophanamide. Fersht (1972) and Fersht and Requena (1971) have presented a mechanism involving rate-determining breakdown of the tetrahedral intermediate at high pH and formation at low pH for α-chymotrypsin and *N*-formyl-L-phenylalanineformylhydrazide. This mechanism is now supported by further $^{14}N/^{15}N$ kinetic isotope experiments on acetyltryptophanamide (O'Leary and Kluetz, 1972) which indicate a change in rate-determining step at low pH. In any case the arguments of Fersht (1971, 1972) and Fersht and Requena (1971) are consistent with the rate constants for the partitioning of the tetrahedral intermediate to either products or reagents being similar and the rate determining step could be altered by the stereochemical nature of the leaving group and acyl portion. For example, *N*-acetyltyrosinealaninamide is more reactive than the other derivatives of similar pK_a (see Table VI, Figure 7). This could affect the rate determining step.

ACCUMULATION OF AN INTERMEDIATE (Caplow, 1969; Lucas and Caplow 1972). The hypothesis that the lowered K_M on increasing electron withdrawal in the anilide ring is indica-

TABLE VI: Catalytic Constants for the Hydrolysis of *N*-Acetyl-L-tyrosinamides and -anilides (*N*-Ac-Tyr-X) by α-Chymotrypsin at 25° and pH 7.8–8.

X	pK_a (X^+H_2)	k_{cat} (sec^{-1})	K_M (mM)	k_{cat}/K_M (sec^{-1} M^{-1})	Ref
4-NO_2PhNH	0.98	0.0385	0.21	182	*a, b*
$H_2CSNHNH$	1.88			64	*c, d*
4-$CH_3COPhNH$	2.19	1.08	4.7	230	*e, b*
4-$(CH_3)_3N^+PhNH$	2.51	0.98	5.1	192	*e, b*
HCONHNH	2.81			1500	*c, d*
3-ClPhNH	3.32	0.0085	0.25	34	*a, b*
4-ClPhNH	3.81	0.0108	0.22	49	*c, d*
$H_2NCONHNH$	3.86			126	*c, d*
3-CH_3OPhNH	4.20	0.04	2.0	20.0	*a, b*
4-CH_3PhNH	5.07	0.077	3.9	17.9	*a, b*
4-CH_3OPhNH	5.29	0.165	3.6	46.0	*a, b*
HONH	5.97	0.22	43.0	51	*f*
4-$(CH_3)_2NPhNH$	6.28	0.195	2.4	81.0	*e, b*
H_2NCOCH_2NH	8.07	0.5	23.0	27.5	*f, g*
$H_2NCOCH_2(CH_3)NH$	8.15	7.5	17.2	436	*g*
H_2NNH	8.10	0.067	30.0	2.2	*f*
NH_2	9.25	0.16	32.0	5.3	*f, g*
CH_3NH	10.62	$<6.6 \times 10^{-4}$	61.0	$<1.09 \times 10^{-2}$	*f*

[a] Inagami *et al.* (1969). [b] Corrected for presence of 5% dimethylformamide by dividing K_M by 3.3 and k_{cat} by 1.3 (see text). [c] Fersht and Requena (1971). [d] Formyl-L-phenyl derivatives normalized to *N*-Ac-L-Tyr derivatives (see text). [e] This work. [f] Hein and Niemann, 1961. [g] Baumann *et al.* (1970).

tive of the build up of an intermediate is no longer tenable; the K_M changes being a consequence of increased nonproductive binding. Tetrahedral intermediates could conceivably accumulate in certain cases if the enzyme–substrate and acyl enzyme complexes are strained.

PRETRANSITION STATE PROTONATION. It was argued that the reaction mechanism involves the preequilibrium transfer of the proton from Ser-195 to the anilide nitrogen of the substrate (Wang and Parker, 1967; Parker and Wang, 1968). The rate-determining step is then the attack of the seroxide ion on the N-protonated substrate. This was based on the finding that the observed values of log k_{cat} for α-chymotrypsin and the methyl, methoxy, and chloro derivatives of *N*-acetyl-L-tyrosineanilide are nearly proportional to the pK_a of the parent anilide. This is now seen to be an artifact. The removal of the experimental basis of this hypothesis reinforces earlier arguments (Fersht, 1971; Fersht and Requena, 1971) that the relevant amide nitrogen is too weakly basic to account for the observed rates. The amide nitrogen is presumably protonated *after* the formation of the tetrahedral intermediate. In this situation the pK_a of the leaving group amine is not lowered by electron delocalization and its pK_a is close to that of the parent amine.

A reasonable mechanism for the chymotrypsin-catalyzed hydrolysis of amides is that previously presented (Fersht, 1971, 1972; Fersht and Requena, 1971). A tetrahedral intermediate occurs between the enzyme–substrate complex and the acyl enzyme but does not accumulate. The rate-determining step of the reaction is probably not unique but depends on the nature of the substrate and pH.

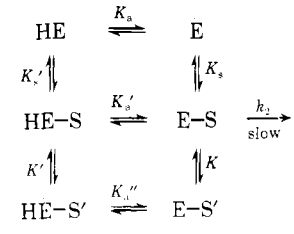
Appendix

Effect of Nonproductive Binding on the pH Dependence of k_{cat} and K_M. In cases where a substrate may bind both productively and nonproductively, the pH dependence of k_{cat} may give an apparent pK_a for the catalytically important group which is far from its real value in the productive complex if the ratio of productive to nonproductive binding changes on the ionization of the group. This is illustrated by the following.

It was suggested that the nonproductive binding of acetyltyrosine-4-chloroanilide to *N*-methyl(His-57)-α-chymotrypsin is lowered due to an unfavorable interaction with the *N*-methyl group of His-57. In the same manner it is likely that the nonproductive binding to α-chymotrypsin at low pH is also inhibited as His-57 is protonated and the positive charge will repel the hydrophobic side chain of tyrosine. In this case, at low pH, the ratio of productive to nonproductive binding is greater than at high pH. The observed k_{cat} is increased proportionately at low pH. k_{cat} will not decrease as rapidly with decreasing pH as the pK_a of His-57 in the productive complex would dictate. The pH dependence of k_{cat} then gives a pK_a value for His-57 which is artificially low. Similarly, K_M will have a sharp pH dependence governed by the same low pK_a, K_M being greater at low pH and smaller at high pH.

This is quantified in Scheme I in which HE–S and E–S are the productively bound enzyme–substrate complexes with

SCHEME I



dissociation constants K_S' and K_S, respectively. K_a' is the ionization constant for His-57 in the productive complex which decomposes with rate constant k_2. K and K' are the equilibrium constants between E–S and E–S′, and HE–S and He–S′, HE–S′ and E–S′ being nonproductively bound. It is easily shown that the observed values for k_{cat} and K_M are given by

$$k_{cat} = \frac{k_2}{K(1 + [H^+]/K_a'') + 1 + [H^+]/K_a'}$$

and

$$K_M = \frac{K_S(1 + [H^+]/K_a)}{K(1 + [H^+]/K_a'') + 1 + [H^+]/K_a'}$$

The relationship between the observed pK_a governing k_{cat} (and K_M) and the microscopic pK_a of His-57 in the productive complex (pK_a') is given by

$$pK_{obsd} = pK_a' - \log \frac{(1 + K)}{(1 + K')}$$

The pH dependence of k_{cat} and K_M for the chymotrypsin-catalyzed hydrolysis of anilides is nicely accounted for by this. The apparent pK_a values governing k_{cat} for the hydrolysis of chloroanilides, which we have shown to bind predominantly nonproductively, are abnormally low, being less than 6 (Caplow, 1969; Inagami *et al.*, 1969). Here $K \gg 1$ and $K < K'$. For a methoxyanilide, which we have shown to bind predominantly productively, the pK_a for k_{cat} is a normal 6.6, *i.e.*, K and $K' \ll 1$.

In general, caution should be exerted in interpreting the pH dependence of k_{cat} in enzyme reactions. Artifactual results may be obtained both from changes in rate-determining step with pH (Fersht and Requena, 1971) and pH-dependent competition between productive and nonproductive binding.

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