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pH Dependence of Chymotrypsin Catalysis†

Appendix: Substrate Binding to Dimeric α-Chymotrypsin Studied by X-Ray Diffraction and the Equilibrium Method

Alan R. Fersht* and M. Renard‡

ABSTRACT: The pK_a of the active site of α-chymotrypsin at 25° and ionic strength 0.1 was found to be 6.80 ± 0.04 from accurate determinations of the pH dependence of k_{cat}/K_M for several substrates in conjunction with the pH dependence of the conformational equilibria for the enzyme. Direct measurement of the proton release on binding certain hydrazide substrates reveals that the pK_a of the active site is definitely and significantly lowered in the enzyme–substrate complex. This is accounted for by extending a recently described model for peptide binding to include a hydrogen bond between the hydrazide leaving group and the oxygen of Ser-195. Rapid stopped-flow experiments show that if an intermediate accumulates on the reaction pathway it must do so with a rate constant of greater than 2000 sec^{-1}. The partitioning of acetylphenylalanine-δ-chymotrypsin between formylhydrazine and water at high ionic strength (to minimize primary salt effects due to varying surface charges) increases by about 30% below pH 6.8. This is consistent with an intermediate being on the reaction pathway. Log (k_{cat}/K_M) for the hydrolysis of AcPheONp by δ-chymotrypsin at high ionic strength decreases linearly with pH from pH 5 to pH 2 with unit slope when the conformational equilibria in the enzyme is taken into account. The first ionization of the charge-relay system must be below pH 2. An X-ray diffraction study of formyl-L-phenylalanine semicarbazide bound to dimeric α-chymotrypsin shows that the substrate is bound nonproductively.

The combination of crystallographic and chemical studies has led to a detailed model for the acylation of the serine proteases by specific polypeptide substrates. It is suggested that for subtilisin (Robertus *et al.*, 1972) and for chymotrypsin and trypsin (Fersht *et al.*, 1973) the substrate forms stronger noncovalent bonds with the enzyme when in the transition state rather than in the initial noncovalent complex. The transition state is thought to resemble the tetrahedral intermediate formed by the addition of the hydroxyl of Ser-195 to the substrate carboxyl. Rühlmann *et al.* (1973) have co-crystallized trypsin and the basic pancreatic trypsin inhibitor to give a complex which is thought to resemble an enzyme–substrate complex. They have interpreted their electron density map from X-ray diffraction studies as showing that the complex is the tetrahedral adduct of the enzyme and the reactive peptide bond. The otherwise unstable tetrahedral intermediate is suggested to be stabilized by the features proposed by Fersht *et al.* (1973) and by the relief of strain built into the native inhibitor. Similar results have also been obtained in this laboratory for the soybean inhibitor (Sweet *et al.*, 1974). The question is whether or not a tetrahedral intermediate occurs in the acylation of the enzyme by a normal substrate and whether or not the intermediate is thermodynamically

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more stable than the noncovalent Michaelis complex and accumulates.

We have recently shown that a tetrahedral intermediate does not accumulate in anilide hydrolysis by using a substrate which generates a chromophoric tetrahedral intermediate (Fastrez and Fersht, 1973a). An earlier study that presented evidence that an intermediate does not accumulate in the hydrolysis of formyl-L-phenylalanine formylhydrazide (FPhe-FH) and semicarbazide (FPheSC) (Fersht and Requena, 1971b) has been challenged (Lucas *et al.*, 1973; Zeeberg *et al.*, 1973).

In this study we present the direct determination of the proton release on binding substrates to chymotrypsin and the correlation with the pH dependence of the Michaelis–Menten parameters, the determination of the partitioning of acetyl-phenylalanine-chymotrypsin between water and certain nucleophiles as a function of pH, a search for the accumulation of intermediates using stopped-flow techniques, and the accurate kinetic determination of the pK_a of the active site of chymotrypsin. New results are obtained concerning the existence and possible accumulation of intermediates, the stereochemistry of substrate binding, and the ionization of the charge-relay system.

Experimental Section

Materials. Three-times-crystallized, lyophilized salt-free α-chymotrypsin was obtained from Worthington (lots CDI 8LK, CDI OBK, and CDI OBF). δ-Chymotrypsin was obtained from Sigma as an ethanol precipitate (lot 20C-0150). Protein concentrations were determined spectrophotometrically using a molar extinction coefficient of 5×10^4. Active-site titrations were performed with *trans*-cinnamoylimidazole (Schonbaum *et al.*, 1961) at pH 6.84 in the stopped-flow spectrophotometer. The δ-chymotrypsin contained 88% and the batches of α-chymotrypsin 89–95% of the theoretical number of active sites. The substrates and reagents were prepared by our previously described procedures (Fersht and Requena, 1971b; Fastrez and Fersht, 1973a,b).

Apparatus. Two stopped-flow spectrophotometers were used. One was a Gilford 2400 spectrophotometer equipped with a stopped-flow mixer (designed by W. P. Jencks) of dead time 4 msec and an optical path length of 1.0 cm. This instrument has excellent long-term stability and reproducibility of absorbance values. The other instrument was superior for the detection of rapid transients, having a dead time of 1.5 msec and, due to using very high photocathode currents, a high signal to noise ratio at low electronic damping. The light source was a 55-W tungsten–iodide lamp in conjunction with a Farrand f/3.5 grating monochromator. The path length of the observation cell was 0.173 cm. Mixing was 99.7% complete after 1.5 msec and greater than 99.9 at 2 msec judged from the neutralization of sodium *p*-nitrophenolate by HCl. The observation chambers and driving syringes of both instruments were thermostatted at 25°. pH measurements and pH-Statting were performed using a Radiometer 26B pH meter equipped with autotitrator and syringe-buret, the sample being thermostatted at 25°. Automatic colorimetric analyses were made using a Technicon peristaltic pump and the Gilford 2400 spectrophotometer equipped with Hellma micro flow cells.

Methods. The determinations of the hydrolysis of amide substrates and proton release on substrate binding were at 25° and ionic strength 0.1. Product ratio experiments and the hydrolysis of the nitrophenyl ester were performed at 25°, ionic strength 0.95 and with 5% Me_2SO.

Determination of Proton Release on Substrate Binding. One syringe of the stopped-flow spectrophotometer (1-cm path length) contained 1.2×10^{-4} M α-chymotrypsin in 0.1 M KCl and *p*-nitrophenol buffer carefully adjusted to the desired pH (6.10 or 6.40). The other syringe contained 0.1 M KCl adjusted to the same pH. The absorbance at 400 nm on mixing was recorded on a storage oscilloscope (20 msec/div sweep rate) and photographed. This was repeated four times and the syringe containing 0.1 M KCl replaced with one containing substrate in 0.1 M KCl at the same pH. The absorbance at 400 nm on mixing this solution with the enzyme was also measured four times in succession. The substrate syringe was removed and replaced by the KCl blank and four measurements were recorded. This procedure was repeated so that a total of three sets of blank measurements and two sets of sample measurements were made in the order blank-sample-blank-sample-blank. Some experiments were performed with the substrate at slower sweep rates. The identical procedure was repeated substituting indole for the substrate.

Titration of Enzyme. A solution of 5×10^{-5} M α-chymotrypsin (lot CDI 8LK) in 0.1 M KCl (10 ml) was rapidly titrated from pH 5.5 to pH 7 by the addition of 0.01 N KOH from a Burroughs-Wellcome "Agla" syringe. The solution was then rapidly back-titrated to pH 5.5 with 0.01 N HCl. A spectrophotometric titration was performed by adding $1–5 \times 10^{-4}$ M *p*-nitrophenol as an indicator to the above solutions and noting the decrease in absorbance at 400 nm on the addition of successive aliquots (5 μl) of 0.01 N HCl to 3.0 ml of solution incubated in a cuvet.

Product Ratios. The partitioning of acetyl-L-phenylalanine-chymotrypsin between nucleophiles and water was determined by adding [^{3}H]AcPheOMe to a buffered solution of δ-chymotrypsin and the nucleophile at 25°, $\mu = 0.95$ and 5% Me_2SO, and separating the products on completion of the reaction by ionophoresis as described in detail by Fastrez and Fersht (1973b). Kinetic determinations were performed in a manner similar to that described in that paper for the partitioning of the acyl-enzyme between methanol and water. One syringe of the stopped-flow spectrophotometer contained a buffered solution ($\mu = 0.95$) of enzyme, the appropriate concentration of nucleophile and 5% Me_2SO. The other syringe contained a freshly prepared solution of 0.1 mM AcPheONp in 5% Me_2SO and 0.95 M KCl. k_{cat} and K_M were determined from the total progress curve for *p*-nitrophenol release at 340 nm or *p*-nitrophenolate at 400 nm.

Hydrolysis of AcPheONp. This was an extension of the study by Renard and Fersht (1973) where the methods are described in detail.

Fraction of δ-Chymotrypsin in Active Conformation. This was determined between pH 2 and 7.6 at 25°, $\mu = 0.95$ and 5% Me_2SO for δ-chymotrypsin by the proflavine titration method (Fersht and Requena, 1971c; Fersht, 1972a).

Additional Stopped-Flow Searches for Intermediates. The stopped-flow spectrophotometer of path length 0.173 cm was used for these experiments.

PROTON RELEASE. One syringe of the mixer contained 10^{-4} M chymotrypsin (α or δ) in 0.1 M KCl and either 1.0 or 1.8 or 3.6×10^{-5} M *p*-nitrophenol at pH 6.10. The other syringe con-

[1] The following abbreviations are used: FPheFH, formyl-L-phenylalanine formylhydrazide; FPheSC, formyl-L-phenylalanine semicarbazide; AcPheAlaNH$_2$, acetyl-L-phenylalanine alaninamide; AcPheOMe, acetyl-L-phenylalanine methyl ester; AcPheONp, acetyl-L-phenylalanine *p*-nitrophenyl ester; AcPheFH, acetyl-L-phenylalanine formylhydrazide.

TABLE I: Experimental Conditions for the Determination of Hydrolysis Rates at 25° and Ionic Strength 0.1.

Substrate	pH	Substrate Concn (mM)	Enzyme Concn (μM)	Buffer[a]	No. of Substrate Concn
AcPheAlaNH$_2$	6.01	3–27	1.2	P	11
	6.45	3–27	0.7	P	11
	7.01	3–27	0.3	P	10
	7.46	3–27	0.2	P	10
	7.78	3–27	1.2	T	11
AcTyr-*p*-acetylanilide	5–5.6	0.1	19	A	1
	6–7.5	0.1	21	P	1
	7.5–7.8	0.1	21	T	1
FPheSC	5.39	0.4–32	74	A	8
	5.6–5.79	1–32	39	A	10
	5.97	0.4–32	39	P	10
	5.99	2–37	15.6	P	9
	6.2–6.9	0.4–24	15.6	P	10
	7–7.2	0.4–32	3	P	10
	7.5–7.8	0.4–24	18	P	11
	8–8.5	0.4–24	8	T	10
FPheFH	5.36	0.2–32	10		10
	5.55–5.56	0.2–40	6		10
	6.2	0.2–30	3.5		10
	7–8	0.2–20	2		10
FPheFH[b]	4.5–5.0	1–20	30		10
	5.5–6.5	0.5–20	7.6		7
	7–7.8	0.2–10	2		9
FPheSC[b]	6.5	0.5–20	30		9
	7–7.8	0.25–20	15		12

[a] A = acetate, P = phosphate, T = tris(hydroxymethyl)aminomethane. [b] δ-Chymotrypsin used instead of α-chymotrypsin for the above.

tained 20 or 27 mM FPheFH in 0.1 M KCl and the same *p*-nitrophenol buffer at pH 6.10. The absorbance changes at 400 nm were recorded as before. The procedure was repeated substituting 2 or 2.5 mM indole for the substrate. Similar experiments were performed at pH 6.40 with 40 mM FPheSC in 2×10^{-5} M *p*-nitrophenol (and indole blanks).

PROFLAVINE DISPLACEMENT. One syringe of the mixer contained 10^{-4} M chymotrypsin (α or δ) and 10^{-4} M proflavine in phosphate buffer (μ = 0.1, pH 6.08). The other syringe contained 24 mM FPheFH–10^{-4} M proflavine in the phosphate buffer. The absorbance changes at 465 nm after mixing were recorded as before. Blank experiments substituting 2 mM indole for the substrate were performed.

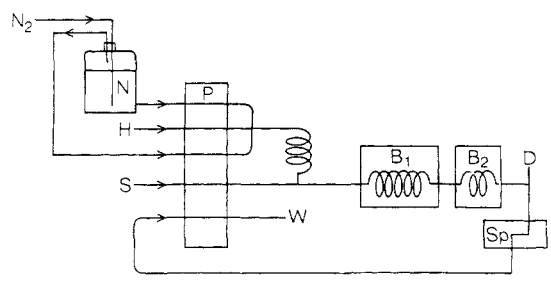


FIGURE 1: Schematics for automated ninhydrin analyzer: P, peristaltic pump; B_1, temperature bath at 95°, B_2, at 25°; Sp, spectrophotometer and flow cell; D, debubbler; N_2, nitrogen (7 psi, 0.16 ml/min); N, ninhydrin (40 g dissolved in 2 l. of methyl Cellosolve, 5 l of 0.8 M sodium acetate and 0.35 M acetic acid; pH 6.30, 0.80 ml/min); H, hydrazine sulfate (0.262 g/l., 0.32 ml/min); S, sample (0.42 ml/min), W, waste, 0.8 ml/min).

There were similar experiments using 40 mM FPheSC at pH 6.36 with 3.2 mM indole as a blank.

Hydrolysis of Amide Substrates. The experimental conditions are summarized in Table I. The hydrolysis of FPheFH was followed by use of the pH-Stat. The hydrolysis of FPheSC was followed using an automated colorimetric assay for the semicarbazide produced (Fersht and Requena, 1971b). The hydrolysis of AcPheAlaNH$_2$ was followed by determining the release of alaninamide by an automated ninhydrin assay. The method of Lenard *et al.* (1965) for the determination of ammonia was not so satisfactory for alaninamide and the method illustrated in Figure 1 was used. The reagents are those recommended by Technicon for use in their amino acid analyzer. k_{cat}/K_M for the hydrolysis of acetyl-L-tyrosine 4-acetylanilide was determined as described by Renard and Fersht (1973).

Results

Proton Release on Substrate Binding. The pH changes on mixing enzyme incubated in a *p*-nitrophenol buffer with FPheFH and FPheSC were monitored spectrophotometrically by the absorbance at 400 nm of the nitrophenolate ion. There was an initial drop in pH on substrate binding, which occurred in the dead time of the apparatus, followed by an initially linear decrease in A_{400} as the substrates hydrolyzed and released protons (see Figure 2).

This initial proton release and subsequent hydrolysis rates were calculated from the changes of pH using a pK_a value of 7.05 and extinction coefficient of 18,000 for *p*-nitrophenolate.

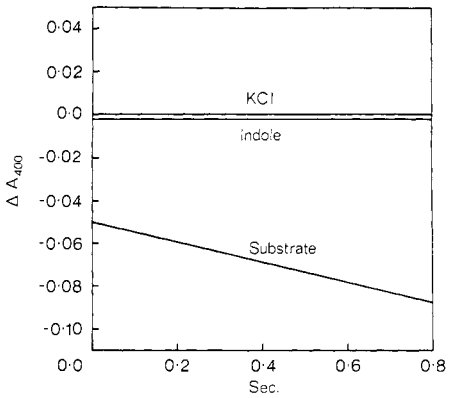


FIGURE 2: Proton release on mixing α-chymotrypsin and FPheFH. One syringe of the stopped-flow spectrophotometer contained 60 μM α-chymotrypsin in 5×10^{-4} M *p*-nitrophenol buffer, the other 20 mM FPheFH, both at pH 6.40, 25°, and 0.1 M KCl. On mixing there was an immediate decrease of 0.05 absorbance unit followed by a linear decrease of absorbance due to the steady state hydrolysis. KCl and indole blank experiments are indicated.

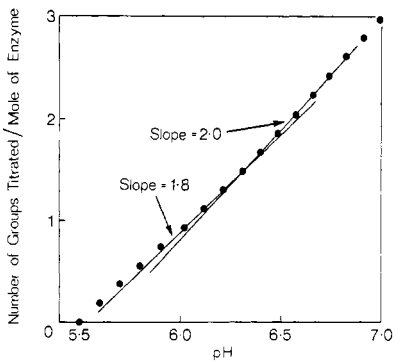


FIGURE 3: Titration of α-chymotrypsin at 25°, μ = 0.1. The slopes of the titration curve at pH 6.10 and 6.40 are indicated.

The following processes were considered in the calculations: proton uptake by the indicator, proton uptake by the substrate (FPheSC, pK_a = 10.04; FPheFH, pK_a = 8.82), proton uptake by the enzyme, proton uptake by the medium and dissolved carbon dioxide, and artifacts due to the perturbation of the conformational equilibria in the enzyme (Fersht and Requena, 1971c) and displacement of *p*-nitrophenol from the active site on ligand binding. The first two terms were obtained by straightforward calculation. The proton uptake by the enzyme was determined by titrating the enzyme. It was found that $d[H^+]/dpH = 1.8$[chymotrypsin] at pH 6.10, and = 2.0·[chymotrypsin] at pH 6.40 (see Figure 3) (*cf.* Edsall and Wyman, 1958, pp 419–420). The buffering due to the enzyme was further checked by a spectrophotometric titration under the same conditions as the substrate binding experiments by noting the A_{400} on adding aliquots of HCl to the enzyme incubated in the *p*-nitrophenol buffer. The proton uptake by the medium and dissolved carbon dioxide was negligible under our conditions, the pH being high and the solutions being prepared in degassed deionized water.

Binding artifacts were hopefully eliminated by performing control experiments replacing the substrate with a solution of indole at the same concentration relative to its K_I as the substrate was relative to its K_M. Indole is most suitable for this as its binding is pH independent (Fersht and Requena, 1971b).

p-Nitrophenol was shown to be a competitive inhibitor of the enzyme with $K_I = 3.3 \times 10^{-4}$ M. The degree of saturation of the enzyme with substrate ($[ES]/[E]_{total}$) was calculated using this value and the measured K_M values for the substrate for each concentration of *p*-nitrophenol and substrate. The initial proton burst on substrate binding was calculated for the amount of enzyme–substrate complex formed. The rate of decrease of A_{400} was calculated using the observed hydrolysis rates from the conventional pH-Stat studies of proton release and the buffering power of the medium calculated as described above. The results are presented in Table II.

It is seen that (a) there is excellent agreement between the calculated and observed hydrolysis rates (dA_{400}/dt) for FPheFH and fair agreement for FPheSC indicating that the calculations of the buffering are correct and (b) there is an initial burst of proton release on binding FPheFH and FPheSC. At low concentration of indicator (2×10^{-4} M) the initial proton burst is not seen for some unknown reason (Fersht, 1972b).

The magnitudes of the bursts are statistically accurate to about ±10–20%. The binding of FPheFH and chymotrypsin gives a burst of 0.3 ± 0.06 proton/mol at pH 6.1 and 0.28 ± 0.06 at pH 6.4. Assuming a pK_a value of 6.8 for the free enzyme pK_a values of 6.16 ± 0.11 and 6.29 ± 0.11 are calculated for the enzyme–substrate complex at the respective pH values. Similarly the binding of FPheSC to chymotrypsin at pH 6.40 gives a burst of protons which requires that the enzyme–substrate complex be about 50% un-ionized at this pH.

Stopped-Flow Search for an Additional Intermediate. PROTON RELEASE. There was no indication of the buildup of an intermediate of perturbed pK_a in any of the above experiments. In some experiments there were transient decreases in A_{400} ($t_{1/2}$ = 10–200 msec) on mixing enzyme incubated in *p*-nitrophenol with substrate. But in every case identical observations were recorded with the indole controls.

Further experiments were performed using a faster stopped-flow spectrophotometer with FPheFH incubated in the same *p*-nitrophenol buffer as the enzyme (pH 6.1)) or with FPheSC at pH 6.40. Again, using three different batches of α-chymotrypsin and one of δ-chymotrypsin and the substrate and indicator concentrations described in the Experimental Section, there was no indication of a transient over and above that of the indole control experiments. The proton release on substrate binding occurred in the 1.5 msec dead time of the apparatus.

PROFLAVINE DISPLACEMENT. There was no indication of the buildup of an intermediate by the proflavine displacement method (Bernhard *et al.*, 1966; Brandt *et al.*, 1967) at pH 6.10 for FPheFH and α- or δ-chymotrypsin or with FPheSC and chymotrypsin at pH 6.36 in the stopped-flow time range.

The conversion of a noncovalent enzyme–substrate complex to a covalent intermediate which accumulates would lead to a decrease in the absorbance at 465 nm of the enzyme–proflavine complex. The only observation at 465 nm on mixing FPheFH with α- or δ-chymotrypsin is a slow *increase* in absorbance as the inactive conformation is converted to the active (Fersht and Requena, 1971c; Fersht, 1972a). This is illustrated in Figure 4 which is a trace for mixing a 24 mM solution of FPheFH with a 100 M solution of α-chymotrypsin both in a pH 6.08 phosphate buffer and 10^{-4} M proflavine. If there is a process in the stopped-flow time range which consists of FPheFH forming an initial noncovalent complex of K_S value about 20 mM which is subsequently converted to a covalent complex with the overall K_M being the observed value of 7 mM then simple calculation using an extinction coefficient of 1.8×10^4 and a dissociation constant of about 40 μM for the enzyme–proflavine complex (Brandt *et al.*, 1967; Fersht and Requena,

TABLE II: Initial Proton Release and Subsequent Hydrolysis Rates on Binding Substrates to α-Chymotrypsin.[a]

pH	[p-Nitrophenol], × 10⁴ M		ΔA_{400}[b] On Addition of Substrate	ΔA_{400}[b] On Addition of Indole	[ES][c]/[E]$_{total}$	$-dA_{400}/dt$,[d] sec^{-1} Obsd	$-dA_{400}/dt$,[d] sec^{-1} Calcd[e]	[H⁺]/Mole Released[f] on Binding
			a. Formylphenylalanine Formylhydrazide[g]					
6.40	5	*h*	−0.05 ± 0.09	−0.002 ± 0.001	0.38	0.047	0.043	0.27
	4	*h*	−0.051 ± 0.002	−0.003 ± 0.005	0.41	0.044	0.038	0.28
	3	*h*	−0.046 ± 0.003	0.0 ± 0.003	0.446	0.038	0.034	0.29
	2	*h*	−0.022 ± 0.002	+0.005 ± 0.002	0.49	0.028	0.028	0.21
	1	*i*	0.0 ± 0.001	0.0 ± 0.001	0.55	0.005	0.005	0.0
6.10	5	*h*	−0.040 ± 0.001	0.006 ± 0.0005	0.36	0.025	0.020	0.30
	4	*h*	−0.038 ± 0.002	0.006 ± 0.604	0.39	0.027	0.024	0.30
	3	*h*	−0.032 ± 0.001	0.007 ± 0.002	0.43	0.018	0.021	0.29
	2	*h*	−0.003 ± 0.001	0.005 ± 0.003	0.47	0.015	0.017	0.08
	2	*i*	−0.009 ± 0.0003	0.0 ± 0.0001	0.47	0.015	0.013	0.11
	1	*j*	−0.0006 ± 0.0007	0.0 ± 0.0001	0.52	0.0035	0.0036	0.03
			b. Formylphenylalanine Semicarbazide[k]					
6.40	5		−0.067 ± 0.004	0.006 ± 0.002	0.67	0.0055	0.0070	0.20
	4		−0.064 ± 0.004	0.006 ± 0.003	0.70	0.0044	0.0066	0.20
	3		−0.043 ± 0.002	0.007 ± 0.002	0.73	0.0036	0.0059	0.16
	2		−0.030 ± 0.004	0.006 ± 0.003	0.76	0.0028	0.0048	0.14

[a] Determined in the stopped-flow spectrophotometer at 25°, μ = 0.1 (KCl). [b] Initial absorbance at 400 nm relative to 0.1 M KCl. [c] Fraction of enzyme liganded. [d] Rate of decrease of A_{400} as substrate is hydrolyzed. [e] Calculated from slow experiments in the pH-Stat and the known buffering power of the solutions with *p*-nitrophenol. [f] Initial proton release/mole of substrate bound/mole of enzyme. [g] 10 mM F Phe-FH–1.1 mM indole. [h] 59–60 μM chymotrypsin. [i] 10 μM chymotrypsin. [j] 42 μM chymotrypsin. [k] 25 mM FPhe-SC–2.5 mM indole.

1971c) shows that there should be a decrease of about 0.02 absorbance unit. But it is seen in Figure 4 that there is no transient decrease at the scale expansion of 0.001 absorbance unit/division. Therefore, if any intermediate does accumulate, the rate constant for its formation from the Michaelis complex must be greater than 2000 sec^{-1}.

Product Ratios. The partitioning of acetyl-L-phenylalanine-chymotrypsin between either formylhydrazine or semicarbazide and water was determined from the hydrolysis of [^{3}H]-AcPheOMe and by δ-chymotrypsin and separating the [^{3}H]AcPheFH or [^{3}H]AcPheSC from [^{3}H]AcPhe by ionophoresis by the method we have recently established (Fastrez and Fersht, 1973b). This was checked at some pH values kinetically by plotting k_{cat} against [formylhydrazine] or [semicarbazide] for the hydrolysis of AcPheONp by δ-chymotrypsin. It was shown in the above study that the ratio of the slope to the intercept of such a plot gives the partitioning of the acyl-enzyme between the nucleophile and water.

The partition ratios are summarized in Tables III and IV.

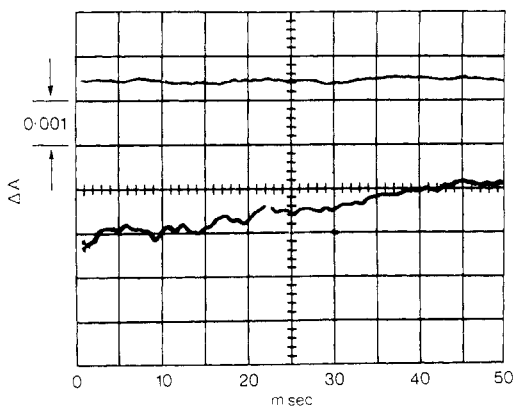


FIGURE 4: Displacement of proflavine from α-chymotrypsin by FPheFH. The absorbance of the enzyme–proflavine complex at 465 nm indicates no transient decrease due to the formation of an intermediate on mixing 100 μM α-chymotrypsin with 24 mM FPheFH both in 10^{-4} M proflavine and pH 6.08 buffer at 25° and μ = 0.1.

TABLE III: Product Distribution on Hydrolysis of AcPheOMe by δ-Chymotrypsin in Presence of Formylhydrazine (FH).[a]

pH	Ratio of AcPheFH:AcPhe[b] FH = 0.5 M	FH = 1.0 M	k_{FH}/k_{H_2O}[c] from Kinetics (M^{-1})
4.46	0.52	1.06	
4.79	0.53	1.10	
5.10	0.52	1.10	
5.73			1.0
6.12	0.49	1.02	
6.35	0.54	1.09	
6.80	0.50	0.98	
7.07	0.46	0.94	
7.32			0.94
7.72	0.45	0.91	
7.80	0.38	0.82	
7.99	0.43	0.95	
8.30	0.42	0.96	
9.58	0.27	0.78	
9.90	0.34	0.84	
10.16	0.40	0.90	
10.69	0.46	0.98	

[a] 25°, μ = 0.95, 5% Me_2SO. [b] From partition ratios. [c] From k_{cat} for the hydrolysis of AcPheONp by δ-chymotrypsin in presence of formylhydrazide.

TABLE IV: Product Distribution on Hydrolysis of AcPheOMe by δ-Chymotrypsin in Presence of Semicarbazide (SC).[a]

pH	Ratio of AcPheSC:AcPhe[b] SC = 1.0 M	SC = 2.0 M	k_{sc}/k_{H_2O}[c] from Kinetics (M^{-1})
4.60	0.80	1.62	0.77
6.00	0.80	1.65	
7.40	0.77	1.70	0.80
9.70	0.67	1.34	
10.30	0.75	1.55	
10.90	0.78	1.46	

[a] 25°, μ = 0.95, 5% Me_2SO. [b] From product ratios. [c] From k_{cat} for the hydrolysis of AcPheONp by δ-chymotrypsin in presence of semicarbazide.

The ratio of AcPheFH:AcPhe formed on the hydrolysis of AcPheOMe in the presence of 1 M formylhydrazine is 1.07 ± 0.02 at pH 6.35 and below, and 0.89 ± 0.03 at pH 7.72 and above. In the presence of 0.5 M formylhydrazine the ratios are 0.52 ± 0.01 and 0.39 ± 0.02, respectively. There is approximately a 26% higher partitioning at low pH, the transition being at approximately pH 6.8. There is a smaller variation with semicarbazide; there is possibly an 8% increase below pH 6, but this is not beyond experimental error.

The kinetic determinations are much less accurate (±20%) but there is fair agreement with the accurately determined product ratios.

pH–Rate Profiles. pK_a values were calculated from the observed rate constants (k_{obsd}) using the equation

$$k_{obsd} = k_{lim} - k_{obsd} \times [H^+]/K_a$$

where k_{lim} is the limiting value at high pH and $[H^+] = A$ log pH, which is analogous to the well-known Eadie (1942) plot. This has the advantage that data over the complete pH range may be plotted on one graph without the values at very low pH dominating the presentation.

The raw data are summarized in Table V. The diagrams and theoretical curves for K_M and k_{cat}/K_M have been corrected for the percentage of the enzyme in the active conformation (Fersht and Requena, 1971c; Fersht, 1972a). We have previously determined the pH dependence of the hydrolysis of FPheFH and FPheSC by α-chymotrypsin (Fersht and Requena, 1971b) but required more accurate data for the present study.

FPheFH. More data at high pH have been obtained with α-chymotrypsin. The theoretical curves allow for the ionization of the substrate which has a pK_a of 8.82. As the pH increases above 8 k_{cat}/K_M decreases as expected (Alberty and Massey, 1954) for this pK_a value but k_{cat} remains constant. The ionization of the FPheFH must therefore affect K_M. The values of K_M and k_{cat}/K_M above pH 7 have been corrected assuming $1/K_M$ tends to zero at high pH with a pK_a of 8.82 (Figures 5, 6, and 7).

The pH dependence with the δ enzyme is similar to that with α. k_{cat} follows a titration curve of pK_a = 6.05 and maximum value 0.27 sec^{-1}, similar to the values of 0.366 sec^{-1} and pK_a = 6.10 with α. Below pH 5.5 the values of k_{cat} are somewhat higher than predicted from the observed pK_a (Figure 5).

FPheSC. The experiments performed in this study with α-chymotrypsin using an automated colorimetric procedure are more accurate than those we previously reported using the pH-Stat (Fersht and Requena, 1971b) enabling K_M and

TABLE V: Catalytic Constants for the Hydrolysis of Amide Substrates at 25°, μ = 0.1.

Substrate	pH	k_{cat} (sec^{-1})	K_M (mM)	k_{cat}/K_M (sec^{-1} M^{-1})
$AcPheAlaNH_2$	6.01	0.53	44	12.2
	6.45	0.93	38	24.3
	7.01	1.8	38	47.2
	7.46	2.43	36	66.7
	7.78	2.0	31	64.6
AcTyr-*p*-acetylanilide	4.97			2.1
	5.33			6.65
	5.58			11.9
	6.01			30.1
	6.37			58.1
	6.45			66.4
	6.46			67.8
	6.66			85.7
	6.75			101.3
	6.85			110.3
	7.01			129
	7.33			139
	7.43			165
	7.46			167
	7.60			166
	7.72			167
	7.78			171
FPheSC	5.39	0.00416	9.84	0.423
	5.60	0.00705	9.75	0.723
	5.79	0.00866	7.15	1.24
	5.97	0.00909	5.47	1.66
	5.99	0.00911	5.45	1.67
	6.18	0.0136	5.39	2.52
	6.36	0.0183	5.16	3.54
	6.45	0.0193	4.64	4.16
	6.84	0.0275	3.83	7.18
	7.00	0.0282	3.49	8.10
	7.16	0.0323	3.28	9.85
	7.48	0.0310	2.77	11.26
	7.78	0.0315	2.68	11.8
	8.14	0.0348	3.22	10.8
	8.46	0.0338	3.57	9.48
FPheFH	5.36	0.057	9.08	6.27
	5.55	0.095	9.0	10.6
	5.65	0.093	10.6	8.73
	6.17	0.194	7.15	27.2
	6.98	0.318	3.33	95.4
	7.55	0.323	2.67	115
	7.68	0.352	2.87	123
	7.85	0.374	3.22	116
	7.98	0.374	3.43	109
FPheSC (δ-chymotrypsin)	6.46	0.0136	3.4	4.0
	6.95	0.0208	2.4	8.64
	7.46	0.0225	1.2	18.8
	7.75	0.0233	1.0	23
FPheFH (δ-chymotrypsin)	4.47	0.013	13.0	1.0
	4.71	0.0124	11.8	1.05
	4.95	0.020	8.25	2.42
	5.45	0.0544	7.0	7.77
	5.95	0.105	5.4	19.4
	6.45	0.165	2.9	57
	6.96	0.19	1.33	143
	7.46	0.229	1.27	180
	7.78	0.236	1.08	218

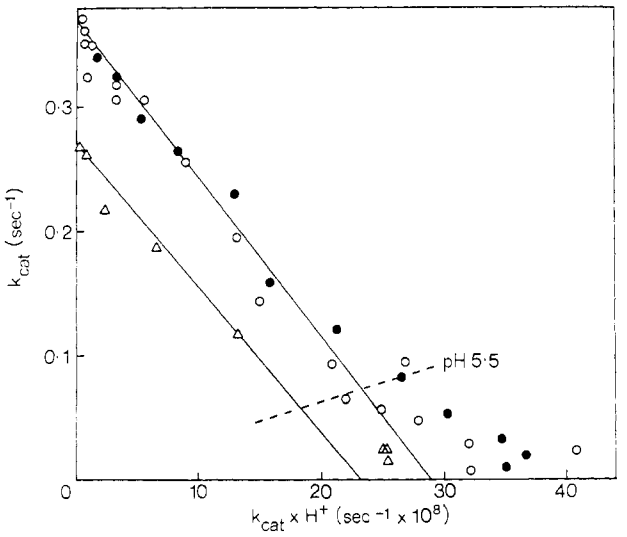


FIGURE 5: k_{cat} *vs.* $k_{cat}[H^+]$ for the hydrolysis of FPheFH: α-chymotrypsin (○), our results; (●) Lucas *et al.* (1973); δ-chymotrypsin (△); 25°, μ = 0.1.

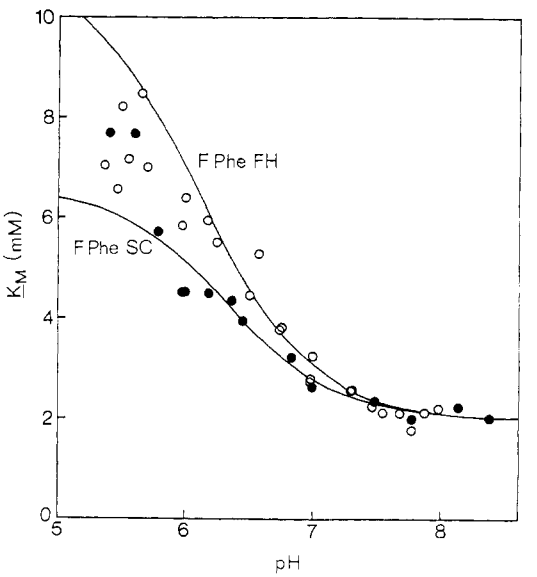


FIGURE 7: K_M *vs.* pH for the hydrolysis of FPheFH (●) and FPheSC (○). With α-chymotrypsin at 25° and μ = 0.1 using the results of this study and Lucas *et al.* (1973). The theoretical curves are calculated assuming a pK_a of 6.84 for the free enzyme, a limiting value of 2 mM at high pH for K_M for both substrates and pK_a values of 6.1 and 6.32 for enzyme–FPheFH and –FPheSC complexes.

k_{cat}/K_M to be determined more precisely. k_{cat}/K_M is seen to follow well an ionization curve of pK_a = 6.84 and maximum value of 17 sec^{-1} M^{-1} (Figure 8). The K_M–pH profile above pH 6.0 fits well a titration curve of pK_a = 6.32 and a limiting value for K_M at high pH of 2.0 mM and at low pH 6.62 mM (Figure 7). k_{cat} fits well a curve of pK_a = 6.32 and limiting value 0.034 sec^{-1}.

pH-Stat experiments with the δ enzyme show that k_{cat} fits a titration curve of about pK_a = 6.38 and limiting value 0.025 sec^{-1}.

k_{cat} for both FPheSC and FPheFH is 26% lower with the δ enzyme but K_M is two times lower. The tighter binding with δ-chymotrypsin has been found also for acetyltryptophanamide (Himoe *et al.*, 1967).

ACETYL-L-TYROSINE *p*-ACETYLANILIDE. The determination of k_{cat}/K_M for this compound is very simple, convenient, and accurate. The K_M value with α-chymotrypsin is 4.7 mM at pH 7.8 and the hydrolysis is accompanied by an increase in absorbance at 340 nm ($\Delta\epsilon$ 5.9 × 10^3). The complete hydrolysis of 10^{-4} M substrate follows first-order kinetics with rate constant (k_{cat}/K_M) × [chymotrypsin] as only 0–2% of the enzyme is bound as the enzyme–substrate complex. It is seen in Figure 9 that there is an excellent fit of k_{cat}/K_M to a theoretical titration curve of pK_a = 6.77 ± 0.01 and limiting value 243 sec^{-1} M^{-1}. The fit is very precise between pH 7.8 and 6.0. The three points at pH values 5.58, 5.33, and 4.97 do in fact deviate from the line when dimerization is taken into account. It is calculated from the data of Aune and Timasheff (1971) that at these values 94.6, 90.6, and 87.4% of the total enzyme is the active monomeric form (see Appendix).

AcPheAlaNH$_2$. This compound is soluble to about 30 mM which is somewhat below the observed K_M values so that the separation of k_{cat}/K_M into k_{cat} and K_M is not very precise. However, the ratio was determined with high precision, the automated ninhydrin assay being very reproducible. The pH dependence of k_{cat}/K_M fits an ionization curve of pK_a = 6.80 ± 0.03 with limiting value 98 sec^{-1} M^{-1}. The value of

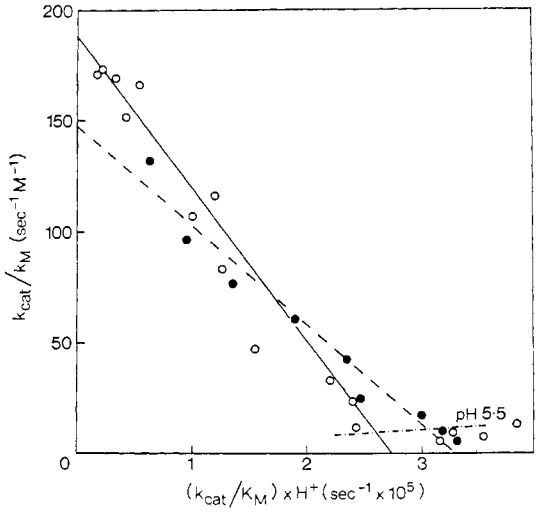


FIGURE 6: k_{cat}/K_M *vs.* $(k_{cat}/K_M)[H^+]$ for the hydrolysis of FPheFH by α-chymotrypsin: our results (○); Lucas *et al.* (1973) (●). Dashed line is for pK_a 6.65 (Lucas *et al.*, 1973); solid line for pK_a = 6.82; 25°, μ = 0.1.

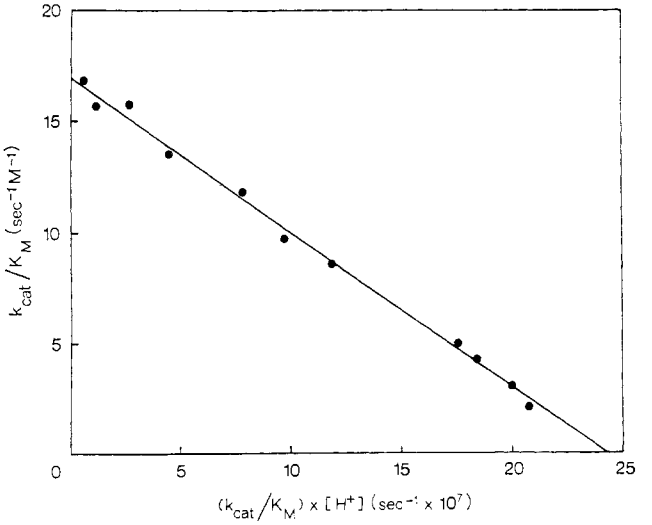


FIGURE 8: k_{cat}/K_M *vs.* $(k_{cat}/K_M)[H^+]$ for the hydrolysis of FPheSC by α-chymotrypsin at 25°, μ = 0.1.

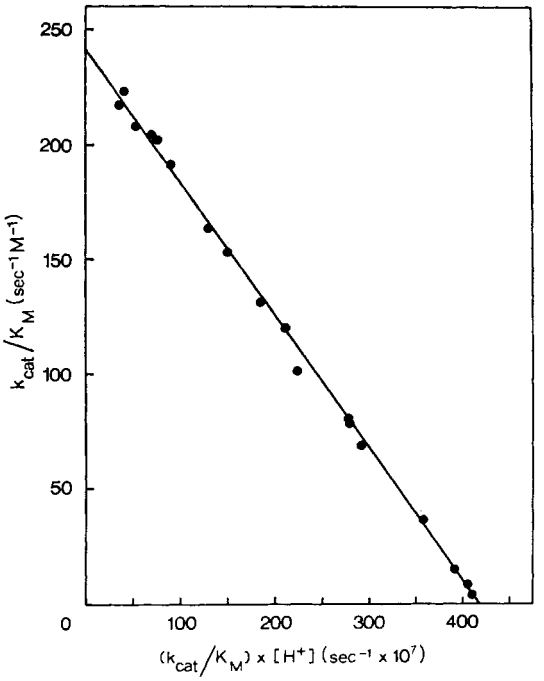


FIGURE 9: k_{cat}/K_M *vs.* $(k_{cat}/K_M)[H^+]$ for the hydrolysis of acetyltyrosine *p*-acetylanilide by α-chymotrypsin at 25° μ = 0.1.

k_{cat}/K_M is considerably lower than that previously found by Baumann *et al.* (1970).

AcPheONp. These results are an extension of those of Renard and Fersht (1973) at μ = 0.95, 5% Me_2SO, and 25° with δ-chymotrypsin. k_{cat}, the rate constant for the attack of water on the acyl-enzyme, follows precisely a titration curve of pK_a = 6.83. At low pH the rate constant falls by a factor of 10 for every pH unit lowered. The point at pH 2 is slightly high but the enzyme denatures at this pH. Log (k_{cat}/K_M), after correction for the fraction in the active conformation (Figure 10), also falls off linearly with pH at low pH with slope 1.0 (Figure 11).

Apart from k_{cat} and K_M for the hydrolysis of $AcPheAlaNH_2$ the quoted catalytic constants are all statistically accurate to a standard error of not greater than ±5%.

The derived pK_a values are summarized in Table VI.

Discussion

The current controversy involving the chymotrypsin mechanism concerns whether or not a tetrahedral intermediate accumulates on the reaction pathway (Lucas *et al.*, 1973; Zeeberg *et al.*, 1973). On the basis of structure–reactivity relationships and the odd pH dependence of k_{cat} and K_M on pH in the hydrolysis of anilides it was postulated that a tetrahedral intermediate accumulated in the chymotryptic hydrolysis of some of these substrates (Caplow, 1969; Lucas and Caplow, 1972). However, it was subsequently demonstrated that an intermediate does not accumulate and that the structure and pH relationships are due to nonproductive binding; the anilide and tyrosine (or tryptophan or phenylalanine) hydrophobic aromatic rings both compete for the substrate binding pocket of the enzyme (Fastrez and Fersht, 1973a).

TABLE VI: Summary of pK_a Values of α-Chymotrypsin.[a]

State of Enzyme	pK_a	Method
Free enzyme	6.77 ± 0.01	Kinetic[b,c]
	6.80 ± 0.03	Kinetic[b,d]
	6.84 ± 0.02	Kinetic[b,e]
FPheSC complex	6.32 ± 0.03	Kinetic[f]
	6.4 ± 0.15	Proton release
FPheFH complex	6.10 ± 0.02	Kinetic[f]
	6.2 ± 0.15	Proton release

[a] 25°, μ = 0. [b] pH dependence of k_{cat}/K_M (corrected for conformational equilibria). [c] Acetyltyrosine *p*-acetylanilide. [d] $AcPheAlaNH_2$. [e] FPheSC. [f] pH dependence of k_{cat}.

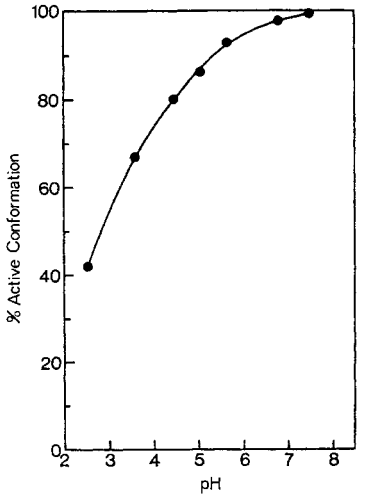


FIGURE 10: Per cent of δ-chymotrypsin in the active conformation at 25°, μ = 0.95, 5% Me_2SO.

The first evidence for an intermediate in the chymotrypsin catalysed hydrolysis of amides was provided by Fersht and Requena (1971b). We suggested that the anomalously low pK_a found from the pH dependence of k_{cat} for the hydrolysis of FPheFH by α-chymotrypsin is due to a combination of the perturbation of the pK_a of the active site and a "kinetic" contribution due to a change in rate-determining step with pH. We proposed that a tetrahedral intermediate occurs on the reaction pathway in a low steady-state concentration and that its partitioning to either acyl-enzyme or Michaelis complex is a function of pH. Subsequently, O'Leary and Kluetz

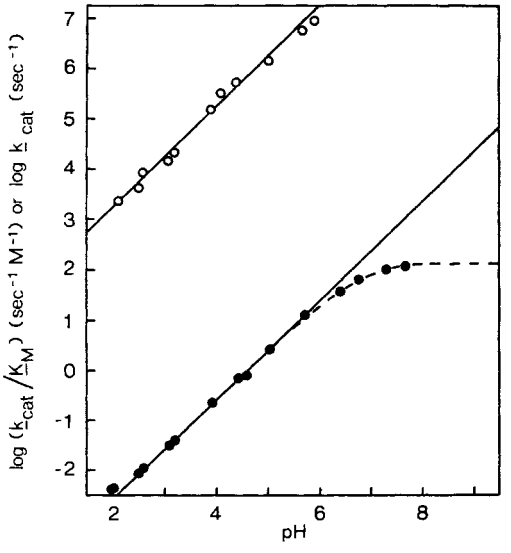


FIGURE 11: Hydrolysis of AcPheONp by δ-chymotrypsin. k_{cat} (●) and k_{cat}/K_M (○) determined at 25°, μ = 0.95 and 5% Me_2SO.

(1972) presented further evidence for a pH-dependent partitioning of an intermediate from kinetic isotope experiments on the hydrolysis of acetyltryptophanamide.

We inferred a "kinetic" contribution to the pK_a of k_{cat} from nonequivalence of pK_a values in the pH dependence of k_{cat} and K_M for the hydrolysis of FPheFH, using FPheSC as a control, since in the absence of a change in rate-determining step the pH dependence of k_{cat} and K_M both depend on the pK_a of the enzyme–substrate complex (Dixon, 1953; Alberty and Massey, 1954). However, Lucas *et al.* (1973) have repeated our measurements and conclude that the pK_a values for k_{cat} and K_M are identical. Also Zeeberg *et al.* (1973) have investigated the partitioning of the acyl-enzyme formyl-L-phenylalanine-chymotrypsin between formylhydrazine and water as a function of pH and conclude that it is pH independent. This implies that the tetrahedral intermediate, which is the same as that for FPheFH and chymotrypsin, does not undergo a pH-dependent partitioning as we proposed. They suggest that the low pK_a of k_{cat} is due to the accumulation of a tetrahedral intermediate of perturbed pK_a.

It is therefore important to accurately establish the pH dependence of k_{cat} and K_M. In the following discussion we point out that the pK_a values obtained by Lucas *et al.* (1973) are in error due to their concentrating on data in the low pH region and ignoring the effects of dimerization of the enzyme and changes in the Linderstrøm–Lang parameter. This latter effect also obscures changes in the pH dependence of partitioning. Because of these difficulties it is difficult to determine unambiguously the equivalence of pK_a values. But it will be seen from the direct determination of proton release on substrate binding that the enzyme substrate complexes of FPheFH and FPheSC with chymotrypsin have similar pK_a values to those determined from the pH dependence of k_{cat}. A small "kinetic" contribution to the pK_a of k_{cat} for AcPheFH and δ-chymotrypsin is indicated from accurate partitioning experiments.

pH Dependence of Michaelis–Menten Parameters. There are the following difficulties in accurately comparing the pH dependence of k_{cat} and K_M. (1) Artifacts may occur due to the high concentrations of substrates required to saturate the enzyme in the measurement of k_{cat} and K_M. The activity coefficient and state of aggregation of the substrate may change at high concentration and also secondary binding to the enzyme may alter its properties by causing substrate activation or inhibition. (2) The accurate determination of the pH dependence of K_M requires a large variation of pH as the limiting values at high and low pH are required. The pK_a values of the groups on the enzyme are sensitive to the charge state of the surface carboxyls and amines (see Edsall and Wyman, 1958, p 510). As the pH falls below 5.5 the carboxyl groups of chymotrypsin are gradually protonated and the effective pK_a of the charge-relay system decreases. This change in the Linderstrøm-Lang parameter causes significant increases in the rate constants for chymotrypsin reactions at low ionic strengths below pH 5.5 (Kézdy *et al.*, 1964). (3) Nonproductive binding causes artefacts in the pH dependence of k_{cat} and K_M (Fastrez and Fersht, 1973a).

We have probably ruled out point (1) in the case of α-chymotrypsin with FPheFH and FPheSC by repeating the experiments with δ-chymotrypsin. The K_M values are a factor of two lower for the δ enzyme and so the substrate concentrations required to determine k_{cat} and K_M are only half those with α. There is little change in the pH dependence of k_{cat} on change of enzyme. However, the pH dependence of K_M cannot be analyzed simply below pH 6 for both point (2) and the existence of a second ionization (Fersht and Requena, 1971b; Lucas *et al.*, 1973). Lucas *et al.* (1973) claim to have fitted the pH dependence of K_M for α-chymotrypsin and FPheFH to two pK_a values; but their theoretical curve does not fit the data above pH 7 and includes data at low pH where the enzyme is dimerized. It is shown in the Appendix that the substrates do not bind productively to the dimer. Above pH 6, K_M for FPheSC and α-chymotrypsin fits fairly well a curve based on the pK_a value of 6.32 which is found from the pH dependence of k_{cat}. Similarly, the K_M for FPheFH may be fitted to a curve of pK_a = 6.10. However, especially in the second case, the assignments are not unique and further criteria must be applied.

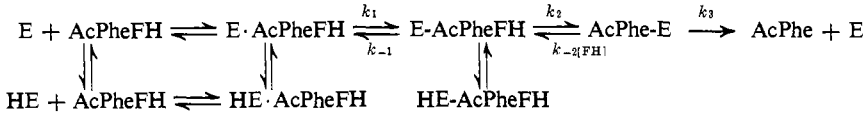
Determination of pK_a of Enzyme–Substrate Complexes by Proton Release. The direct determination by stopped flow spectrophotometry of the proton release on binding FPheFH and PFheSC indicates that the respective enzyme–substrate complexes have pK_a values of about 6.2 ± 0.15 and 6.4 ± 0.15, which are appreciably lower than previously thought (Fersht and Requena, 1971b; Fersht, 1972b). These pK_a values are within experimental error of the values determined from the pH dependence of k_{cat}.

The pK_a of the enzyme is definitely and significantly lowered on the binding of FPheFH and FPheSC. The question now is whether this is due to a substrate-induced perturbation of the pK_a of the active site due to noncovalent interactions or due to the buildup of an intermediate of perturbed pK_a. The proton release is completed in the 1.5-msec dead time of the stopped-flow spectrophotometer. Similarly, more sensitive experiments using proflavine displacement as a probe for intermediates (Bernhard *et al.*, 1966; Brandt *et al.*, 1967) indicate that the binding is at least 97% complete in 1.5 msec (Figure 4). Any intermediate that accumulates must do so with a rate constant of greater than 2000 sec^{-1}. In view of this and the following stereochemical evidence we feel that the pK_a values of the enzyme–substrate complexes are due to substrate-induced perturbations and not due to the buildup of intermediates.

Stereochemical Explanation of Substrate-Induced pK_a Perturbations. Fersht *et al.* (1973) have shown that there is a binding site for the leaving group of specific polypeptide substrates. An important feature of this is a binding locus for the carbonyl oxygen of the leaving group. When this is occupied the α carbon of the leaving group makes an unfavorable contact with the hydroxyl of Ser-195. Formylhydrazide and semicarbazide are sterically similar to specific leaving groups except for the important feature that there is no α carbon but an α nitrogen. Instead of involving an unfavorable contact the α nitrogen and its hydrogen may form a hydrogen bond with the Ser-195 oxygen. The oxygen is a stronger acceptor at high pH when the charge-relay system is negatively charged. Protonation of the active site is inhibited as this weakens the hydrogen bond. Thiosemicarbazide will perhaps not fit into the leaving group site due to the large radius of the sulfur atom.

pH Dependence of k_{cat}/K_M. As the comparison of the pH dependence of k_{cat} and K_M was not sufficiently precise to detect small changes in rate determining step due to the attendant complications outlined, we turned our attention to the pH dependence of k_{cat}/K_M. This does not suffer from points 1 and 3 as k_{cat}/K_M is determined for low substrate concentration and nonproductive binding effects do not affect k_{cat}/K_M. Point 2 is also not important as the pK_a of k_{cat}/K_M may be determined accurately by using the pH range 6–8 in which

SCHEME I



the net charge on the protein does not change as dramatically as at extreme pH values. Furthermore, as the pH dependence of k_{cat}/K_M gives the pK_a of the free enzyme this is substrate independent so that control experiments are easy to perform.

We find that acetyltyrosine *p*-acetylanilide, AcPheAlaNH$_2$, and FPheSC are hydrolyzed by α-chymotrypsin with k_{cat}/K_M giving pK_a values of 6.77, 6.80, and 6.84, respectively. Lucas *et al.* (1973) report a pK_a of 6.65 for the hydrolysis of FPheFH under the same conditions, which, taken in conjunction with our measurements, could indicate a change in rate-determining step. However, examination of Figure 6 reveals that their data extend only to pH 7.3 and the fit relies heavily on values below pH 5.5. in the region in which Kézdy *et al.* (1964) have shown the values are artifactually high at low ionic strengths. A least-squares fit to the data between pH 5.5 and 8 gives a pK_a value of 6.82, but there is sufficient spread to mask small effects.

Partitioning of Acetylphenylalanine-δ-chymotrypsin between Nucleophiles and Water. It may be easily demonstrated that in Scheme I the partitioning of the acyl-enzyme, AcPhe-E, between water and formylhydrazine is pH independent and given by

$$[\text{AcPheFH}]/[\text{AcPhe}] = [k_{-1}k_2/(k_{-1} + k_2)]([\text{FH}]/k_3)$$

where AcPheFH and AcPhe are the amounts of amide and substrate initially produced when AcPhe-E is introduced into a solution of formylhydrazine of concentration [FH] (*cf.* Zeeberg *et al.*, 1973).

We investigated the partitioning of acetylphenylalanine-δ-chymotrypsin between water and either formylhydrazine or semicarbazide as a function of pH under conditions of high ionic strength. This minimizes the changes in the Linderstrøm–Lang parameter with pH. At low ionic strength the changes in this parameter are significant and could introduce primary salt effects on the deacylation reactions. Goldstein (1972) has shown that the rates with amide substrates are more sensitive than esters to the surface charge of the protein and to salt effects. δ-Chymotrypsin was used rather than α as it dimerizes less at low pH.

It is seen in Figure 11 that the deacylation reaction under these conditions follows a good titration curve unlike that of acetyltryptophan-α-chymotrypsin at ionic strengths 0.05 and 0.25 (Kézdy *et al.*, 1964).

Under these conditions the partitioning of the acyl-enzyme between water and formylhydrazine increases by 26% in favor of the amine at low pH. The transition occurs at about pH 6.8 (Table III). The partitioning with semicarbazide is relatively pH insensitive (Table IV). As pointed out by Zeeberg *et al.* (1973) this is required in the mechanism we proposed for the hydrolysis of FPheFH which involved an increase in partitioning of the tetrahedral intermediate toward formylhydrazine expulsion at low pH (Fersht and Requena, 1971b). Zeeberg *et al.* (1973) did not find this increase using α-chymotrypsin at ionic strength 0.1 with formylphenylalanine methyl ester and formylhydrazine.

The increase is certainly consistent with our proposed mechanism but it could be caused by other features such as the binding of the formylhydrazine to the enzyme or the specific perturbation by the substrate of an unknown pK_a on the enzyme. A change of 26% implies that there should be a decrease of 0.1 unit in the pK_a of k_{cat}/K_M for the hydrolysis of AcPheFH by δ-chymotrypsin relative to other substrates. It would be difficult to unambiguously establish this considering that there is a spread of 0.07 unit with our control compounds.

In conclusion, there is no evidence for the accumulation of a tetrahedral intermediate in the chymotrypsin-catalyzed hydrolysis of any small substrate yet examined. Even if k_{cat} and K_M for the hydrolysis of FPheFH have identical pK_a values this is certainly not evidence for the accumulation of an intermediate of perturbed pK_a as there is a reasonable alternative stereochemical explanation available. In the case of anilides there is strong evidence against accumulation (Fastrez and Fersht, 1971a). There is an evolutionary argument against the accumulation of an intermediate in the hydrolysis of peptides. There is pressure on enzymes whose function is to maximize rate to evolve to increase k_{cat} at the expense of increasing K_M (Fersht, 1974). The accumulation of intermediates involves a mutual decrease in k_{cat} and K_M.

There is evidence consistent with a tetrahedral intermediate occurring on the reaction pathway (but not necessarily accumulating) from our experiments on the partitioning of acetylphenylalanine-chymotrypsin with formylhydrazine and water as a function of pH and also from the kinetic isotope experiments of O'Leary and Kluetz (1972).

Ionization of the Charge-Relay System. Fersht and Sperling (1973) have shown that the charge-relay system in chymotrypsin (Blow *et al.*, 1969) is negatively charged above pH 7 and either neutral or zwitterionic below this. The system of Asp-His-Ser has the possibility of a further protonation to give a positively charged entity. The pK_a for this was shown to be less than 4.5 and it was suggested that it might even be as low as 2. The pH dependence of k_{cat}/K_M for the hydrolysis of AcPheONp (Figure 11) appears to confirm this lower value. If a second ionization occurred above pH 2 then the line of slope 1 in Figure 11 should exhibit downward curvature at the pK_a of the ionization and lead to a slope of two at lower pH values. This, of course, refers to the pK_a in the active conformation as the values of k_{cat}/K_M are corrected for this (Fersht and Requena, 1971c; Fersht, 1972a). Without this correction the plot in Figure 11 would be curved.

Appendix: Substrate Binding to Dimeric α-Chymotrypsin Studied by X-Ray Diffraction and the Equilibrium Method

It is generally not possible to study enzyme–substrate complexes which usually react with half-times of milliseconds by techniques which rely on the slow acquisition of data. However, in the case of proteolytic enzymes an equilibrium system may be set up which is time independent.

$$\text{RCONHR}' \rightleftharpoons \text{RCO}_2^- + \text{RCO}_2\text{H} + \text{R}'\text{NH}_2 + \text{R}'\text{NH}_3^+$$

The equilibrium between amide, RCONHR′, and acid may be forced toward the amide by addition of R′NH$_2$. We examined the feasibility of using this approach by determining the free energies of hydrolysis of several classes of amides (Fersht and Requena, 1971a). Semicarbazide sub-

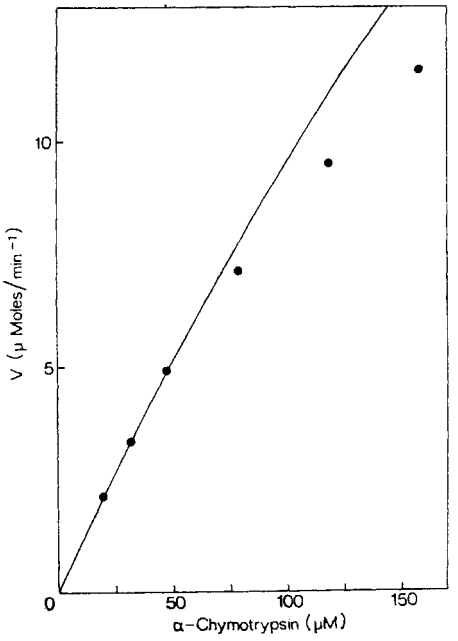


FIGURE 12: Effect of dimerization on hydrolysis of FPheSC by α-chymotrypsin. The initial rate of hydrolysis was determined at μ = 0.1, 25° and pH 5.6 for 3.8 mM substrate. The solid curve is that calculated from the dimerization results of Aune and Timasheff (1971) assuming only the monomer is active.

strates are particularly favorable on energetic grounds and also because of their high solubilities. At pH 4.2 and in the presence of 1 M semicarbazide the equilibrium constant between FPhe and FPheSC is about 10:1 in favor of the *synthesis* of the hydrazide. Similarly, between pH 4 and 7.5 the equilibrium constants between AcPhe-AlaNH$_2$ and AcPhe-GlyNH$_2$ and their hydrolysis products are respectively about 8:1 and 3:1 in favor of *hydrolysis* (Fastrez and Fersht, 1973b).

A 5.5-Å difference Fourier of FPheSC bound to crystalline α-chymotrypsin (enzyme prepared as described by Steitz *et al.* (1969) and soaked in a solution of 40 mM FPheSC, 1.0 M semicarbazide, 65% saturated ammonuim sulfate, and 0.07 M citrate at pH 4.2) revealed no electron density in the substrate binding pocket but only in a nonproductive mode at the mouth of the pocket between the two molecules of the dimer (Wright, 1974). This is consistent with recent observations on the inaccessability of the binding site of the dimer in solution toward certain substrates and inhibitors (Shiao and Sturtevant, 1969; Faller and LaFond, 1971; Faller, 1971; Nichol *et al.*, 1972). Also we find that the initial rate of hydrolysis of FPheSC at pH 5.6 by α-chymotrypsin does not increase linearly with enzyme concentration but follows the concentration of the monomer calculated from the dimerization data of Aune and Timasheff (1971) (although at high enzyme concentrations the rate falls off faster than expected (Figure 12) possibly due to further polymerization). K_M values determined at low pH and high enzyme concentration are therefore unreliable. It may be easily shown that under such circumstances *V vs.* [S] plots are *sigmoid* due to the equilibrium between monomers and dimer.

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