Mechanism of the α-Chymotrypsin-Catalyzed Hydrolysis of Amides. pH Dependence of k_c and K_m.[1] Kinetic Detection of an Intermediate

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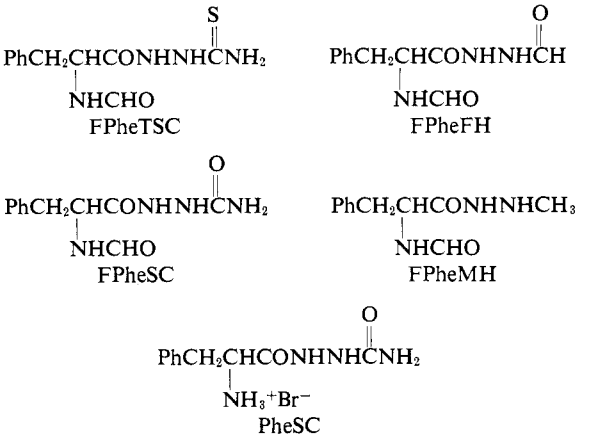
Abstract: A new series of water-soluble amides of *N*-formyl-L-phenylalanine has been synthesized and the pH dependence of the hydrolysis by α-chymotrypsin has been studied. Plots of k_c and K_m against pH for these specific substrates reveal apparent ionization constants; k_c is controlled by a pK_a which varies from 6.1 to 7.1 and K_m by two pK_a values of ~6.7 and ~5. The noncoincidence of pK_a values for k_c and K_m is interpreted as evidence for a change in the rate-determining step of the reaction as a function of pH and for the "ionization" controlling k_c being a composite function of rate constants and a real ionization of His-57. This implies that there is an additional intermediate between the noncovalently bound Michaelis complex and the acyl enzyme. This intermediate does not accumulate but exists in a low steady-state concentration. A possible interpretation is that this intermediate is the tetrahedral addition compound of Ser-195 with the substrate; the rate-determining step in the reaction is, at high pH, its breakdown and, at low pH, its formation. The pretransition-state protonation theory is incompatible with these results. The binding of substrates to α-chymotrypsin is controlled by three groups of pK_a values, 5.3, 7.0, and 8.8.

The delineation of the mechanism of action of chymotrypsin requires a knowledge of all the intermediates on the reaction pathway. In the particular case of the specific reaction of chymotrypsin, the hydrolysis of peptides, an intriguing question is whether or not a tetrahedral intermediate is formed and is part of the catalytic process. Tetrahedral intermediates have been implicated in some nonenzymatic reactions of amides,[2] but even here there is some question as to the ubiquity of a discrete intermediate. There are indications that the aminolysis of some active acyl compounds may proceed *via* a single transition state; the distinction between an intermediate and a transition state becoming blurred as the activation energies for the breakdown of the intermediate tend to zero.[3] Structure–reactivity relationships are now available for the nonenzymatic alcoholysis of amides to compare with the enzymatic reactions[4] and this reference system will have more meaning if it is known whether or not the enzymatic reaction involves a discrete intermediate.

An important contribution to this problem has been made by Caplow[5] who noticed that the pK_a governing the first-order rate constant k_c for the acylation of α-chymotrypsin by some anilide substrates is variable and has postulated that this is due to an accumulation of a tetrahedral intermediate as part of the enzyme–substrate complex which has a perturbed pK_a.

This raises a dilemma as the pK_a governing the acylation step should manifest itself in the pH dependence of K_m, but this was not found. However, accurate data were difficult to obtain in Caplow's experiments due to the poor solubility of his substrates.

We have synthesized the following new substrates which are soluble to well above the K_m values for the hydrolytic reactions and have obtained accurate experimental data: *N*-formyl-L-phenylalanine thiosemicarbazide (FPheTSC); *N*-formyl-L-Phe formylhydrazide (FPheFH); *N*-formyl-L-Phe semicarbazide (FPheSC); *N*-formyl-L-Phe methylhydrazide (FPheMH); and L-Phe semicarbazide hydrobromide (PheSC).



The questions we wish to answer are: (a) what is the cause of this pK_a anomaly; (b) is there an intermediate; (c) what is its nature; (d) does it accumulate; (e) what is the rate-determining step of the reaction; and (f) is the binding of substrates pH dependent below pH 8?

Experimental Section

Materials. Chymotrypsin crystallized (three times), lyophilized, and salt free was lot no. CDI 8LK from Worthington. Organic and inorganic reagents were commercial products unless otherwise stated. *N*-Formylhydrazine was prepared by the method of Blackburn and Jencks.[6] *N*-Formyl-L-phenylalanine was prepared according to Greenstein and Winitz.[7]

(1) The kinetic parameters mentioned in this paper are defined by $v = k_cES/(K_m + S)$ where v is the initial velocity, E and S the initial enzyme and substrate concentrations, K_m the Michaelis constant, and k_c the turnover number of the enzyme. Abbreviations used are ES for the enzyme–substrate complex and accepted notation for amino acids, *e.g.*, Phe = phenylalanine.

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(3) A. R. Fersht and W. P. Jencks, *J. Amer. Chem. Soc.*, **92**, 5442 (1970).

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Semicarbazide was prepared from the hydrochloride as follows: 111.5 g of semicarbazide hydrochloride was dissolved in 250 ml of 4 *N* NaOH and the pH was adjusted to 7.0; the solution was evaporated to dryness and extracted with hot dimethylformamide. On cooling semicarbazide crystallized from the DMF and further material was obtained by precipitation with ether. Pure compound, mp 95–96° (lit.[8] 96°), was obtained by recrystallization from acetonitrile.

***N*-Formyl-L-phenylalanine semicarbazide** was prepared from *N*-formyl-L-phenylalanine and semicarbazide by the dicyclohexylcarbodiimide coupling procedure. The acid (4.82 g, 0.025 mol) and 1.876 g (0.025 mol) of semicarbazide were dissolved in 75 ml of tetrahydrofuran and 75 ml of acetonitrile. Dicyclohexylcarbodiimide (5.7 g, 0.277 mol) was added and the mixture was stirred for 5 hr and then 0.5 ml of glacial acetic acid was added. The mixture was left at 0° overnight and filtered and the precipitate was extracted with hot water. The extract was evaporated to dryness and this residue was recrystallized several times from absolute ethanol to give colorless crystals; mp 177–179°; α_D +20°.

Anal. Calcd: C, 52.74; H, 5.64; N, 22.37. Found: C, 52.76; H, 5.59; N, 22.48.

***N*-Formyl-L-phenylalanine formylhydrazide** was prepared from the acid and formylhydrazine as above except that the recrystallization was from water; mp 191–193°; α_D +3°.

Anal. Calcd: C, 56.16; H, 5.57; N, 17.86. Found: C, 55.88; H, 5.60; N, 17.69.

***N*-Formyl-L-phenylalanine thiosemicarbazide** was prepared as above except that dimethyl sulfoxide was used as the solvent. After the addition of glacial acetic acid the DMSO was distilled off under high vacuum at 60–80° and the residue was extracted with hot water. The extract was evaporated to dryness and this residue was recrystallized from water. During the recrystallization when the solution was sufficiently cool a little concentrated HCl was added to retain in solution any thiosemicarbazide as the more soluble hydrochloride; mp 193.5–194.5°; α_D +16°.

Anal. Calcd: C, 49.61; H, 5.30; N, 12.04. Found: C, 49.52; H, 5.21; N, 12.18.

***N*-Formyl-L-phenylalanine methylhydrazide** was prepared by dissolving 2.1 g (0.0109 mol) of the acid in 70 ml of acetonitrile and adding 0.5 g (0.0109 mol) of methylhydrazine rapidly followed by 2.5 g (0.0121 mol) of dicyclohexylcarbodiimide. The mixture was stirred for 5 hr, 0.2 ml of glacial acetic acid was added, and it was cooled at 0° overnight. The mixture was filtered, the filtrate was evaporated to dryness, and the residue was extracted with hot water. The extract was evaporated to dryness and recrystallized from absolute alcohol; mp 154.5–155.5; α_D +60°.

Anal. Calcd: C, 59.71; H, 6.83; N, 18.99. Found: C, 59.38; H, 6.80; N, 19.07.

L-Phenylalanine Semicarbazide Hydrobromide. Carbobenzoxy-L-phenylalanine semicarbazide was prepared from carbobenzoxy-L-phenylalanine and semicarbazide as described for the formyl compound. The dicyclohexylurea was removed by filtration and the tetrahydrofuran acetonitrile by rotary evaporation. After recrystallization from ethanol–ether or water the hydrazide was debenzylated in 38% HBr in glacial acetic acid. The product was characterized by the procedure recommended by Sheehan[9]—the formyl derivative was prepared[9] and shown to be formyl-L-phenylalanine semicarbazide (ir, melting point, mixture melting point, and optical rotation).

The optical impurities of the first three compounds were subjected to a further check by noting the release of hydrazide (assayed either colorimetrically or by acid release) after prolonged incubation with α-chymotrypsin. All three gave within experimental error the theoretical amount. The less reactive methylhydrazide was not subjected to this test. Sheehan and Yang have previously noted that the diimide coupling of *N*-formyl-L-phenylalanine with glycine ethyl ester does not lead to racemization.[10]

N-Formyl-L-phenylalanine ethylamide was a gift from Dr. D. Kosman.

The above melting points were recorded on a Reichert heating block and microscope stage (Kofler block). Microanalyses were kindly performed by the Cambridge University Chemical Laboratories. Optical rotations were performed in aqueous solution in a Bellingham and Stanley visual matching polarimeter at room temperature.

Apparatus. The spectrometer and stopped-flow mixing device have been described elsewhere.[11] The pH-stat apparatus was a conventional Radiometer 26B pH meter coupled with a TTT-11 unit, recorder, and motorized micrometer syringe buret. Automatic colorimetric analyses were performed using a Technicon peristaltic pump and a flow cell in conjunction with the Gilford 2400 spectrophotometer in an arrangement similar to that described by Lenard, *et al.*,[12] for automated ninhydrin assays. Solutions were thermostated at 25.0 ± 0.05°

The hydrolyses of *N*-formyl-L-phenylalanine semicarbazide and the formylhydrazide were followed at 25° and ionic strength 0.1 (added KCl) by means of the pH-stat. Reactions were initiated by adding enzyme from a Hamilton 100-μl syringe to the stirred reaction mixture. Kinetic runs were corrected for a small enzyme blank reaction—much smaller than that previously reported.[13] Reaction volumes were from 10 to 50 ml according to substrate concentration, and the titrant was 10^{-2} *N* NaOH. Runs at higher pH values for the formylhydrazide were corrected for the ionization of the substrate and at lower pH values for the ionization of the *N*-formyl-L-Phe. The hydrolyses of the methylhydrazide and thiosemicarbazide were performed by automatic colorimetric assaying as follows.

Automatic Assay for Thiosemicarbazide. We have previously shown[14] that 1,2-naphthaquinone-4-sulfonic acid (NSA) provides an excellent assay for thiosemicarbazide. Stock solutions of NSA (130 mg/500 ml of H_2O) were prepared daily and stored in the dark. The peristaltic pump mixed equal volumes of NSA solution and carbonate buffer (0.8 *M* $KHCO_3$, 0.2 *M* K_2CO_3) and then mixed 3.75 vol of this with 1 vol of reaction mixture and then circulated it through a flow cell monitored at 460 nm. The apparatus was calibrated by using standard solutions of thiosemicarbazide as the reaction mixture. A solution of 5×10^{-4} *M* thiosemicarbazide gives an absorbance of 1.3–1.4 at 460 nm under these conditions. The reaction mixture consisted of 10 ml of substrate dissolved in aqueous buffer at ionic strength 0.1 and the reaction was initiated by the addition of enzyme.

Automatic Assay for Semicarbazide. The hydrolysis rates of *N*-formyl-L-Phe semicarbazide measured on the pH-stat were in excellent agreement with those measured colorimetrically by the following modification of the thiosemicarbazide assay. The assay was monitored at 460 nm as above but the carbonate buffer used consisted of 0.8 *M* K_2CO_3 and 0.2 *M* $KHCO_3$. A 5×10^{-4} *M* solution of semicarbazide hydrochloride gives an absorbance of about 0.81 under these conditions.

Automatic Assay for Methylhydrazine. Niemann's[15] assay for hydrazine was modified for use in the Technicon apparatus. The peristaltic pump mixed 1 vol of the reaction mixture with 1.9 vol of the assay mixture (2.4 g of dimethylaminobenzaldehyde, 300 ml of DMSO, 60 ml of 5 *N* HCl, and 40 ml of H_2O) and then circulated the combined solutions through a flow cell which was monitored at 470 nm. Under these conditions 2×10^{-4} *M* methylhydrazine gives an absorbance of about 1.1 at 470 nm. A very slow run (over 3 days) at pH 4.52 was performed by taking aliquots and assaying.

Enzyme solutions were always prepared daily in 10^{-3} *N* HCl and stored on ice. The enzyme was assayed as described previously[11] (94% of theoretical active sites).

The relatively high solubility of the substrates enabled purely aqueous solutions to be used and concentrations going far above the K_m values in most cases. This, combined with the excellent reproducibility of the assay techniques, afforded accurate kinetic data. The results are presented in Tables I and II.

pK_a Determinations. The hydrazides FPheTSC, FPheFH, and FPheSC ionized as acids due to the activation of the amide nitrogens. The pK_a values were determined by titration of 10 ml of 0.02 *M* solutions in 0.1 *M* KCl at 25.0 ± 0.05° with 1.0 *N* KOH. *N*-Formyl-L-phenylalanine was titrated twice in like manner except at ionic strength ~0 and 1.0 *M*. The pK_a of formylhydrazine ionizing as a base was measured by the titration of 20 ml of 10^{-2} *M*

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

Substrate	pH	Substrate concn, m*M*	Enzyme concn, μM	Buffer	No. of substrate concns
FPheFH[a]	4.48	1.5–20	18–36		8
	4.74	1.4–20	36		7
	4.98	0.5–20	18		8
	5.24	1–20	8		7
	5.47	0.5–20	9		8
	6–8.6	0.5–20	2.3–2.5		9–13
FPheSC[a]	5.47	1–20	90		9
	5.95	1–20	36		7
	6.47	0.5–20	18–25		7
	7–7.8	0.5–20	9		7–12
FPheTSC[b]	5.64	1–16	34	Acetate	6
	6.5–7.1	1–16	34	Phosphate	6
	7.4–7.8	1–16	34	Tris	5
FPheMH[b]	4.52	4–32	60	Acetate	5
	6.3–6.8	4–32	84	Phosphate	6–8
	7.8	4–32	84	Tris	9
PheSC[a]	5.7	4–95	320		6

[a] Assayed in pH-stat, ionic strength maintained with added KCl. [b] Assayed by automated calorimetric procedure, ionic strength maintained by buffer alone.

Table II. Catalytic Constants for Hydrolysis of Hydrazides by α-Chymotrypsin at 25° and Ionic Strength 0.1

Substrate	pH	k_c, sec^{-1}	K_m, m*M*	k_c/K_m, $sec^{-1}\ M^{-1}$
FPheFH	8.58	(0.044)[a]	(7.6)[a]	(59)[a]
	7.78	0.362	2.6	139
	7.47	0.348	2.7	127
	6.98	0.306	3.3	88.3
	6.73	0.306	4.46	68.7
	6.48	0.256	6.3	39.4
	5.98	0.143	7.3	19.2
	5.47	0.065	8.4	7.66
	5.24	0.048	11.6	4.19
	4.98	0.029	12.5	2.3
	4.74	0.022	24	0.936
	4.48	0.0097	22	0.444
FPheSC	7.79	0.0288	2.28	12.6
	7.48	0.0306	2.24	13.7
	6.98	0.0244	3.45	7.1
	6.47	0.0192	5.6	3.4
	5.95	0.00957	6.7	1.46
	5.47	0.0032	7.0	0.46
PheSC	5.7	0.0038	49	0.077
FPheTSC	7.80	0.0301	6.0	5.02
	7.39	0.0235	3.85	6.27
	7.05	0.0179	3.57	5.01
	6.79	0.00974	3.12	3.15
	6.58	0.00914	2.67	3.42
	6.24	0.00352	2.0	1.77
	5.65	0.00139	2.86	0.487
FPheMH	7.82	3.2×10^{-4}	8.6	0.0376
	7.16	3.04×10^{-4}	11.1	0.0274
	6.84	2.68×10^{-4}	11.8	0.0227
	6.36	1.7×10^{-4}	12.2	0.0139
	4.58			2.5×10^{-4}

[a] Substrate 36.5% ionized.

formylhydrazine in 1.0 *M* KCl using 1.0 *N* HCl as titrant. A Burroughs-Wellcome "Agla" syringe was used for titrant. The results were corrected for H^+ and OH^- contributions where appropriate. The results are presented in Table III.

Determination of Dissociation Constants. The dissociation constants, K_s, were determined for a few substrates and inhibitors by means of the proflavin displacement method of Hess.[16] Approximate values only were obtainable at pH 4.5, reproducibility being low. The results are presented in Table IV.

Stopped-Flow Search for Intermediates. Presteady-state kinetics with chymotrypsin are obscured by the existence of two slowly interconvertible conformations of α-chymotrypsin present at all pH values.[11] Preliminary experiments mixing either *N*-formyl-L-Phe formylhydrazide or semicarbazide with various concentrations of proflavin and enzyme (proflavin displacement method of Bernhard[17]) revealed no relaxation times in the region 10^{-2}–10^{2} sec other than the enzyme isomerization. More refined experiments were performed by incubating the enzyme with an excess of proflavin and mixing with the substrate in the stopped-flow spec-

Table III. pK_a Values at 25°

Compd	Ionic strength[a]	pK_a
FPheTSC[b]	0.1	8.38 ± 0.01
FPheFH[b]	0.1	8.82 ± 0.01
FPheSC[b]	0.1	10.03 ± 0.01
N-Formyl-Phe	0.005	3.30 ± 0.01
	1.0	3.10 ± 0.01
Formylhydrazine[c]	1.0	2.81 ± 0.01

[a] Maintained with KCl. [b] The hydrazino group between the two carbonyl groups ionizes as an acid. Tentative assignments are that the ionizing proton is on the nitrogen adjacent to the Phe carbonyl for FPheSC, while that for FPheFH is adjacent to the formyl carbonyl. [c] Ionizing as a base.

Table IV. Dissociation Constants (K_s) of Substrates and Inhibitors with α-Chymotrypsin at 25° and Ionic Strength 0.1[a]

Compound	K_s, m*M* pH 4.5	5.6	7.8
N-Acetyl-L-tryptophanamide	15	6	4.1
Indole	0.8[b]		0.8
Proflavin	0.14	0.039	0.022
N-Formyl-L-Phe TSC	7[b]	2.9[c]	3.6[c,d]
N-Formyl-L-Phe FH	21[c]	8[c]	2.2 (2.6)[c]
N-Formyl-L-Phe SC	17[b]	7[c]	2.0 (2.3)[c]
N-Formyl-L-Phe MH	60–200[b,c]		8.6
N-Formyl-L-Phe ethylamide	>40[b]		20
L-Phe SC		49[c]	

[a] By proflavin displacement unless otherwise stated; ionic strength maintained by Tris at pH 7.8 and acetate at lower pH. [b] These measurements are approximate. [c] Obtained kinetically in 0.1 *M* KCl. [d] Substrate is 21% ionized under these conditions; this value is extrapolated from lower pH measurements.

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Table V. Summary of Kinetic Data at 25° and Ionic Strength 0.1

Substrate	pK_a[a] of amine	k_c, sec^{-1}	k_c/K_m,[b] sec^{-1} M^{-1}	K_m,[b,c] min mM
FPheTSC	1.88[d]	0.036 ± 0.002	11 ± 1	3.3
FPheFH	2.81	0.366 ± 0.006	225 ± 15	1.6
FPheSC	3.86[e]	0.0316 ± 0.002	22 ± 2	1.44
FPheMH	8.20[f]	$(3.4 \pm 0.1) \times 10^{-4}$	$(5.6 \pm 0.2) \times 10^{-2}$	6.1
FPhe ethylamide	10.97[e]			~15

[a] pK_a of the amine displaced on hydrolysis; determined for 25° and ionic strength 1.0 (KCl). [b] Corrected for contribution of inactive conformation (A. R. Fersht and Y. Requena, *J. Mol. Biol.*, **60,** 279 (1971). [c] The dissociation constant for binding to the enzyme at higher pH (not protonated on His-57). [d] J. Sayer and W. P. Jencks, *J. Amer. Chem. Soc.*, **91,** 6353 (1969). [e] W. P. Jencks and M. Gilchrist, *ibid.*, **90,** 2622 (1968). [f] T. C. Bruice, A. Donzel, R. W. Huffman, and A. R. Butler, *ibid.*, **89,** 2106 (1967).

Table VI. Summary of pK_a Values

Substrate	pK_a[a] of amine	pK_a[b] k_c	pK_a[c] k_c/K_m	pK_a[d] ES
FPheTSC	1.88	7.1 ± 0.1	~6.9	
FPheFH	2.81	6.08 ± 0.06	7.0 ± 0.1	~6.8 (~5)[e]
FPheSC	3.86	6.37 ± 0.07	7.1 ± 0.1	~6.7
FPheMH	8.20	6.25 ± 0.1	~6.9	

[a] pK_a of amine constituent of substrate at 25° and ionic strength 1.0; see Table V. [b] pK_a governing k_c—if this were a "true" ionization, it would be the pK_a of the enzyme–substrate complex (ES). [c] pK_a governing k_c/K_m and the pK_a of enzyme (E). [d] pK_a of enzyme–substrate complex from midpoint of K_m against pH plot. [e] A second value observed at lower pH.

trophotometer, the concentrations calculated so that there was insignificant change in the enzyme isomerization equilibrium. For example, 100 μM (5 × K_s) proflavin was incubated at pH 7.84 with 36 μM enzyme and then mixed with FPheFH or FPheSC at 10–20 mM (5–10 × K_s). No detectable relaxations were observed that could indicate the formation and accumulation of an intermediate with a rate constant slower than ~100 sec^{-1}.

Results

The observed parameters are given in Tables I–VII. In Figures 1 and 2 K_m and k_c/K_m are calculated for the *active* conformation of chymotrypsin since we have previously shown that two conformations exist over the entire pH range,[11] *e.g.*, at pH 7.8, $K_m = 0.745(K_m)_{obsd}$ and $k_c/K_m = 0.745^{-1}(k_c/K_m)_{obsd}$.[11] These corrections hardly affect the calculated pK_a values. The substrate concentrations were generally in the range of $K_m/5$–5 × K_m enabling k_c and K_m to be accurately determined. High reproducibility was attainable and where the substrate concentration could be taken to ~4 × K_m the internal accuracy is about ±5% and not worse than ±10%. The convenience of the active-site titration[18] and the high activity of the enzyme batch (94% of theoretical active sites) provide a high absolute accuracy. The most careful studies were with FPheFH and then FPheSC. Experimentally, the assays with FPheMH were the most difficult, the hydrolysis rates being slow and total absorbance changes of only ~0.07 being followed. The more reactive FPheTSC could usually be monitored over absorbance changes of 0.2–1.0.

The hydrazides provide an excellent series of substrates. The solubilities vary from about 25 mM for the FPheTSC to ~100 mM for the FPheSC with the other two greater than 40 mM. This is in great contrast to the anilide and amide substrates. The disadvantage is the ionization at higher pH. However, for studies below about pH 7.8 the FPheFH is extremely useful; the low pK_a of formylhydrazine enables use of the pH-stat in the same way as for an ester substrate, and the reactivity is high. The FPheSC may be used at higher pH values, its pK_a being 10.03. As semicarbazide has a pK_a of 3.86 it too may be assayed easily in the pH-stat. The available pH range may be extended by using the colorimetric assay procedure.

Semicarbazide is an excellent solubilizing group, itself having high solubility in water and very low solubility in organic solvents such as ether or chloroform.

The important observations are given in Figures 1 and 2. Above pH 5.5 the plot of K_m against pH exhibits a sigmoid curve which has its midpoint, *i.e.*, its pK_a, at pH 6.7–6.8 for both FPheFH and FPheSC. The plot for k_c against pH exhibits a pK_a of 6.08 for FPheFH and 6.37 for FPheSC The pK_a of the ionizing group on the enzyme (pK_{a_E}) is found from the k_c/K_m plot[19] to be 7.0–7.1. The pK_a values were determined graphically from plots of the observed k_c or k_c/K_m against $k_c[H^+]$ or $(k_c/K_m)[H^+]$, analogous to the well-known plot of v against $v/[S]$ for the determination of K_m values.

Table VII. Ionizing Groups on α-Chymotrypsin at 25° and Ionic Strength 0.1

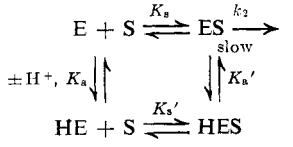
Group	pK_a	$pK_{a_{ES}}$[a]
Ile-16 / Asp-194 salt bridge[b]	8.76 ± 0.06	>10
His-57[c]	7.0 ± 0.1	6.6–7.1
d	~5.3	~4.9–5.3

[a] pK_a of enzyme–ligand complex. [b] This is an "apparent" ionization constant, composed of equilibrium and ionization constants. See A. R. Fersht and Y. Requena, *J. Mol. Biol.*, **60,** 279 (1971). [c] Part of H-bonded system. [d] Occurs in the binding of proflavin, FPheFH, and formanilide; see text.

Theory

Scheme I, the simplest Michaelis–Menten system used

Scheme I



to describe the acylation of chymotrypsin by amides, requires that the pH dependence of k_c and K_m be gov-

(18) M. L. Bender, M. L. Begué-Canton, R. L. Blakeley, L. J. Brubacher, J. Feder, C. R. Gunter, F. J. Kézdy, J. V. Killheffer, Jr., T. H. Marshall, C. G. Miller, R. W. Roeske, and J. K. Stoops, *J. Amer. Chem. Soc.*, **88,** 5890 (1966).

(19) R. A. Alberty and V. Massey, *Biochim. Biophys. Acta*, **13,** 347 (1954); M. L. Bender, G. E. Clement, F. J. Kezdy, and H. d'A. Heck, *J. Amer. Chem. Soc.*, **86,** 3680 (1964).

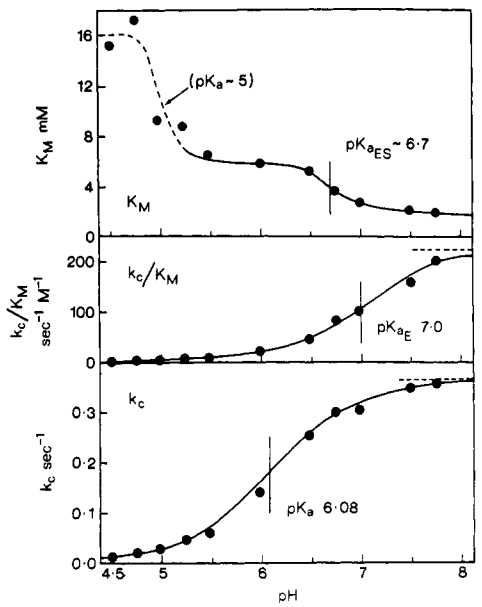


Figure 1. Plots of k_c, k_c/K_m, and K_m against pH for the hydrolysis by α-chymotrypsin of *N*-formyl-L-phenylalanine formylhydrazide (FPheFH) at 25° and ionic strength 0.1. k_c/K_m is calculated for the "active" conformation of α-chymotrypsin as two conformations exist at all pH values (A. R. Fersht and Y. Requena, *J. Mol. Biol.*, in press); the solid curve is that calculated for a pK_a of 7.0 for the ionization of the enzyme. The K_m plot is similarly calculated for the "active" conformation. The solid line in the k_c plot is that calculated for a *single* ionization of pK_a 6.08 (if Scheme II and Figure 4 are the mechanistic pathway this implies $k_2 \sim k_2'$).

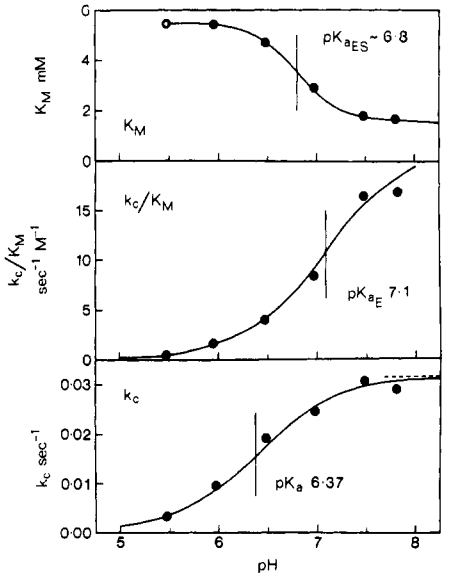


Figure 2. Plots as in Figure 1 for the hydrolysis of *N*-formyl-L-phenylalanine semicarbazide (FPheSC) using the pK_a values as indicated for the ionizations.

erned by the same ionization constant, K_a'. K_m may be affected by ionizations other than at the active site and exhibit these also in the pH profile. But if the binding of a substrate to the active site causes a perturbation of the pK_a of a catalytically active group then this must be reflected in K_m; K_m will be pH dependent and governed by the same ionization constant as k_c.

If varying substrates on binding to the enzyme cause varying perturbations in the pK_a of a catalytically active group, then the same pK_a variations must occur in the pH dependence of K_m. Similarly Scheme II

Scheme II

$$\begin{array}{ccccccc} E + S & \overset{K_s}{\rightleftharpoons} & ES & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES' & \xrightarrow{k_2} & \\ \pm H^+,\ K_a \uparrow\downarrow & & K_a' \uparrow\downarrow & & \uparrow\downarrow K_a'' & & \\ HE + S & \overset{K_s'}{\rightleftharpoons} & HES & \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} & HES' & \xrightarrow{k_2'} & \end{array}$$

is subject to the same constraints. However, if in Scheme II k_1' and k_{-1}' are extremely slow, the pK_a for k_c may apparently vary independently of that of K_m. Scheme II is then kinetically equivalent to Scheme III.

Scheme III

$$\begin{array}{ccccccc} E + S & \overset{K_s}{\rightleftharpoons} & ES & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES' & \xrightarrow{k_2 + k_3[H^+]} & \\ \pm H^+,\ K_a \uparrow\downarrow & & K_a' \uparrow\downarrow & & & & \\ HE + S & \underset{K_s'}{\rightleftharpoons} & HES & & & & \end{array}$$

In Schemes II and III an element of asymmetry has been introduced. There are paths depending on [HE] and [E] for the breakdown of the intermediate

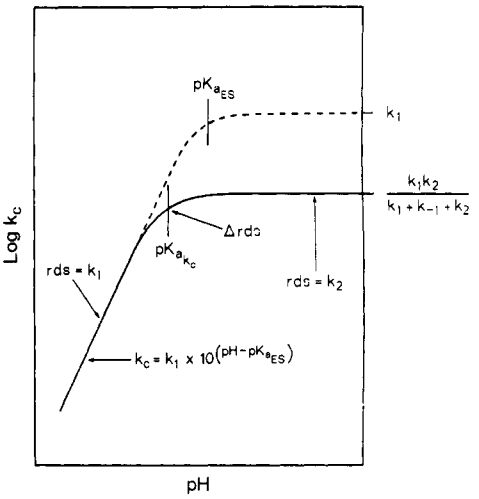


Figure 3. Diagrammatic representation of how a change in rate-determining step (Δrds) from formation of an intermediate at low pH to its breakdown at high pH leads to a pK_a in the k_c–pH profile lower than that of the ionization of the base catalyst. The notation of Scheme II and Figure 4 is used. The solid line is that for the observed k_c against pH, while the dotted line is that calculated for the first step k_1.

to give products, but the formation of the essential intermediate depends only on [E] and not on [HE]. In this way a change of rate-determining step is possible. If k_{-1} is greater than k_2, the rate-determining step at high pH is the breakdown of ES'. However, at low pH the rate of the step k_{-1} decreases with pH but the pathway *via* HES' in Scheme II or *via* $k_3[H^+]$ in Scheme III increases and may become greater than k_{-1} so that at low pH the formation of ES', rather than its breakdown, is rate determining. This introduces a "kinetic pK_a" into the pH dependence of k_c, as illustrated in Figure 3.

The analytical solutions for k_c and K_m in Schemes II and III lead to each having two common ionization constants. But where ES' does not accumulate, but exists in a low steady-state concentration, K_m is a

true binding constant and exhibits just the ionization constant of the ES complex, K_a'. k_c shows mainly an ionization constant which is greater than K_a'. Where ES′ does accumulate, its concentration makes an important contribution to the observed K_m. K_m at high pH is now much less than K_s, but at low pH, where the formation of ES′ is rate determining and this intermediate does not accumulate, the observed K_m is equal to K_s. Clearly, as the rate-determining step changes from formation to breakdown of the intermediate there is a large decrease in K_m, and so K_m and k_c have a common pK_a value.

In summary, to accommodate a change of rate-determining step with pH in an enzymatic reaction which involves an initial rapid reversible enzyme–substrate complex formation, at least one extra intermediate is required. Also, if the substrate is varied and the pK_a for k_c changes without a corresponding change in the pH dependence of K_m, then this is evidence for a change in rate-determining step as the pK_a for K_c must be a kinetic, rather than a real, ionization.

Discussion

pK_a Values of k_c and K_m. Evidence for Change in Rate-Determining Step. There is now considerable evidence that the following scheme is the minimal reaction pathway for the chymotrypsin-catalyzed hydrolysis of amides and peptides, and that the rate-determining

$$E + RCON< \overset{K_s}{\rightleftharpoons} E\cdot RCON< \xrightarrow{k_2} EC(=O)-R + >NH_2 \xrightarrow{k_3} E + RCO_2H$$

step is the acylation of the enzyme, k_2[16,20,21] (*i.e.*, $k_c = k_2$). The pH dependence of the rate constants k_c, k_c/K_m, and k_3 is governed by ionization constants associated with the imidazole of His-57.[22] However, in several studies of the chymotrypsin-catalyzed hydrolysis of anilides of *N*-acetyl-L-tryptophan or tyrosine[5,23,24] the pK_a for k_c has been found to vary by more than can be reasonably expected. The only explanation offered so far is from Caplow[5] who has postulated an accumulation of a tetrahedral intermediate of perturbed pK_a in a scheme analogous to II where ES′ is the tetrahedral intermediate and also $k_2' = 0$.

In the present study the pK_a for k_c varies from 6.1 to 7.1. Some of the variation is due to the genuine perturbation of the pK_a of His-57 and this will be discussed first.

pH Dependence of Binding of Inhibitors. It is sometimes argued that the dissociation constants for the binding of substrates and inhibitors to α-chymotrypsin are independent of pH in the range 6–8.[5] However, binding is pH dependent and is a function of the nature of the bound species. For example, Glick[25a] has shown that at 20° the binding of anisole causes a pK_a shift of 6.7–6.4 presumably associated with His-57.[25a] Formanilide causes a pK_a shift from 5.3 to 5.0. As binding constants and ionization constants are thermodynamically linked, this implies that both anisole and formanilide bind less well to the lower pH enzyme by 0.3 log unit. The binding of the charged dye proflavin exhibits three plateau regions against pH.[11,16] These values in conjunction with our pK_a data show that the pK_a of His-57 drops from 7.0 to ~6.7 on binding of proflavin and there is another change from about 5.1 to 4.8. The identity of this latter group is unknown but is presumably the same as that observed in formanilide binding.[25a,b] The binding of the dye 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) also exhibits a sigmoid dependence on pH governed by a pK_a of 6.65.[26] Both proflavin and TNS bind better by a factor of about 2 to the higher pH form of the enzyme; protonation of His-57 decreases the binding constant.

Indole, which presumably binds deep in the aromatic binding pocket,[27] appears to be affected less by the state of ionization of the protein (below pH 8). *N*-Acetyltryptophanamide is affected slightly by the ionization of His-57, binding some 20–40% better to the higher pH enzyme.[16]

In this study FPheFH exhibits the 7–6.7 and 5.3–5 pK_a shifts in the K_m–pH profile. Similarly, FPheSC and FPheMH bind 2–3 times better to the higher pH enzyme. FPheTSC appears to be unaffected by the ionization of His-57.

In summary, the binding of FPheFH appears not to be abnormal. Protonation on His-57 decreases the binding of some inhibitors and substrates. The substrates affected most are those with larger groups in the amino portion. This is to be expected. One role of the imidazole of His-57 is to protonate the displaced amino group of the peptide; the two groups must be in close proximity so that the amine interacts with the positive charge on the protonated His-57. Peptides with small leaving group amines, such as *N*-acetyltryptophanamide, interact less.

In general, ligand binding to α-chymotrypsin is expected to show K_s *vs.* pH plots with three plateau regions of decreasing K_s as pH increases from 4 to 8, as found for FPheFH. In addition, the conformational equilibria of α-chymotrypsin will be superimposed upon this.[11] Above pH 8, the ionization of Ile-16 prohibits binding of aromatic ligands.[11]

The pH 8.8 ionization involves a major conformational change in the molecule.[11] The His-57 represents presumably an unfavorable direct interaction with the substrate without a concomitant conformational change. The pH 5.3 ionization does not cause a slow isomerization change as noted for the pH 8.8 transition.[11]

pH Dependence of k_c. k_c is governed by a pK_a of 6.08 for FPheFH and 6.37 for FPheSC. These values are clearly different from the 6.7–6.8 observed in the K_m *vs.* pH plots. It is highly unlikely that 6.08 and 6.37 represent high perturbations of the pK_a of His-57 and 6.7–6.8 is the pK_a of the ionization of another residue.

The lowering of pK_a for k_c must represent a "kinetic ionization constant" due to a change of rate-deter-

(20) H. Gutfreund and J. M. Sturtevant, *Proc. Nat. Acad. Sci. U. S.*, **42**, 719 (1956).
(21) M. L. Bender and F. J. Kezdy, *J. Amer. Chem. Soc.*, **86**, 3704 (1964).
(22) For a summary see M. L. Bender, M. J. Ghibian, and D. J. Whelan, *Proc. Nat. Acad. Sci. U. S.*, **56**, 833 (1966).
(23) T. Inagami, A. Patchornik, and S. S. York, *J. Biochem. (Tokyo)*, **65**, 809 (1969).
(24) L. Parker and J. H. Wang, *J. Biol. Chem.*, **243**, 3729 (1968).
(25) (a) D. M. Glick, *Biochemistry*, **7**, 3391 (1968); L. M. Olm and D. M. Glick, *J. Biol. Chem.*, **245**, 814 (1970). (b) A referee has suggested that this ionization is related to the dimerization of the enzyme (D. J. Winzor and H. A. Scheraga, *J. Phys. Chem.*, **68**, 338 (1964)).
(26) W. McClure and G. M. Edelman, *Biochemistry*, **6**, 559 (1967).
(27) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, **46**, 337 (1969).

mining step in the reaction. Similar evidence has been quoted for the existence of tetrahedral intermediates in nonenzymatic acyl transfer reactions.[2,6]

In the enzymatic reaction the change of rate-determining step is unlikely to be due to a conformational change of the enzyme as the rates observed here are slower than observed with esters or some amide reactions such as the hydrolysis of *N*-acetyl-L-tyrosinehydroxamic acid.[28] The change in rate-determining step implies the existence of another intermediate on the reaction pathway between the ES complex and the acyl enzyme.

FPheMH, with a more basic leaving group, also exhibits a perturbed pK_a for k_c, and, although a detailed pH dependence for k_c and K_m was not obtained, a change in rate-determining step is probably indicated. FPheTSC, the least basic compound examined, is hydrolyzed with k_c being governed by an apparently unperturbed pK_a of about 7. The most simple explanation is that rate-determining breakdown of the intermediate is being observed over the entire pH region studied and that the change in rate-determining step is outside this region.

The data for the chymotryptic hydrolysis of the anilides[5,23,24] are poor but, in that case also, the pK_a values for k_c and K_m do not appear to coincide. The variation of the pK_a for k_c in that series must also represent a change in rate-determining step thus implying the existence of a further intermediate.

Identity of the Intermediate. Recently it has been suggested[29] that the chymotryptic hydrolysis of amides involves cyclization of the amide to form an oxazolinone in a step intermediate between the enzyme–substrate complex and the acyl enzyme. Oxazolinone formation involves the *N*-acyl carbonyl group of the peptide. However, the *N*-acyl group is not essential for catalytic activity. The protonated forms of L-tyrosinehydrazide and L-tyrosinehydroxamic acid have similar reactivities to the *N*-formyl or *N*-acetyl derivatives as calculated from the data of Niemann.[28,30–33] The unprotonated forms are less reactive and, as these compounds have pK_a values of 7.1 and 7.0, respectively,[31,32] at pH 7.8, the usual assaying pH, they appear to have low reactivity.[30] As for these compounds k_c and K_m were determined in a pH region where the substrate exists about equally in protonated and unprotonated forms, we prepared L-phenylalanine semicarbazide and followed the hydrolysis at a lower pH to obtain these parameters for the protonated compound. The k_c's for this and FPheSC are similar at pH 5.7.

The similar reactivity of the *N*-acyl and the deacylated amino acid hydrazides and hydroxamates shows that the *N*-acyl group is not essential to the catalytic action and oxazolinone formation is probably unimportant in the hydrolysis of peptides or amides. It still may be important in ester hydrolysis.

In nonenzymatic acyl transfer reactions the kinetically detected intermediate would automatically be assumed to be the tetrahedral addition compound by chemical intuition as no reasonable alternative is available. In the enzymatic reaction the situation is more complex and there is the possibility of additional intermediates. But, by analogy with the nonenzymatic reactions, a tetrahedral addition intermediate is chemically reasonable, and, until another intermediate is shown to exist, we shall assume that a tetrahedral intermediate is the kinetically important species observed on the chymotrypsin reaction pathway (see Figure 4).

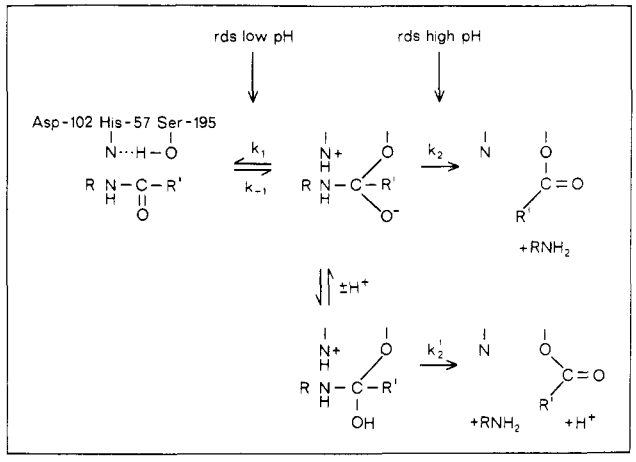


Figure 4. A mechanism for the acylation of α-chymotrypsin by amides consistent with the observed experimental kinetic evidence. The covalently bound tetrahedral intermediate does not accumulate but is present at low concentration. The imidazole of His-57 acts as a general base for the k_1 step and the resultant imidazolium ion acts as a general acid for the k_2 and k_{-1} steps.

Nature of the Rate-Determining Step. At high pH the rate-determining step must be the breakdown of the tetrahedral intermediate as the apparent pK_a for k_c is *lower* than that of the catalyzing His-57. A change in rate-determining formation of intermediate at high pH to breakdown at low pH would cause an apparent *increase* in the pK_a of His-57 as manifested in the pH dependence of k_c.

Consistent with this is the kinetic nitrogen isotope effect $(k_{14N})/(k_{15N})$ recently determined by O'Leary and Kluetz[34] for the hydrolysis of *N*-acetyltryptophanamide. This kinetic isotope effect is not in itself evidence for the rate-determining breakdown of a tetrahedral intermediate but is just indicative of a large degree of C–N bond cleavage in the transition state of the reaction.

Studies[6,35] on the partitioning of tetrahedral intermediates that occur in the aminolysis of esters and the hydrolysis of imidates show that at high pH there is preferential expulsion of alkoxide ion from an anionic intermediate, at a lower pH there is a transition to a neutral (or zwitterionic) intermediate in which the partitioning increases toward amine expulsion, and at still lower pH a positively charged intermediate tends to favor amine expulsion. The acylation of chymotrypsin also requires the expulsion of an amine from a tetrahedral intermediate. If the nonenzymatic studies are a good model for the enzymatic reaction, decreasing pH will favor the partitioning of the tetrahedral inter-

(28) A. N. Kurtz and C. Nieman, *Biochemistry*, **1**, 238 (1962).
(29) M-A. Colettil-Previero, C. Axelrud-Cavadore, and A. Previero, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **11**, 213 (1970).
(30) R. Lutwack, H. F. Mower, and C. Niemann, *J. Amer. Chem. Soc.*, **79**, 2179 (1957).
(31) R. J. Foster, R. R. Jennings, and C. Niemann, *ibid.*, **76**, 3142 (1954).
(32) A. N. Kurtz and C. Niemann, *ibid.*, **83**, 3309 (1961).
(33) R. Lutwack, H. F. Mower, and C. Niemann, *ibid.*, **79**, 5690 (1957).
(34) M. H. O'Leary and M. D. Kluetz, *ibid.*, **92**, 6089 (1970).
(35) G. L. Schmir, *ibid.*, **90**, 3478 (1968); W. P. Jencks and M. Gilchrist, *ibid.*, **90**, 2622 (1968); T. C. Pletcher, S. Koehler, and E. H. Cordes, *ibid.*, **90**, 7072 (1968).

mediate toward amine expulsion and this is manifested in the change of rate-determining step at low pH.

The rate-determining step changes from breakdown at high pH to formation of the tetrahedral intermediate at low pH. At high pH $k_2[ES'] < k_{-1}[ES']$ so that k_2 is rate determining, but at low pH $k_2'[HES'] > k_{-1}[ES']$ and k_1 is rate determining.

High pH

$$ES \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES' \xrightarrow[k_2]{\text{slow}}$$

Low pH

$$ES \underset{k_{-1}}{\overset{\text{slow}\ k_1}{\rightleftharpoons}} ES' \quad \begin{matrix} \downarrow \\ \text{fast} \\ \uparrow \end{matrix}$$

$$HES' \xrightarrow{k_2'}$$

Does a Tetrahedral Intermediate Accumulate? Increasing electron withdrawal in the aniline moiety of acetyltyrosine and acetyltryptophan anilides is accompanied by a decreasing K_m at pH ~8 for the chymotryptic hydrolysis.[4,23,24] *N*-Acetyl-L-tyrosine *p*-methoxyanilide and *p*-chloroanilide hydrolyses involve a K_m of 12 and 0.82 m*M*, respectively, at 25° and pH 8.[23] This variation has been interpreted as due to the build up of a tetrahedral intermediate in the reactions of the derivatives of the less basic anilides,[5] the enzyme–substrate complex for the *p*-methoxyanilide being mainly the noncovalently bound Michaelis complex while that of the *p*-chloroanilide is mainly a tetrahedral intermediate covalently bound to Ser-195 of chymotrypsin.[5]

The dissociation constants for the formyl-L-Phe derivatives tend to increase also with increasing amine pK_a (Table IV). We consider that there is no build up of a tetrahedral intermediate for the following reasons. First, the rate constant for the conversion of the Michaelis complex ES to the tetrahedral intermediate ES′ can be calculated and shown to be in the accessible time range but is not observed. For example, if at high pH the enzyme–substrate complex exists mainly as the tetrahedral intermediate than the observed k_c is the rate constant for its breakdown, k_2. k_1 may be calculated from the knowledge that this step is rate determining at low pH and the pK_a of the enzyme–substrate complex is known from the pH dependence of K_m. Also, as the intermediate accumulates, $k_1 \gg k_{-1} + k_2$. The rate constant for the conversion of ES to ES′ in the presteady state is easily shown to be $k_1 + k_{-1} + k_2$. For FPheFH this is calculated to be ~3 sec^{-1} and for FPheSC ~0.3 sec^{-1}. k_1 may also be estimated for a scheme involving a build up of a second intermediate from the observed k_c/K_m (which is equal to k_1/K_s[19]) by assuming a value for K_s; *e.g.*, if the K_m for FPheSC and FPheFH, which is ~2 m*M*, represents a K_s of ~20 m*M*, as found for *N*-formyl-L-phenylalanine ethylamide, combined with a subsequent equilibrium constant of ~10 for the formation of the second intermediate, then substitution of this K_s value in the observed values for k_c/K_m ($\equiv k_1/K_s$) gives values of k_1 in agreement with those from the previous calculation. The presteady-state studies using the proflavin displacement method show that there is *no* accumulation of an intermediate with a rate constant < 100 sec^{-1}.

Second, as the rate-determining step changes from rate-determining formation of the intermediate to rate-determining breakdown of the accumulated intermediate there should be a dramatic change in K_m (*i.e.*, a sigmoid region in the K_m *vs.* pH curve as discussed in the Theory section) but this is not observed; k_c and K_m do not exhibit a common pK_a in the pH profile.

This latter argument suggests also that there is no accumulation of a tetrahedral intermediate in the anilide hydrolysis.[5,23,24]

Structure–Reactivity Relationships. Plots of k_c and k_c/K_m against the pK_a of the hydrazine leaving group are not meaningful because of specific binding effects as previously demonstrated for the reaction of amines with furoylchymotrypsin.[36] Indeed, model building at our laboratory[37] suggests that there is a specific H-bonding site that may interact with the carbonyl oxygen of semicarbazide or formylhydrazide. Also, small changes in the nature of the leaving group amine can drastically alter the value of k_c; for example, Ac-Phe-Ala-NH_2 is turned over 20 times faster than Ac-Phe-Gly-NH_2[38] despite the almost identical basic strengths of Gly-NH_2 and Ala-NH_2.[38] For this reason the structure–reactivity relationships in acetyltyrosine[23,24] anilides have more meaning as substitution in the aniline ring is a less drastic change than is the variation thiosemicarbazide–formylhydrazine–semicarbazide–methylhydrazine of the present study. We have previously shown by comparison with nonenzymatic reactions[4] that the structure–reactivity relationship of increasing k_c with increasing aniline basic strength is consistent with rate-determining breakdown of a tetrahedral intermediate.

The variation of K_m with structure is not obviously clear. A possible explanation is that although the substrate is not covalently bound to the enzyme the reacting carbonyl group is in juxtaposition with the nucleophilic serine oxygen and electron withdrawal from the carbonyl group decreases nonbonded electron repulsion.

Pre-Transition-State Protonation Theory. We have previously shown that this theory[24,39] of the mechanism of the acylation step of chymotrypsin involving a preequilibrium transfer of a proton from Ser-195 of chymotrypsin to the nitrogen of the amide substrate is inadequate on the grounds of calculation.[4] For example, for FPheFH the pK_a for the protonation of the leaving group nitrogen calculated from our earlier equation[3] is ~ -17.[40] Substituting this value into the above theory[39] requires impossibly large rate constants, *e.g.*, a constant of $\sim 10^{30}$ sec^{-1} for the attack of seroxide ion on the N-protonated substrate and also $\sim 10^{30}$ sec^{-1} for the proton transfer from the N-protonated substrate to that seroxide ion in the reverse reaction. Even allowing a pK_a of 7[41] for the serine as part of an H-bonded "charge relay" system, the required specific rate constants are impossibly high. Williams[42] has

(36) P. W. Inward and W. P. Jencks, *J. Biol. Chem.*, **240**, 1986 (1965).
(37) C. Schubert Wright, personal communication.
(38) W. K. Baumann, S. A. Bizzozero, and H. Dutler, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **8**, 257 (1970).
(39) J. H. Wang and L. Parker, *Proc. Nat. Acad. Sci. U. S.*, **58**, 2451 (1967).
(40) Utilizing eq 45 of ref 3 and allowing 1.4 units for the additional electron withdrawal in *N*-formylphenylalanine (pK_a 3.3) above that in acetic acid (pK_a 4.7).
(41) D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature (London)*, **221**, 337 (1969).
(42) A. Williams, *Biochemistry*, **9**, 3383 (1970).

recently queried this mechanism on similar, but qualitative, grounds.

Summary

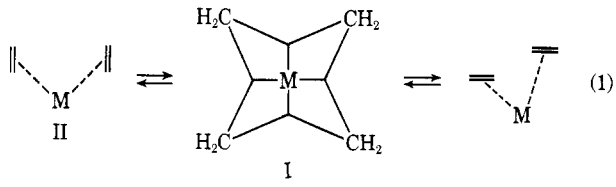
(1) A change of rate-determining step with pH occurs in the acylation of chymotrypsin by some specific hydrazide substrates and also by some anilide substrates. (2) This implies the existence of an additional intermediate between the Michaelis complex and the acyl-chymotrypsin. (3) This intermediate does not appear to be an oxazolinone. (4) A possible interpretation consistent with experimental observations, model studies, and structure–reactivity considerations is that a tetrahedral intermediate is formed in a low steady-state concentration; at high pH its breakdown is rate determining and at low pH its formation is the rate-determining step. (5) The binding of ligands to α-chymotrypsin is controlled by three ionizing groups on the enzyme of pK_a values ~5.3, 7.0, and 8.8. (6) The pre-transition-state protonation theory is inadequate in its description of these reactions.

Communications to the Editor

On the Mechanism of the Metal-Catalyzed Disproportionation of Olefins

Sir:

The disproportionation reaction of olefins, effected by various transition metal catalysts, is one of the unique types of transformations discovered in recent times and considerable interest lies in the mechanism of the process, particularly the role played by the metal atom. We have recently proposed that the reaction proceeds *via* reversible transformation of two coordinated olefinic bonds into a multi-three-centered species I in which four CH_2 units of sp^3-hybridized carbon atoms are involved (eq 1).[1] For convenience the species I is referred to as the tetramethylene complex and is to be equated with the terms "quasicyclobutane"[2] and "pseudocyclobutane"[3] used during the early studies of this reaction. Two virtues of this mechanistic scheme are that it avoids invoking a cyclobutane ring structure as an intermediate (cyclobutanes are not products of the reaction, and olefins are not converted into cyclobutanes under the disproportionation reaction conditions).[4] In addition, the scheme offers an explanation of the role played by the metal atom in removing the "forbiddenness," according to the Woodward–Hoffmann rules of orbital symmetry, associated with the overall reaction.[5]



(1)

In each of the systems discovered so far,[6] in no case has the active catalyst species been identified; they all involve metal systems of unknown character deposited on metal oxide surfaces, or substances derived from metal complexes together with Lewis acid or base co-catalysts. We have discovered that $W(CO)_6$, $Mo(CO)_6$, and arene–$W(CO)_3$ complexes can act as homogeneous catalyst precursors for the disproportionation of olefins, and although they are not notably effective as catalysts, their simplicity has allowed for a more detailed mechanistic investigation. With these catalysts we now report data consistent with the proposed mechanism given by eq 1.

An interesting feature of the proposed mechanism is that in proceeding from the bisolefin metal system II to the tetramethylene complex I the number of donor electrons provided by the organic ligands increases from 4 to 8. It is widely recognized that the most thermodynamically stable complexes of metals in zero or low oxidation states are those in which the effective atomic number rule (EAN), or inert gas rule, is obeyed.[7] Complexes in which the number of electrons about the metal falls short of the next inert gas are known, but species in which the number of donor electrons causes the next inert gas structure to be exceeded do not appear to exist. They are certainly high-energy species and are unlikely to be species involved in any catalytic reaction. It is significant that in none of the reactions catalyzed by transition metals in low-oxidation states are species proposed in which the number of electrons about the metal exceeds that of the next inert gas (the hydroformylation of olefins catalyzed by $HCo(CO)_4$[8] and the cyclooligomerization of butadiene catalyzed by Ni complexes[9] are good examples).

If the scheme depicted in eq 1 is correct and the transformation II → I involves an increase in donor electrons from 4 to 8 then only two other CO ligands can be present on the W atom in the tetramethylene complex I if the next inert gas configuration is not to be exceeded. That is, in addition to toluene, one molecule of CO must be removed from the tungsten atom in toluene–$W(CO)_3$ in order to generate the true catalytic species. A more detailed mechanistic scheme can

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