Chymotrypsin Catalysis. Evidence for a New Intermediate, III[1]

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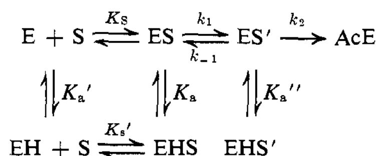
Abstract: V_{max}, K_m, and V_{max}/K_m for the chymotrypsin-catalyzed hydrolysis of *N*-formyl-L-phenylalanine formylhydrazide in the neutral pH range are dependent upon ionizations with p*K* values of 6.0, 6.1, and 6.7, respectively. The equivalence of the p*K* values influencing V_{max} and K_m is required by the previously proposed mechanism in which a tetrahedral intermediate is formed and accumulates in a preequilibrium reaction prior to acyl-enzyme formation. These results are not in agreement with those reported previously.

We have previously found that in reactions of chymotrypsin with anilide substrates the K_m and enzymic p*K* affecting activity (V_{max}) are influenced by para substituents in the aniline moiety.[2] These results were accounted for in terms of Scheme I.

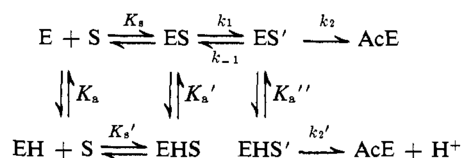
Scheme I



ES is the Michaelis complex, AcE is the acyl-enzyme, ES′ is a tetrahedral intermediate formed by attack of Ser-195 on the substrate, and the site of proton addition in EH, EHS, and EHS′ is on His-57. The new element in this reaction scheme is the interposition of a tetrahedral intermediate, formed in a preequilibrium reaction (*i.e.*, $k_{-1} > k_2$), between the Michaelis complex and acyl-enzyme. In the case of anilide substrates with a strongly electron withdrawing group, such as the *p*-chloro function, it is proposed that the tetrahedral intermediate accumulates ($k_1/k_{-1} \geqslant 1$). The observed pH dependence of substrate structure effects on the apparent binding (K_m) and V_{max}[2] further requires that the p*K* of the active site histidine residue (His-57) is lower in the tetrahedral intermediate than in either the free enzyme or Michaelis complex ($K_a'' > K_a$ and K_a'). The increased acidity of His-57 in the tetrahedral intermediate makes for a lower apparent p*K* for activity (V_{max}) and for a pH dependence for binding. Modulation of the influence of the low value of pK_a'' is effected by substrate structural effects on the equilibrium constant for tetrahedral intermediate formation (k_1/k_{-1}). However, for substrates where the k_1/k_{-1} equilibrium is favorable, the dissociation constant affecting V_{max} and K_m will be *identical*.[3]

An alternate mechanism has been offered (Scheme II).[4] The components are defined as in Scheme I.

Scheme II



Again the mechanism includes a tetrahedral intermediate. However, in this case the intermediate is formed with catalysis by a neutral His-57, while breakdown of the tetrahedral intermediate proceeds with catalysis by the neutral His-57 *and* the corresponding conjugate acid. The apparent p*K* for activity (V_{max}) is presumed to be influenced by a change in rate-determining step, from rate-determining tetrahedral intermediate formation at low pH (where His-57 is largely protonated, and the k_2'(EHS′) step in Scheme II is rapid compared to the k_{-1}(ES′) reaction), to breakdown at high pH (where His-57 is deprotonated, and the k_2'(EHS′) and k_2(ES′) reactions are slow compared to the k_{-1}(ES′) reaction). The apparent p*K* is, therefore, a "kinetic constant" which varies with changes in the substrate's leaving group because of the leaving group's influence on the tendency for the change in rate-determining step to occur. It is also required that there be a substrate specific p*K* perturbation in His-57 of the Michaelis complex (K_a') to account for substrate structural effects on the pH dependence of K_m. The origin of this perturbation is unknown.

The two schemes may be distinguished in certain cases. As indicated above, Scheme I requires that the pH dependence of K_m and V_{max} be affected by an identical proton dissociation constant in the case of substrates in which a tetrahedral intermediate is presumed to accumulate. Scheme II permits different values or identity of the p*K*'s influencing V_{max} and K_m. We report here results obtained in a study of *N*-formylphenylalanine formylhydrazide hydrolysis by chymotrypsin. Hydrolysis of this substrate is associated with a very low apparent p*K* affecting V_{max} and a severe pH dependence for K_m. The apparent p*K*'s influencing V_{max} and K_m in the neutral pH range are indistinguishable. These results differ from those previously reported.[4] They are consistent with both Schemes I and II.

Experimental Section

Materials. Three times crystallized α-chymotrypsin, lyophilized and salt free, was Lot No. CDI OLC from Worthington. *N*-Formyl-L-phenylalanine formylhydrazide was prepared as described by Fersht and Requena;[4] mp 192.5–194° (lit.[4] 191–193°). *Anal.*

(1) This investigation was supported by a Public Health Service Research Career Development Award (1-K4-GM-10,010-01) from the National Institute for General Medical Sciences and by a grant (DE-03246) from the National Institutes of Health.

(2) (a) M. Caplow, *J. Amer. Chem. Soc.*, **91**, 3639 (1969); (b) E. C. Lucas and M. Caplow, *ibid.*, **94**, 960 (1972).

(3) In the earlier studies of anilide hydrolysis,[2] the p*K*'s for V_{max} and K_m could not be precisely determined. However, the results were in general agreement with this requirement.

(4) A. R. Fersht and Y. Requena, *J. Amer. Chem. Soc.*, **93**, 7079 (1971).

Table I. Chymotrypsin Hydrolysis of *N*-Formyl-L-phenylalanine Formylhydrazide at 25°[a]

pH	K_m, mM[b]	K_m, mM[b,c]	K_m lit.[c,d]	V_{max}, sec^{-1} [b]	V_{max}/K_m, M^{-1} sec^{-1} [b]	Enzyme concn, μM
7.30	3.20 ± 0.12	2.60 ± 0.11		0.329 ± 0.005	127 ± 7	1.8
7.00	3.94 ± 0.08	3.28 ± 0.06		0.319 ± 0.002	97.2 ± 2	2
			2.77			
7.00	4.14 ± 0.10	3.44 ± 0.09		0.319 ± 0.002	92.4 ± 3	1.3
6.75	4.56 ± 0.23	3.78 ± 0.19	3.75	0.288 ± 0.005	76.3 ± 5	2.2
6.50	5.41 ± 0.28	4.47 ± 0.24		0.267 ± 0.004	59.7 ± 4	2.5
			5.3			
6.50	5.15 ± 0.16	4.37 ± 0.14		0.262 ± 0.001	60.1 ± 2	2.5
6.25	6.59 ± 0.49	5.47 ± 0.39		0.229 ± 0.006	41.8 ± 4	6
6.00	7.78 ± 0.18	6.38 ± 0.14		0.159 ± 0.002	24.9 ± 0.9	4
			6.03			
6.00	7.78 ± 0.09	6.38 ± 0.08		0.157 ± 0.001	24.6 ± 0.5	6
5.75	8.76 ± 0.29	7.00 ± 0.23		0.120 ± 0.001	17.1 ± 0.6	6
5.50	10.22 ± 0.29	7.96 ± 0.23		0.0845 ± 0.0010	10.6 ± 0.4	8
			6.67			
5.50	10.85 ± 0.24	8.46 ± 0.19		0.0833 ± 0.0008	9.80 ± 0.3	8
5.25	11.96 ± 0.53	9.10 ± 0.45	9.05	0.0536 ± 0.0013	5.89 ± 0.5	18
5.00	15.1 ± 0.75	11.17 ± 0.55	9.37	0.0348 ± 0.0008	3.12 ± 0.2	18
4.75	16.7 ± 0.75	12.00 ± 0.54	17.4	0.0207 ± 0.0004	1.72 ± 0.1	27
4.50	20.8 ± 1.2	14.18 ± 0.9	15.2	0.0111 ± 0.0003	0.783 ± 0.07	39

[a] The substrate concentration range was 2–20 mM, except for the reactions at pH's 7.3, 7.0, 4.75, and 4.50 where this range was 1–10, 1–20, 5–40, and 5–40 mM, respectively. The ionic strength was 0.10. Reactions were followed 1–5% to completion. [b] The reported values are followed by the computed standard error. [c] Corrected for the fraction of the enzyme present in the active form: A. R. Fersht and Y. Requena, *J. Mol. Biol.*, **60**, 279 (1971). [d] Results from ref 4.

Calcd: C, 56.16; H, 5.57; N, 17.86. Found: C, 55.93; H, 5.70; N, 17.63. *p*-Nitrophenyl acetate was prepared by a modification of Chattaway's procedure,[5] mp 78–79° (lit.[5,6] 81–82°, 79.5–80°).

Kinetic Studies. Hydrolysis of the hydrazide was followed at 25° and ionic strength 0.1 (maintained by KCl) by means of a pH-stat apparatus, which consisted of a Radiometer TTT1c titrator, an SBR2c Titrigraph recorder, an SBU1a motorized syringe buret, and a Radiometer GK2351B combination electrode. The reaction vessel was thermostated by means of a water jacket and could be sealed to enable reactions at pH's above 6.5 to be carried out under argon. The blank rate due to enzyme autolysis was measured and found to be negligible compared with the rate of substrate hydrolysis. The results at low pH were corrected for the protonation of the *N*-formyl-L-phenylalanine produced, using the published pK_a value.[4]

Enzyme solutions were prepared daily in 10^{-3} M HCl and stored on ice. The reaction was initiated by addition of 0.1 ml of enzyme solution to the reaction solution (usually 15 ml). Hydrolysis was followed by the addition of 9 mM or 4.5 mM sodium hydroxide solution. Good linear initial rates were obtained. The V_{max} for enzyme-catalyzed hydrolysis of the hydrazide at pH 7.00 and 25° was determined using an enzyme solution which had been assayed by titration with *p*-nitrophenyl acetate at pH 7.9 and 25°. For convenience the hydrolysis of the hydrazide at pH 7.00 was then used as the routine assay of the concentration of enzyme solutions. Six to eight substrate concentrations were used for each determination of V_{max} and K_m, and the results were processed by a computer program which fitted the data directly to the Michaelis–Menten equation.[7a] Analysis of the pH *vs.* K_m curve was done by a logistic plot[7b] of the data obtained at alkaline pH's, where the lower pK has no influence.

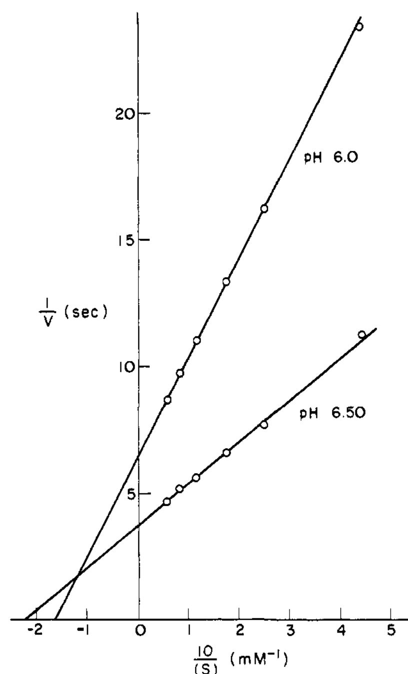


Figure 1. Chymotrypsin hydrolysis of formylphenylalanine formylhydrazide at pH 6.0 and 6.5; reciprocal plot.

Results

Results obtained in a typical series of rate determinations are illustrated in Figure 1 and the calculated kinetic constants are summarized in Table I. Values of K_m and V_{max}/K_m are corrected for the active–inactive enzyme equilibrium described by Fersht and Requena.[8] In general, the standard errors are small, and duplicate determinations show good reproducibility. The agreement with the previously published data[4] is good. The pH dependences of V_{max}, K_m, and V_{max}/K_m are graphically illustrated in Figures 2–4. V_{max} depends upon the ionization of a group with a pK of 6.0, and is equal to 0.35 sec^{-1} for the fully ionized enzyme; the literature value is 0.366 sec^{-1}.[4] V_{max}/K_m (Figure 4) depends upon an ionizable group with a pK of 6.65 with a limiting rate equal to 147 M^{-1} sec^{-1}; the literature value is 225 M^{-1} sec^{-1}.[4] Substrate ionization prevents rate determinations over the full pH range where activity varies and there is, therefore, probably more uncertainty in these kinetics parameters than those for V_{max}.

K_m is influenced by two ionizations, as outlined in

(5) F. D. Chattaway, *J. Chem. Soc.*, 2495 (1931).
(6) F. J. Kezdy and M. L. Bender, *Biochemistry*, **1**, 1097 (1962).
(7) (a) K. R. Hanson, R. Ling, and E. Havir, *Biochem. Biophys. Res. Commun.*, **29**, 194 (1967); (b) W. M. Clark, "Oxidation-Reduction Potentials of Organic Systems," Williams and Wilkins Co., Baltimore, Md., 1960.
(8) A. R. Fersht and Y. Requena, *J. Mol. Biol.*, **60**, 279 (1971).

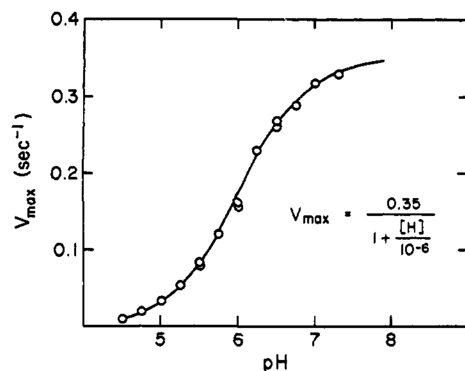


Figure 2. pH dependence of V_{max} for the chymotrypsin-catalyzed hydrolysis of formylphenylalanine formylhydrazide. The solid line was drawn to fit the equation given.

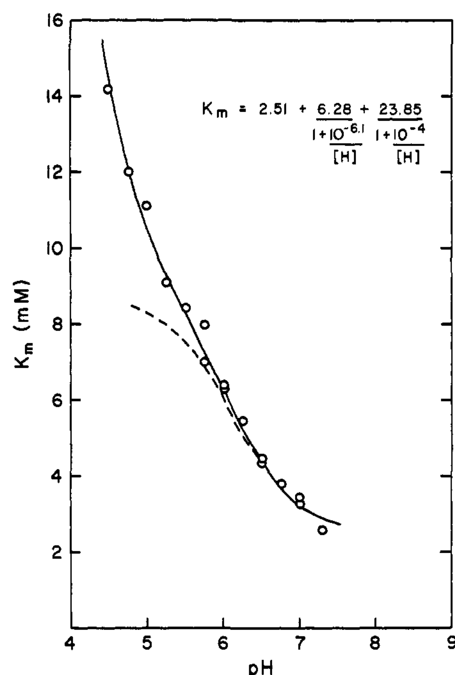
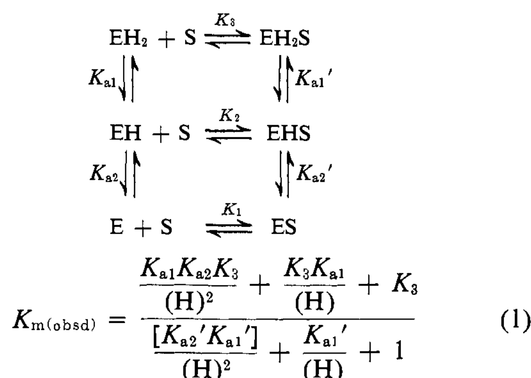


Figure 3. pH dependence of K_m for formylphenylalanine formylhydrazide. The solid line was drawn to fit the equation given and the dotted line shows the influence of K_{a2}' (Scheme III) only. The lower pK only significantly influences K_m at pH's below 5.

Scheme III and described by eq 1. The pK's, equal to

Scheme III



$$K_{m(obsd)} = \frac{\frac{K_{a1}K_{a2}K_3}{(H)^2} + \frac{K_3K_{a1}}{(H)} + K_3}{\frac{[K_{a2}'K_{a1}']}{(H)^2} + \frac{K_{a1}'}{(H)} + 1} \qquad (1)$$

pK_{a1}' and pK_{a2}' in Scheme III, are 4.0 and 6.10, respectively. Since only a limited portion of the low pH range was accessible (the K_m becomes so high at low

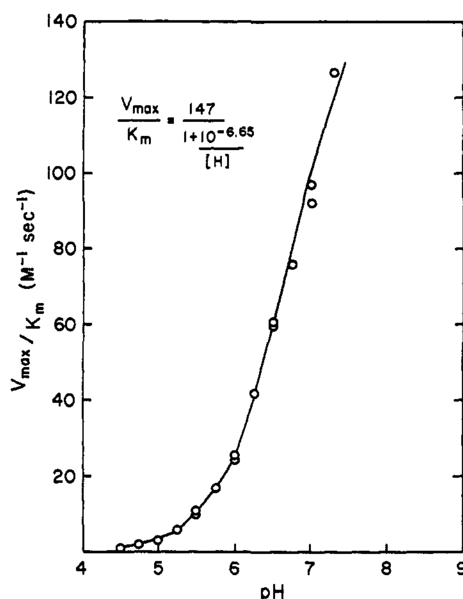


Figure 4. Dependence of V_{max}/K_m on pH for the chymotrypsin-catalyzed hydrolysis of formylphenylalanine formylhydrazide.

pH that saturation of the enzyme is impossible) the uncertainty in pK_{a1}' and K_3 is quite large; only the ratio K_3/K_{a1}' can be precisely determined at pH's distant from the pK. However, in the neutral pH range a major change in the estimate for K_{a1}' with an equivalent change in K_3 will not affect the fit of the available data to the curve given. For example, in the pH range 7.5–4.5 the theoretical curve shown in Figure 2 is virtually indistinguishable using a value of pK_{a1}' equal to 3.0, if a tenfold larger value for K_3 is assumed. On the other hand, there is relatively little uncertainty in pK_{a2}', K_2, and K_1. As shown by the dotted line in Figure 2 at pH's above 5, the effect of the pK_{a2}' ionization is only slightly overlapped by that produced by the lower pK. The pH dependence of K_m fits extremely well to the assigned pK; a change in pK_{a2}' by 0.1 unit gave an appreciably poorer fit of the data to the theoretical curve. The curve in Figure 2 shows little indication of leveling in the pH 5 region because of the very substantial effect of the low pK on substrate binding. The values of K_1, K_2, and K_3 in Scheme III are 2.51, 8.79, and 32.64 mM, respectively. As required by Scheme III the ratio $K_{a2}'/K_{a2} = K_2/K_1$, with K_{a2} obtained from the pH dependence of V_{max}/K_m. The pK of 4.0 is probably not related to the protein conformational change associated with breaking the Ileu-16–Asp-194 salt bond[9] since this ionization was accounted for in the determination of the fraction of the enzyme present in the active form at different pH's[8] (compare columns 2 and 3 in Table I). Some other ionization, perhaps that of Asp-102, plays a role here. The pK of 6.10 is attributed to His-57.

Discussion

For Scheme I[2] the pK influencing V_{max} *and* K_m is identically equal to $K_aK_a''[1 + (k_1/k_{-1})]/[K_a'' + (k_1/k_{-1})K_a]$. For Scheme II there are two pK's affecting both V_{max} and K_m, which are equal to $[k_1K_a' + k_{-1}K_a'' + k_2'K_a' + k_2K_a'']/k_2'$ and $(k_1 + k_{-1} + k_2)K_a''K_a'/[k_1K_a' + k_{-1}K_a'' + k_2'K_a' + k_2K_a'']$. These are equal

(9) G. P. Hess, *Enzymes, 3rd Ed.*, 3, 213 (1971), and references therein.

to $(k_{-1}/k_2')K_a''$ and K_a', respectively, if tetrahedral intermediate formation is unfavorable ($k_{-1} > k_1$), and if $k_{-1} > k_2$ and k_2'. The observation of only a single pK influencing V_{max} does not controvert Scheme II since only a relatively narrow pH range was studied. Also, the two pK's influencing V_{max} and K_m need not both be visible, depending upon the relative size of the various rate and equilibrium constants. We find here equivalent pK's in the neutral pH range, for V_{max} (6.0) and K_m (6.1). This result does not permit a distinction between Schemes I and II.

The results obtained here do not agree with those reported previously.[4] In earlier work a pK of 6.08 was calculated for V_{max}, and pK's of 6.7 and 5 were presumed to influence K_m. However, the earlier published data do not fit a theoretical curve calculated from these pK's. They fit moderately well to the pK assignments given here.

The pK's reported here for K_m are in serious conflict with results obtained in a study of proton release with chymotrypsin binding to formylphenylalanine formylhydrazide.[10] If the pK of the enzyme–substrate complex is equal to 6.08[4] and the free enzyme has a pK of 7.0[4] (our V_{max}/K_m results suggest a pK of 6.65 for the free enzyme; this value would result in a lower theoretical yield of protons) there should be a release of protons on substrate binding. The release will be extremely fast if substrate complexation to the enzyme involves no covalent bond formation and relatively slow if the noncovalent complexation step is followed by the equilibrium formation of a tetrahedral intermediate in which the pK of His-57 is low. However, no significant release of protons was found on substrate binding. We are unable to account for this observation, which is in conflict with the pH–K_m results described here and previously.[4]

The numerous differences in kinetic behavior between anilides[2] and the formylhydrazide, and related compounds such as the semicarbazide and thiosemicarbazide,[4] make us reluctant to apply the mechanistic conclusions derived in our studies of anilides to the latter class of compounds. For example: (A) K_m values for anilides are quite well correlated with amine basicity[2] while no such correlation was found with the hydrazide derivatives;[4] (B) V_{max} with anilides is dependent upon the basicity of the aniline leaving group[2] while no clear dependence upon basicity is seen with the hydrazide compounds;[4] (C) the apparent pK influencing V_{max} with anilides is made lower with decreasing basicity in the aniline leaving group,[2] while an opposite effect was reported for the hydrazide derivatives.[4] These discrepancies strongly suggest that there are important differences between the anilides and hydrazide derivatives. All that can be said is that the results obtained with formylphenylalanine formylhydrazide are not inconsistent with the previously proposed mechanism[2] involving the accumulation of the tetrahedral intermediate (Scheme I).

Scheme II is similar in part to a possible mechanism we previously discussed[2a] in which it was assumed that the tetrahedral intermediate does not accumulate and the substrate-dependent pH effects result from a substrate specific pK perturbation in the Michaelis complex. We are unable to envision a mechanism for such a perturbation. Fersht and Requena[4] have suggested that there is a His-57 pK-perturbing interaction of the amine portion of amide and anilide substrates which is highly sensitive to the amine's structure. This interaction is required to have a rather strange specificity, it being important with a formylhydrazine leaving group and insignificant with thiosemicarbazide, ammonia, and methylhydrazine leaving groups.[4] However, there appears to be no evidence for a specific interaction of amines in the aminolysis of furoyl-chymotrypsin[11] and acetyltyrosyl-chymotrypsin.[12] This reaction is the exact reverse of the reaction in which an amide acylates the enzyme. Of course the proposed interaction with the amine leaving group could be present in the amide Michaelis complex only, and not in the ground state and transition state for acyl-enzyme aminolysis. The observation that there is no deuterium isotope effect on the K_m for anilides[13] suggests that hydrogen bonding between the anilide and His-57 is not responsible for the substrate induced perturbation of the His-57 pK.

(10) A. R. Fersht, *J. Amer. Chem. Soc.*, **94**, 293 (1972).

(11) P. W. Inward and W. P. Jencks, *J. Biol. Chem.*, **240**, 1986 (1965).

(12) B. Zeeberg, unpublished data.

(13) L. Parker and J. H. Wang, *J. Biol. Chem.*, **243**, 3729 (1968).