THE ALKALINE PH DEPENDENCE OF CHYMOTRYPSIN REACTIONS: POSTULATION OF A PH-DEPENDENT INTRAMOLECULAR COMPETITIVE INHIBITION*

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For chymotrypsin reactions which follow equation (1), the pH dependence k_2/K_s (or the equivalent k_{cat}/K_m) is a bell-shaped curve with pK's of 6.8 ± 0.3 and 8.9 ± 0.3, while the pH dependence of k_3 is a sigmoid pH-rate constant profile whose pH is 7.0 ± 0.3.[1]

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

The two groups observed in k_2/K_s may both belong to k_2 (acylation) or to K_s (binding), or one may belong to k_2 and the other to K_s. Several studies[2] indicate that the pH of 6.8 is part of the k_2 step. This finding is consistent with the presence of pK 7.0 in the k_3 step, since one would expect that the same catalytic group should be operative in both steps, if k_2 and k_3 are the microscopic reverse of one another. Both these pK's can be assigned to the imidazole group of histidine 57 which has been demonstrated by a stoichiometric inhibition reaction to be necessary for enzymatic activity.[3] The ionization of pK 8.9 cannot as easily be assigned, however. Since it does not appear in the k_3 step,[1] it is presumably not a catalytic group. In an earlier paper this ionization was assigned to the k_2 step,[1] and was postulated to control a conformational change of the enzyme in that step.[6] Recently, we have found that the ionization of pK 8.9 occurs in the pH dependence of the reaction of L-1-chloro-3-tosylamido-4-phenylbutanone with chymotrypsin,[4] a reaction which results in the simple alkylation of histidine 57 of the enzyme.[3] Since the nonenzymatic reaction of this reagent with imidazole cannot account for a pH dependence of 8.9, this finding implies that this pH dependence may be involved in the binding step of chymotrypsin and casts doubt on previous assignment of this pH dependence to the k_2 step. Because of these doubts and because the function of the group of pK 8.9 has not been completely elucidated, we have carried out further experiments on the pH dependence of chymotrypsin-catalyzed reactions and experiments designed to probe the action of the group of pK 8.9

The kinetics of the acylation of α-chymotrypsin by p-nitrophenyl acetate were repeated and again show (Table 1) a binding constant (K_s) which is independent of pH and a rate constant, k_2, which shows a bell-shaped pH-rate constant profile with pK's of 6.8 and ~9, as stated previously.[1]

However, the kinetics of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophanamide show a K_m dependent on an acidic group of pK ~9 and a k_{cat} dependent only on a basic group of the pK ~ 6.8, and independent of pH up to pH 9.4.[5] These data are shown in Table 2. Good agreement between the pH dependence of K_m and the K_i of N-acetyl-L-tryptophanamide [as determined

TABLE 1

ACYLATION OF CHYMOTRYPSIN BY p-NITROPHENYL ACETATE*

pH	Buffer	I (M)	k_2† (sec^{-1})	K_s (mM)	$k_2/K_s \times 10^{-3}$ (M^{-1} sec^{-1})
7.53	Tris-HCl	0.12	3.0	1.15	2.62
7.62	Phosphate	0.15	3.84	1.17	3.29
8.81	Tris-HCl	0.06	1.78	1.02	1.75
9.20	Borate	0.06	1.52	1.02	1.48
9.58	Carbonate	0.10	1.0	0.73	1.36

* Acetonitrile-water; 1.6% (v/v) 25.0 ± 0.2°C. The reactions were followed at 400 mμ using a Beckman spectrophotometer equipped with a stopped-flow mixing device.
† The error in these values is approximately 20%.

TABLE 2

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF N-ACETYL-L-TRYPTOPHANAMIDE*

pH	k_{cat}/K_m (M^{-1} sec^{-1})	$k_{cat} \times 10^2$ (sec^{-1})	K_m (mM)
6.25	[2.52]	[4.30]	[5.17]
7.35	10.5 ± 1.0	4.68 ± 0.50	4.47 ± 0.50
7.96	9.02 ± 0.20	5.67 ± 0.23	6.3 ± 0.3
8.87	6.93 ± 0.20	5.54 ± 0.27	8.2 ± 0.4
9.20	2.01 ± 0.10	5.38 ± 0.60	26.8 ± 5.0
9.43	1.78 ± 0.03	6.7 ± 1.0	38 ± 5

* 25.0 ± 0.2°C. Aqueous solution, 0.25 M KCl, 0.2 M phosphate, Tris or carbonate buffer, initial rates, $S_0 = 2.5 \times 10^{-2}$ to 1×10^{-3} M; $E_0 = 1 \times 10^{-4}$ M.

TABLE 3

HIGH pH DEPENDENCE OF SOME α-CHYMOTRYPSIN-CATALYZED HYDROLYSES*

pH	k_{cat} (sec^{-1})	$K_m \times 10^5$ (M)	$k_{cat}/K_m \times 10^{-5}$ (M^{-1} sec^{-1})
	Methyl N-Acetyl-L-tryptophanate		
10.24	64 ± 6	69 ± 8	0.93 ± 0.06
10.88	57 ± 4	206 ± 15	0.28 ± 0.01
10.93	53 ± 2	178 ± 10	0.29 ± 0.01
11.52	>50	≥620	0.082 ± 0.002
11.52	>33	≥500	0.066 ± 0.011
	Ethyl N-Acetyl-L-tryptophanate		
10.14	42 ± 4	70 ± 6	0.606 ± 0.03
10.80	45 ± 3	194 ± 13	0.230 ± 0.01
11.22	47 ± 10	468 ± 22	0.101 ± 0.01
11.36	>40	>640	0.090 ± 0.01
11.64	>25	>600	0.040 ± 0.01
11.65	>40	>800	0.047 ± 0.04

* Acetonitrile-water, 1.6% (v/v), carbonate or phosphate buffers, $S_0 = 2.46 \times 10^{-3}$ M, $E_0 = 1$–8×10^{-6} M.

TABLE 4

pH DEPENDENCE OF SOME INHIBITION CONSTANTS OF CHYMOTRYPSIN*

N-Acetyl-L-tryptophanamide†		Indole‡		Benzamide§	
pH	K_i (mM)	pH	K_i (mM)	pH	K_i (mM)
8.00	6.7 ± 0.6	7.50	0.86 ± 0.10	7.5	7.3 ± 0.9
8.45	5.6 ± 0.7	8.54	0.72 ± 0.10	8.8	15 ± 3
8.61	8.8 ± 0.5	9.50	4.9 ± 1.0	9.45	26 ± 5
9.64	>17	10.04	>8.3	9.93	39 ± 5
9.65	~62	11.14	>10	10.54	57 ± 9
10.16	>61				

* Versus methyl N-acetyl-L-tryptophanate; 3.8×10^{-4} M, $E_0 = 2.4 \times 10^{-7}$ M; 25°. All compounds showed strict competitive inhibition. Acetonitrile-water, 1.6% (v/v), $I = 1 \times 10^{-2}$ M.
† [N-Acetyl-L-tryptophanamide] = 1×10^{-2} M.
‡ [Indole] = 3×10^{-3} M.
§ [Benzamide] = 45.3×10^{-3} M.

versus methyl N-acetyl-L-tryptophanate (Table 4)] was found. For this reaction, $K_m = K_s$, and $k_{cat} \equiv k_2$.[6]

For a methyl or ethyl ester, $k_{cat} = k_2k_3/(k_2 + k_3)$[6] and thus the pH dependence of k_{cat} is a function of both k_2 and k_3. Table 3 indicates that the α-chymotrypsin-catalyzed hydrolysis of methyl and ethyl N-acetyl-L-tryptophanate possesses a k_{cat} which is independent of pH from 8 to 11.6, although the value of k_{cat}/K_m decreases markedly above pH 8.[7] These data are consistent with the pH-independent k_2 reaction at high pH although our lack of knowledge of the k_2/k_3 ratio and its pH dependency prevents any definitive statement.

The pH dependence of the inhibition of the methyl N-acetyl-L-tryptophanate hydrolysis by benzamide and indole have also been determined, as shown in Table 4. As with the tryptophan derivative, the binding constants increase with pH above pH 9.

The pH dependence of the K_i of N-acetyl-L-tryptophanamide agrees with that determined independently by R. Lumry,[8] and the K_m values agree qualitatively but not quantitatively with those determined by Himoe and Hess.[9]

The results reported above indicate that for specific substrates such as N-acetyl-L-tryptophan derivatives, the binding, K_s, is dependent on a group of pK_a 8.9 while k_2 and k_3, the acylation and deacylation steps, are dependent on a basic group of pK 6.8. This revised pH dependence eliminates one point previously considered to be a contradiction: the pH dependence of the acylation and deacylation steps of specific substrates are now identical to one another in agreement with the prediction from symmetry considerations.[4]

Mechanistic Hypothesis for the pK 8.9 Dependency.—Although the binding of many enzyme-substrate combinations containing ionizable substrates is pH dependent, viz., the fumarase-furmarate system, the binding of a neutral substrate to an enzyme would be expected to be pH independent. It is therefore of interest to delve into the meaning of the pH-dependent binding of chymotrypsin.

The pH dependence of binding requires that the native enzyme exists in two forms, controlled by an (apparent) ionization of the enzyme, only one of which forms will bind the substrate. The catalytic mechanism on both sides of the ionization must be the same since k_{cat} (V_{max}) is independent of the ionization.

A rapid equilibrium must exist between the two forms of the enzyme since (1) the enzyme can be transformed from pH 5 to 10 to 5 with complete reversibility as determined kinetically; (2) the concentration of enzyme active sites as determined by titration with either N-*trans*-cinnamoylimidazole or with p-nitrophenyl acetate is the same at pH 9.5 and 7; (3) Michaelis-Menten kinetics are observed at all pH's; and (4) V_{max} values are independent of the pK 8.9 ionization. These four observations imply that a substrate rapidly drains all of the nonbinding form of the enzyme into the form that does bind. Thus, disregarding at the moment the pK_a 6.8 dependence of the catalytic rate steps, the pH dependence of the enzymatic reaction can be written as:

$$\begin{array}{ccccccc} E^*H^+ & \overset{K_{TH}}{\rightleftharpoons} & EH^+ & & & & \\ K^*_H \Big\uparrow\downarrow K_T & & \Big\uparrow\downarrow K_H & \overset{S}{\underset{K_S}{\rightarrow}} & E(H^+)S & \xrightarrow{k_{cat}} & E + P, \\ E^* & \rightleftharpoons & E & & & & \end{array} \qquad (2)$$

where E and E^* are the binding and nonbinding forms of the enzyme, $K_T = E^*/E$, and $K_{TH} = \frac{E^*H^+}{EH^+}$.

The existence of two forms of chymotrypsin, only one of which will bind substrate, implies that a change of the enzyme occurs, a suggestion that has been made before.[10, 11] It is of interest to probe the nature of this change.

The change cannot be an aggregation process since large variations in enzyme concentration produce no effect on the pK 8.9 dependency.[12] Thus, the change must be of intramolecular nature.

This enzymatic change may be described as a chemical modification such as the unmasking of a nucleophile which can participate in a covalent reaction with the substrate either directly or indirectly, or may be described as a conformational change. At least two examples of the former possibility may be ruled out. If the pK 9 were the ionization of an amine, leading above pH 9 to a free amine which could react as a nucleophile with the substrate to divert the product, then V_{max} would decrease above pH 9, a result which is not observed. If the pK 9 were the ionization of an amine, leading above pH 9 to a free amine which could react with an (aspartate) carboxylate ion of the active site to form an amide, blocking the site, the inhibition of the binding should be time-dependent and quite slow, a result not found. Thus by process of elimination, it appears that the change occurring with a pK of 9 is a conformational change.

What is the nature of the conformational change? It must affect the active site since it affects binding. However, it is not mandatory that it affect any other part of the molecule. Some information concerning the conformational change is given below.

The driving force of the conformational change may be due to a positive assistance from the protonated form of the group of pK_a 9 or due to a negative effect of the unprotonated form of this group. The protonated form of this group could assist by stabilizing a particular conformation necessary for binding or by catalyzing the binding. The unprotonated form could hinder binding by leading to an unfavorable conformation. Below, it is suggested that the driving force of the conformational change is due to a negative effect of the unprotonated form of the group of apparent pK_a 9.

The specific rotation of chymotrypsin solutions is pH-dependent above neutrality;[15–17] in particular, the specific rotation of acetylated δ-chymotrypsin in the region of pH 6–10.5 is dependent on a single ionizable group of pK 8.3.[10] The specific rotation changes thus appear to be a good measure of the conformational change occurring in this region, if one uses the criterion of pH dependency. The difference in specific rotation between pH 10 and pH 8 is in the same direction as the difference between 8 M urea and water, but is not nearly as extensive a change.[18] The first-order rate constant of the irreversible denaturation of α-chymotrypsin decreases with pH with a pK_a of 9.[1] Since the reaction is a first-order process, the rate-determining step cannot be described as an autolysis of one chymotrypsin molecule by another, but may be described as a rate-determining unfolding of one chymotrypsin molecule. The latter process is apparently inhibited by the conformational change with a pK of 9.

Lowering the temperature to 4° perturbs the pK 9 to 9.5, as expected for the

ionization of a cationic group, but does not exhibit a transition melting temperature in the region of study. The introduction of organic solvents perturbs the pK to much higher values (with 30% dioxane to pK 11).[19] This result cannot be accounted for by a single protonic equilibrium, indicating rather that a second equilibrium perturbs the true pK under these conditions. As shown above, the pK 8.9 dependency appears to be substrate-dependent, specific substrates exhibiting this pH dependency, but some nonspecific substrates not exhibiting it. This result indicates that the conformational change may be such that it does not remove the whole active site from binding, but only a part of it.

The description of the conformation change given above resembles competitive inhibition: the binding of substrates is lowered, but the rate constant of their subsequent catalytic reaction is unaffected. This is the classical criterion for establishing competitive inhibition. The description of the conformational change also resembles the intramolecular competitive inhibition seen in catalyses by chymotrypsin which had been alkylated on methionine-192. Such catalyses exhibit binding which is markedly reduced and a rate constant which is only slightly altered. Furthermore, the perturbed binding with that enzyme is substrate-dependent in the same way that the conformation change is.[20] Thus, it is attractive to suggest that the nature of the *conformational change seen in the binding of chymotrypsin involves a pH-dependent intramolecular competitive inhibition.*

Applying the steady-state approximation to equation (2) leads to equation (3) as the description of the velocity of the intramolecular competitive inhibition.

$$v = \frac{k_{cat} E_0 S_0}{S_0 + K_s\left(1 + \dfrac{K_T}{1 + H/K_H}\right)}. \qquad (3)$$

A significant point of equation (3) is the perturbation of K_s by a quantity $(1 + K_t/(1 + (H/K_H)))$. This equation differs from that of an ordinary simple ionization process which would show a perturbation of K_s by the quantity $(1 + K_t/(H/K_H))$. Data on ethyl-N-acetyl-L tryptophanate could conceivably differentiate between equation (3) involving intramolecular competitive inhibition and a simple ionization process. However, even though the above data show a decrease in k_{cat}/K_m(app) of approximately 100-fold up to pH 11.6,[14] differentiation between equation (3) and a simple ionization process depends on knowing the value of K_T. According to the hypothesis of intramolecular competitive inhibition, the value of $K_T(= E^*/E)$ should be greater than 1; if it is greater than 100, a plausible value, equation (3) and the simple ionization process will essentially give the same fit to the data.

The description of the conformational change as an intramolecular competitive inhibition is consistent with essentially all the presently known information concerning this phenomenon. It is consistent with the observation of a pH-dependent change in optical rotation and a pH-dependent change in the rate of denaturation of the enzyme around pH 9. It is consistent with the perturbation of pK 9 by 30 per cent dioxane to pK 11. In a similar fashion, 30 per cent dioxane decreases the binding of a substrate to chymotrypsin by 300-fold;[21] it would therefore be expected to perturb the apparent pK of an intramolecular competitive inhibition

(K_T/K_H) (ignoring the term 1) by two orders of magnitude by decreasing K_T by that amount. In this connection, it should be noted that the pK 9 is perturbed to *ca.* pK 10 in reactions catalyzed by chymotrypsin alkylated on methionine-192, a perturbation which is accompanied by approximately a tenfold decrease in the binding of this enzyme.[20]

The most significant feature of the pK 9 phenomenon is its substrate dependence. The binding of the specific substrate, N-acetyl-L-tryptophanamide, to α-chymotrypsin is dependent on a group of pK 9, but the binding of the nonspecific substrate, p-nitrophenyl acetate, is not. In addition, the binding of the nonspecific substrate, ethyl acetyl-L-valinate, to α-chymotrypsin is not dependent on a group of pK 9.[22] These results are consistent with the hypothesis of an intramolecular competitive inhibition if one assumes that the intramolecular competitive inhibitor does not fill the entire site. For example, indole is a competitive inhibitor toward specific substrates but is a noncompetitive inhibitor toward the nonspecific substrate, methyl hippurate. The latter results may be most easily rationalized by saying that indole blocks only a portion of the enzymatic active site, and that with a nonspecific substrate, the binding of both substrate and inhibitor are possible. Thus, with nonspecific substrates in the present situation, the binding of both the intramolecular competitive inhibitor and the substrate may occur simultaneously. But with p-nitrophenyl acetate and ethyl N-acetyl-L-valinate,[22] acylation exhibits a dependence on a group of pK 9. This result may simply mean that although the nonspecific substrate may bind at the same time as the intramolecular competitive inhibitor, it cannot undergo the catalytic reaction because of steric interference from the inhibitor.[23]

The intramolecular competitive inhibitor may involve the N-terminal isoleucine group. This group has been implicated in chymotrypsin action since reacetylation of acetylated δ-chymotrypsin (made from the acetylated zymogen and containing only one amine moiety) results in a 90 per cent decrease in k_{cat} of the hydrolysis of ethyl N-acetyl-L-tyrosinate.[10] In addition, nitrosation of chymotrypsin leads to concomitant loss of activity and loss of N-terminal isoleucine.[24]

Cationic substrates are known to bind to the active site of chymotrypsin with more difficulty than neutral compounds.[25] N-terminal isoleucine must be near the active site (since it is liberated on activation of the enzyme) and it must be in a fairly mobile state (since it is a terminus). It is then reasonable to suggest that the isoleucine group in its neutral form may bind to the active site of chymotrypsin, and thus block the site toward external substrate, but the isoleucine group in its cationic form may not bind and thus free the site. In this connection, it is interesting to note that although trypsin contains an equivalent N-terminal isoleucine, its binding does not exhibit the dependence on a pK 9 shown with chymotrypsin. This result may reflect the fact that trypsin does not bind neutral compounds better than cationic ones; in fact, the reverse is the case.

From equation (3), the apparent pK of the group controlling the conformational change is $-\log K_H K_T$ (ignoring the term 1). From the k_{cat}/K_m(app) data of ethyl N-acetyl-L-tryptophanate, K_T is at least 100. Since the apparent ionization constant is 10^{-9}, K_H must then be 10^{-11} or smaller. This result is not that expected of an N-terminal isoleucine, but it should be noted that the N-terminal isoleucine is near the sequence Asp.Glu.Glu, groups whose combined negative

charge may perturb the pK of this group to a value significantly higher than its normal one in solution.

The inactivity of chymotrypsinogen may be related to the intramolecular competitive inhibition described here. In the zymogen, the intramolecular competitive inhibitor would presumably be the elements of the dipeptide later removed rather than the N-terminal isoleucine or other group in the free enzyme, but the principle may be the same.

At the present time, the reader should view this proposal as a working hypothesis, and await the tests of this hypothesis currently being pursued.

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[5] These results disagree with those previously recorded.[1] We have found that the spectrophotometric conditions used previously resulted in serious deviations from Beer's law because of the very high backgrounds employed. By using 1-mm cells rather than the 10-mm ones used previously, carefully maintaining the given narrow slit-width, and determining the $\Delta\epsilon$ (= 80 at 306 mμ) under simulated reaction conditions, we were able to perform initial rate studies with quite high precision.

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[11] This enzymatic change is not to be confused with any change that may occur on acylation.

[12] The change also cannot be due to the trivial reason of an impurity since chymotrypsin gel-filtered with Sephadex G-25[13] shows the same properties as untreated chymotrypsin. The change also cannot be due to a multiplicity of enzymes in the preparation such as α, β, γ, and δ-chymotrypsins since all these isozymes have the same pH 9 dependence.

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