A Kinetic Analysis of the Papain-Catalyzed Hydrolysis of α-*N*-Benzoyl-L-citrulline Methyl Ester*

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ABSTRACT: The hydrolysis of α-*N*-benzoyl-L-citrulline methyl ester catalyzed by papain was investigated from pH 3 to 9. This noncharged ester is isosteric to α-*N*-benzoyl-L-arginine ethyl ester which is the best synthetic substrate for papain previously studied. It is an excellent substrate for papain, a poor substrate for chymotrypsin, and an extremely poor substrate for trypsin. The kinetic constants for the papain-catalyzed hydrolysis, $K_{M\,app}$, k_{cat} (turnover rate constant), k_2 (acylation rate constant), k_3 (deacylation rate constant), and K_s, and their pH dependencies, were estimated. The pH dependency of acylation is similar to previously reported substrates above pH 4 but differs in acid where it was found that $k_{cat}/K_{M\,app}$ and k_2 reached a maximum at pH 4. On the other hand, deacylation is affected by the ionization of a single group with a pK_a of 7 which has been interpreted as the formation of a second active form of acyl-enzyme.

It is possible that this ionizing group is the imidazole moiety of a histidine residue. Recalculation of previously published data on the hydrolysis of α-*N*-benzoyl-L-arginine amide has revealed the presence of this same group. It may also control the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester, but in this case the pK_a is much higher.

The proteolytic enzyme papain has been under investigation for many years. Extensive studies on the covalent structure from the laboratory of E. L. Smith showed that it consists of a single polypeptide chain with a molecular weight of approximately 20,700. The covalent structure is now almost completely established, and the current status has been published (Light *et al.*, 1964). Studies of the mechanism of action and specificity have been reviewed by Smith and Kimmel (1960) who proposed that the reaction consists of two steps: (1) an acylation of the active site which is dependent upon an ionized carboxyl group of $pK_a = 4.3$ and a protonated sulfhydryl group of $pK_a = 8$ and (2) a deacylation step dependent on an ionized carboxyl group of $pK_a = 3.5$. Recently, the mechanism of action has been reinvestigated in several laboratories, and the following general scheme depicted in eq 1 has been suggested (Whitaker and Bender, 1965; Lowe and Williams, 1965b; Kirsch and Igelstrom, 1966).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2 \quad (1)$$

where

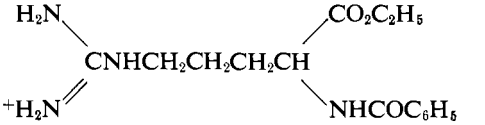
$$K_{M\,app} = (k_3/(k_2 + k_3))K_s \qquad K_s = k_{-1}/k_1 \quad (2)$$

and

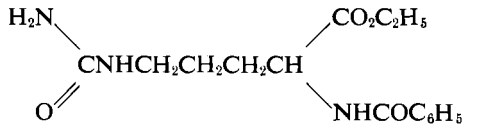
$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (3)$$

ES is the Michaelis–Menten complex and ES′ is the acyl intermediate shown to be a thioester (Lowe and Williams, 1965a). P_1 and P_2 represent the alcohol and acid formed when esters are used as substrates.

Papain exhibits a broad specificity (Smith and Kimmel, 1960) in contrast to trypsin which catalyzes the hydrolysis of derivatives of the basic amino acids, arginine and lysine. The best substrates for papain, however, also are derivatives of arginine and lysine. Since a positively charged group is required for trypsin substrates and apparently not for papain substrates, the properties of α-*N*-benzoyl-L-citrulline methyl



BAEE



BCME

ester,[1] which is essentially isosteric to BAEE, should

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[1] Abbreviations used: BCME, α-*N*-benzoyl-L-citrulline methyl ester; BAEE, α-*N*-benzoyl-L-arginine ethyl ester; BAA, α-*N*-benzoyl-L-argininamide; BGEE, benzoylglycine ethyl ester.

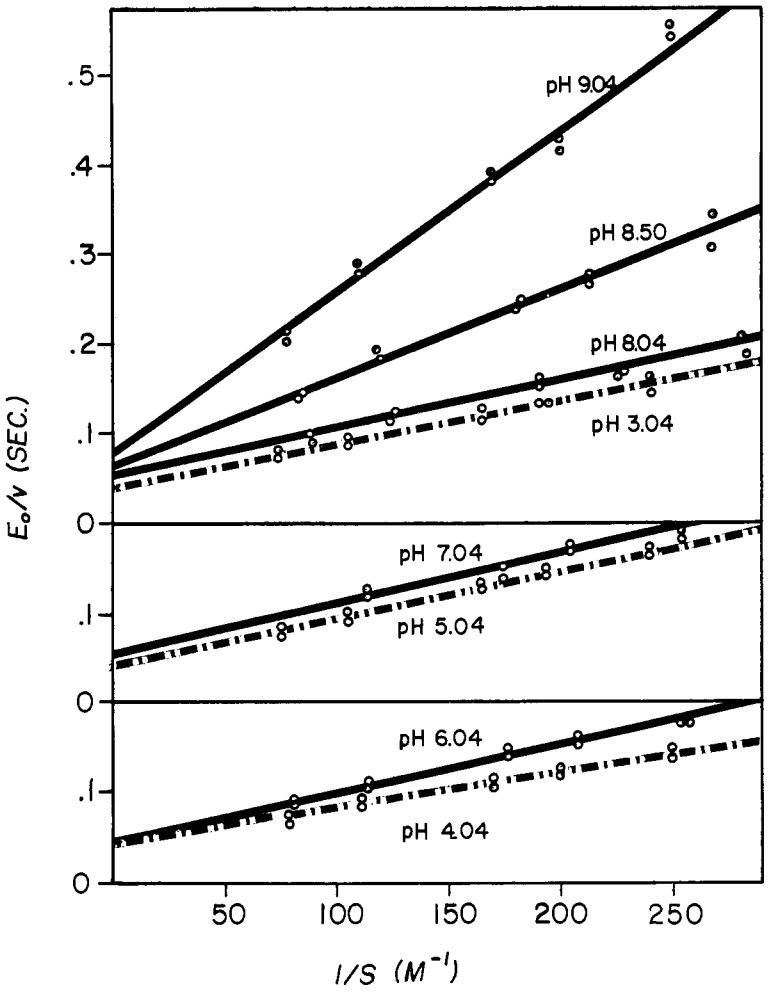

FIGURE 1: Some typical Lineweaver–Burk plots for the papain-catalyzed hydrolysis of α-*N*-benzoyl-L-citrulline methyl ester at 25°.

be of interest. BCME differs from BAEE by virtue of the substitution of an oxygen atom for a nitrogen atom in the guanidino group of BAEE; this eliminates the positive charge from the molecule. It was found that BCME was a poor substrate for trypsin but was an excellent substrate for papain; it was, in fact, superior to BAEE. Investigation of the Michaelis–Menten parameters of BCME from pH 3 to 9 has revealed hitherto unobserved pH dependency upon a functional group with a pK_a of 7 as well as other pH dependencies which have not been resolved.

Materials and Methods

Enzymes. A fresh stock solution of twice-crystallized papain (Worthington Biochemical Corp., batch papain 5611) containing 0.005 M L-cysteine hydrochloride and 0.001 M EDTA (pH 5.15) was prepared daily and stored in an ice bath. The enzyme concentration was calculated from its absorbance at 280 mμ using ϵ 5.10 × 10^4 M^{-1} cm^{-1}. The active enzyme concentration was determined at pH 5.15, 25°, ionic strength 0.03, by the use of BAEE as substrate and assuming k_{cat} = 15.7 (Whitaker and Bender, 1965). The enzyme was found to be 81.8% pure using an average of ten determinations and assuming a molecular weight of 20,700. Twice-crystallized trypsin (lyophilized batch TRL 6259) and three-times-crystallized-chymotrypsin (batch CD 16108-9) were purchased from Worthington Biochemical Corp.

Substrates. α-*N*-BENZOYL-L-ARGININE ETHYL ESTER HYDROCHLORIDE (Mann Research Laboratories, no. 1441, mp 127–129°) was used without further purification.

SYNTHESIS OF α-*N*-BENZOYL-L-CITRULLINE. Benzoyl chloride (23.3 ml, 200 mmoles) was added slowly with stirring to a solution (0°) of L-citrulline (20 g, 114 mmoles) (Mann Research Laboratories, chromatographically purified) in 80 ml of 2 N sodium hydroxide. Additional 2 N sodium hydroxide was added as needed to maintain the pH near 8. The reaction was considered finished when no more acid was produced. The pH was then adjusted to 3, and the product, which precipitated as a thick yellowish oil, was washed

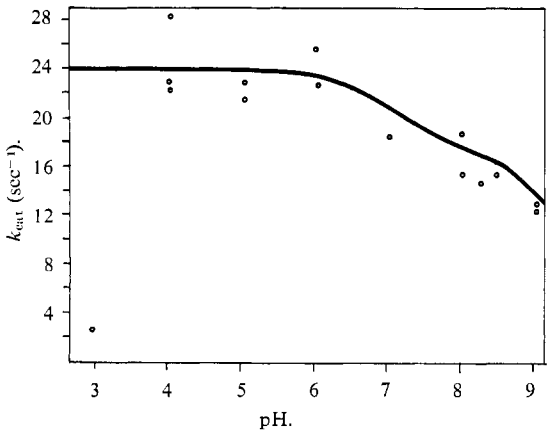

FIGURE 2: k_{cat} as a function of pH for the papain-catalyzed hydrolysis of α-*N*-benzoyl-L-citrulline methyl ester at 25°. The solid line represents a theoretical curve calculated by using eq 3, 8, and 9.

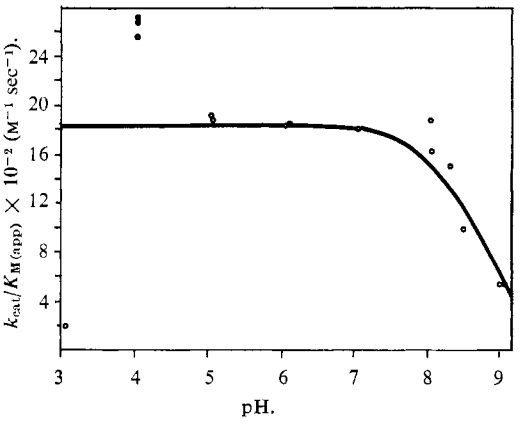

FIGURE 4: $k_{cat}/K_{M\ app}$ as a function of pH for the papain-catalyzed hydrolysis of α-*N*-benzoyl-L-citrulline methyl ester at 25°. The solid line represents a theoretical curve calculated from eq 7 and the data in Table II.

several times with deionized water. It was then suspended in water, redissolved by adjusting to pH 8, and slowly acidified to pH 3 with stirring. Crystals formed which were recrystallized from absolute ethanol, mp 178–179°, 80% yield.

SYNTHESIS OF α-*N*-BENZOYL-L-CITRULLINE METHYL ESTER. Dry HCl gas was bubbled for 3 hr through 10 g (35.76 mmoles) of α-*N*-benzoyl-L-citrulline which was dissolved in approximately 120 ml of anhydrous methanol. Oily crystals were obtained by evaporation of the solvent and removal of excess acid. A good crystalline product was isolated by suspending the oily crystals in 30 ml of deionized water and adjusting to pH 6 with 2 N sodium hydroxide. The crystals obtained were washed several times with cold water, collected by centrifugation, and recrystallized from absolute ethanol, mp 146–148°, yield 72%.

Anal. Calcd for $C_{14}H_{19}N_3O_4$: C, 57.27; H, 6.52; N, 14.41. Found: C, 57.23; H, 6.51; N, 14.24.

Kinetic Measurements. Initial rates (less than 5% of the substrate was hydrolyzed when the lowest concentration was used) of hydrolysis were determined employing a pH-Stat (Radiometer titrator, type TTT1c; Radiometer titrigraph, type SBr2c) and a scale expander attachment (type PHA 630Ta). A syringe buret was used which delivered 0.25 ml of 0.01 N sodium hydroxide for full-scale deflection on the recorder. A reaction volume of 10 ml, maintained at 25°, was used for all rate measurements. All rate data were analyzed with an IBM 1410 computer by stepwise regression analysis. The kinetic constants, k_{cat} and $K_{M\ app}$, were calculated from the Lineweaver–Burk (1934) form of the Michaelis–Menten equation. Rates were corrected for slow nonenzymic hydrolysis and for the presence of undissociated α-*N*-benzoyl-L-citrulline which has a pK_a of 4.16. The pK_a was determined at an ionic strength of 0.03. At pH values higher than 7, the initial velocity measurements were performed under a nitrogen atmosphere. The concentrations of α-*N*-benzoyl-L-citrulline and α-*N*-benzoyl-L-citrulline methyl ester stock solutions were calculated from absorbance measurements using ϵ 1.10×10^4 and 9.75×10^3 M^{-1} cm^{-1} at 232 mμ, respectively.

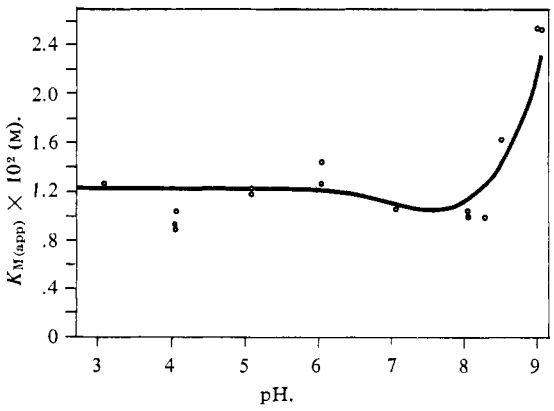

FIGURE 3: $K_{M\ app}$ as a function of pH for the papain-catalyzed hydrolysis of α-*N*-benzoyl-L-citrulline methyl ester at 25°. The solid line represents a theoretical curve calculated by using eq 2 substituting values of k_2 and k_3 obtained from eq 8 and 9, respectively, and K_s from eq 6.

Results

The kinetic constants for the papain-catalyzed hydrolysis of BCME were calculated from the data of two consecutive series of experiments. Some of the data showing the rates of hydrolysis from pH 3 to 9 are plotted in Figure 1. The constants and their standard deviations are reported in Table I. The pH dependencies of k_{cat}, $K_{M\ app}$, and $k_{cat}/K_{M\ app}$ are shown in Figures 2–4, respectively.

TABLE I: Kinetic Constants of the Papain-Catalyzed Hydrolysis of α-*N*-Benzoyl-L-citrulline Methyl Ester[a] at 25°.

pH	$K_{M\ app}$ ($\times 10^2$ M)	k_{cat} (sec^{-1})	$k_{cat}/K_{M\ app}$ ($\times 10^{-2}$ M^{-1} sec^{-1})
3.04	1.25 ± 0.12	2.46 ± 0.24	2.03 ± 0.09
4.04	0.88 ± 0.05	22.33 ± 0.25	25.66 ± 0.90
4.04	0.87 ± 0.07	22.91 ± 0.75	27.01 ± 1.17
4.04	1.05 ± 0.04	28.30 ± 1.07	26.97 ± 0.54
5.04	1.23 ± 0.09	22.91 ± 0.58	18.89 ± 0.60
5.04	1.17 ± 0.10	21.53 ± 1.82	18.77 ± 0.73
6.04	1.44 ± 0.15	25.60 ± 2.55	18.34 ± 0.74
7.04	1.07 ± 0.11	18.55 ± 1.99	18.08 ± 1.01
8.04	1.04 ± 0.12	18.79 ± 2.01	18.93 ± 1.07
8.04	1.01 ± 0.11	15.54 ± 1.62	16.16 ± 0.90
8.3	1.01 ± 0.09	14.72 ± 1.24	14.97 ± 0.62
8.5	1.63 ± 0.24	15.35 ± 2.12	9.97 ± 0.51
9.04	2.55 ± 0.50	13.00 ± 2.78	5.51 ± 0.27
9.04	2.46 ± 0.59	12.50 ± 2.74	5.69 ± 0.37

[a] The substrate concentration was varied from 0.35 to 1.33 × 10^{-2} M. The active enzyme concentration was varied from 6.68 to 130 × 10^{-8} M. The higher enzyme concentrations were required at pH 3 and 9. The concentrations of cysteine and EDTA were 5 and 1 × 10^{-3} M, respectively. Ionic strength was maintained at 0.03 with potassium chloride.

Equation 4 can be derived by combining eq 2 and 3.

$$k_{cat}/K_{M\ app} = k_2/K_s \quad (4)$$

The influence of pH on the ratio $k_{cat}/K_{M\ app}$ applies directly to the acylation step, assuming that K_s is constant over the pH range; this appears to be true from pH 5 to 9. $k_{cat}/K_{M\ app}$(lim) as well as the ionization constant, K_1, which appears to control the pH dependency in the alkaline pH range can be calculated from eq 5 (Laidler, 1958).

$$k_{cat}/K_{M\ app} = k_{cat}/K_{M\ app}(\text{lim}) - (k_{cat}/K_{M\ app})(K_1/(H^+)) \quad (5)$$

The values for $k_{cat}/K_{M\ app}$(lim) and pK_1 are reported in Table II.

In order to obtain further information on the mechanism of action from kinetic data, it becomes necessary to separate and calculate the rate constants k_2 and k_3 as well as the equilibrium constant K_s. This can be done for steady-state conditions by first combining eq 2 and 3 and then rearranging them to give eq 6 (Whitaker and Bender, 1965).

$$1/K_{M\ app} = 1/K_s + (k_{cat}/K_{M\ app})(1/k_3) \quad (6)$$

A plot of the data obtained from experiments carried out at pH 8–9, shown in Figure 5, was found to be a straight line from which k_3(lim) and K_s could be calculated (Table II). Knowing K_s and assuming it to be constant over the entire pH range of investigation, the rate constant for acylation, k_2, at all pH values was calculated from eq 4, and the values were plotted in Figure 6. The deacylation constant k_3, at all pH values, was calculated using eq 6 with the experimental k_{cat} and $K_{M\ app}$, assuming that K_s is constant. The results are shown in Figure 7.

TABLE II: Kinetic Constants of Papain-Catalyzed Hydrolysis of α-*N*-Benzoyl-L-citrulline Methyl Ester at 25°.

$k_{cat}/K_{M\ app}$(lim)[a]	1836 ± 266 M^{-1} sec^{-1}
k_2(lim)[b]	139.7 ± 13.6
k_3'(lim) (pH 8–9)[c]	20.15 ± 2.41 sec^{-1}
k_3''(lim) (pH 4.6)[d]	28.82 ± 2.75 sec^{-1}
K_s[c]	0.0727 ± 0.0278 M
pK_1[a,e]	8.69 ± 0.06
pK_2[d]	7.0

[a] Equation 5. [b] k_{cat}/K_M(lim) and K_s using eq 4. [c] Equation 6. [d] Equation 9. [e] Equation 8. The kinetic constants were determined by using the values given above.

Theoretical curves, represented as solid lines in Figures 2–7, were constructed from the values reported in Table II and from the appropriate theroetical functions. The theoretical $k_{cat}/K_{M\ app}$ or k_2/K_s curve was calculated using eq 7 and the values for K_s, k_2(lim), and pK_1 of 0.0727 M, 139.7 sec^{-1}, and 8.69, respectively.

$$k_{cat}/K_{M\ app} = k_2/K_s = [k_2(\text{lim})/(1 + (K_1/(H^+)))]/K_s \quad (7)$$

k_2(lim) and pK_1 were estimated from k_2, which was calculated from eq 4 and 8. Theroetical k_2 values were obtained by using eq 8 with the same k_2(lim) and pK_1.

$$k_2 = k_2(\text{lim})/[1 + (K_1/(H^+))] \quad (8)$$

The theoretical curve for k_3 was constructed by using eq 9, a modification of the expression employed by Alberty and Bloomfield (1963). The possible application of this equation to explain our data will be discussed later.

$$k_3 = [k_3''(\text{lim})(K_2/(H^+)) + k_3'(\text{lim})]/[1 + (K_2/(H^+))] \quad (9)$$

The k_3'(lim) and k_3''(lim) and pK_2 values are given in Table II. The theoretical k_{cat} curve was calculated using eq 3 with k_2 and k_3 values obtained from eq 8

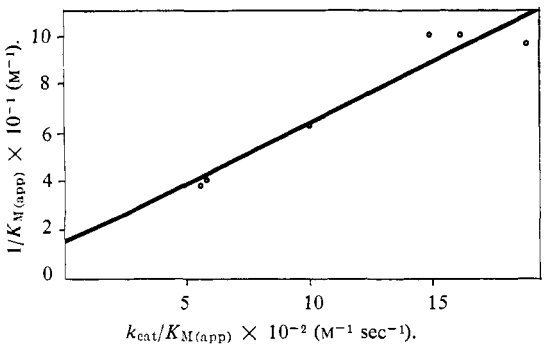

FIGURE 5: Determination of K_s and k_3(lim) at pH 8–9. The data are plotted according to eq 6.

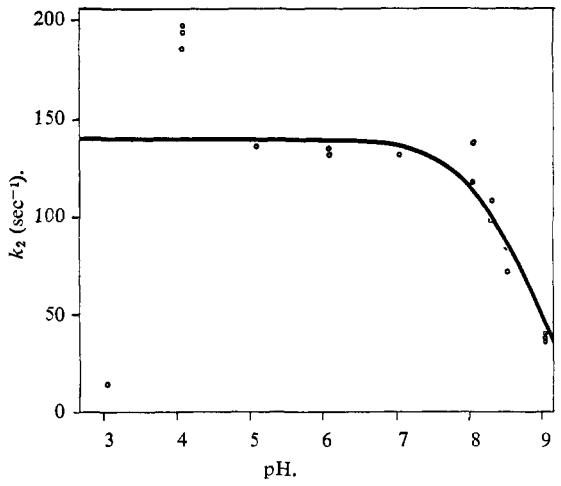

FIGURE 6: k_2 as a function of pH for the papain-catalyzed hydrolysis of α-*N*-benzoyl-L-citrulline methyl ester at 25.° The solid line represents a theoretical curve obtained from eq 8 using the data in Table II.

and 9; the theoretical K_M curve was obtained by using eq 2 and the same k_2 and k_3 values. There is good agreement between the experimental data and the theoretical curves. The last curve indicates that K_s is constant at least down to pH 5.

Hydrolysis of α-N-benzoyl-L-citrulline Methyl Ester by Trypsin and Chymotrypsin. The rates of hydrolysis of BCME by trypsin and chymotrypsin (at pH 7.04, using the same procedure employed for papain) were studied at a single substrate concentration, and the results are recorded in Table III. Trypsin hydrolyzes BCME very slowly, whereas chymotrypsin hydrolyzes BCME at approximately one-fifth the rate of papain. If one compares these rates to those with BAEE as the substrate, it may be noted that the positive charge contributes a factor of approximately 100 to the rate with trypsin in contrast to chymotrypsin and papain; the latter makes little distinction between the substrates.

TABLE III: Specificity of Proteolytic Enzymes.

Enzyme	BCME[a] ($\times 10^2$ M)	v/E_0 (sec^{-1})
Papain	1.168	9.97
	1.285	10.41
Trypsin	1.168	5.55×10^{-2}
Chymotrypsin	1.285	1.89
	BAEE[b]	
Papain	1.168	5.90
	1.285	6.38
Trypsin	1.168	8.93
Chymotrypsin	1.285	1.03

[a] The hydrolysis of BCME was performed at pH 7.04, 25°. Papain, $E_0 = 6.54 \times 10^{-8}$ M; trypsin, $E_0 = 1.56 \times 10^{-5}$ M; chymotrypsin, $E_0 = 1.56 \times 10^{-7}$ M. [b] The hydrolysis of BAEE was performed at pH 8.0, 25°. The data for papain were taken from Whitaker and Bender (1965) and the data for trypsin and chymotrypsin from Inagami and Sturtevant (1960).

Discussion

The data presented in this paper were found to be consistent with the following mechanism for the papain-catalyzed hydrolysis of α-*N*-benzoyl-L-citrulline methyl ester.

$$\begin{array}{ccccccc} E + S & \underset{k_{-1}''}{\overset{k_1''}{\rightleftharpoons}} & ES & \xrightarrow{k_2''} & ES' + P_1 & \xrightarrow{k_3''} & E + P_2 \\ \downarrow\uparrow K_1 & & \downarrow\uparrow K_1 & & \downarrow\uparrow K_2 & & \\ EH + S & \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} & ESH & \xrightarrow{k_2'} & ESH' + P_1 & \xrightarrow{k_3'} & EH + P_2 \end{array} \quad (10)$$

A general mathematical expression for the deacylation reaction for steady-state enzyme kinetics involving multiple intermediates has been described by Alberty and Bloomfield (1963) and is shown in eq 11.

$$k_3 = [k_3' + k_3''((H^+)/K_3) + k_3''(K_2/(H^+))]/ [1 + ((H^+)/K_3) + (K_2/(H^+))] \quad (11)$$

where the middle terms of the numerator and denominator correspond to a second ionization of the acylated intermediate. This second equilibrium could not be characterized in our system; therefore. the equation was reduced to the form of eq 9 which was used directly to construct the theoretical curve.

The theoretical values agree well with the experimental data for deacylation, indicating that the rate is dependent upon the presence of a protonated group having a pK_a of 7. The change in the rate of deacylation

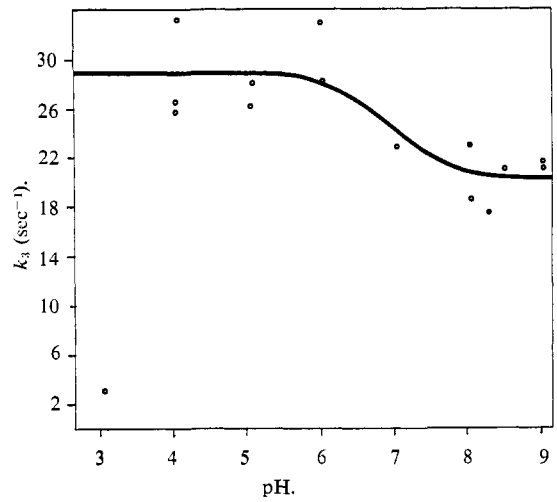

FIGURE 7: k_3 as a function of pH for the papain-catalyzed hydrolysis of α-*N*-benzoyl-L-citrulline methyl ester at 25°. The solid line represents a theoretical curve calculated from eq 9 using the data in Table II.

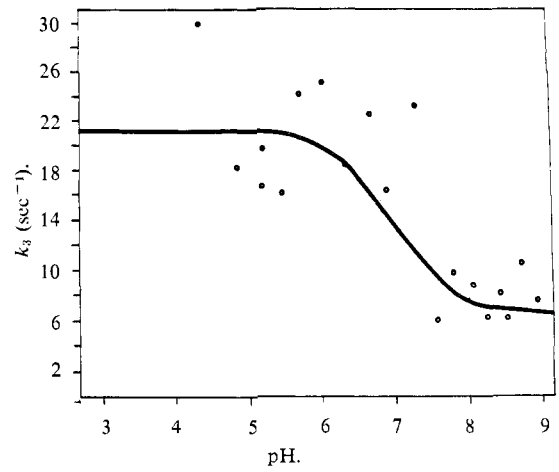

FIGURE 8: k_3 as a function of pH for the papain-catalyzed hydrolysis of α-*N*-benzoyl-L-argininamide at 25°. Data taken from Whitaker and Bender (1965) was recalculated and plotted according to eq 9.

is not due to the ionization of a prototropic group on the substrate since no such group in the acyl-enzyme can ionize in the neutral pH range. The ionization observed is, therefore, attributed to a prototropic group on the enzyme. It is tempting to identify this group with a histidine residue, which was suggested as a participating group in the mechanism of hydrolysis by Lowe and Williams (1965b), but this assignment would be premature. This type of pH dependency for k_3 was not reported for BAEE; but, if the published data are replotted, as outlined above, it may be seen that k_3 = 12.0 at pH 9.22 and 8.1 at pH 9.56, whereas k_3(lim) = 20.2 ± 1.7 sec^{-1} (Whitaker and Bender, 1965). The investigators showed that no denaturation occurs under the conditions employed; this is in accord with the data of Stockell and Smith (1957). This decrease of k_3 may be the result of the ionization of the same functional group observed with BCME but with pK_a shifted.

The dependency of acylation on (H^+) in the acidic region for the papain-catalyzed hydrolysis of BCME is markedly different from that observed with BAEE as substrate. Our data do not permit us to describe acylation or deacylation on a theoretical level below pH 5.

k_2/K_s, which is essentially constant from pH 5 to 7, rises to a maximum near pH 4, indicating that k_2 is increased with respect to K_s. This can result from a decrease in K_s or from an increase of k_2. It must be pointed out that the constants in eq 10, k_2' and k_2'', are not necessarily different. However, the studies of Sluyterman (1966) indicate that K_s for BAEE does increase in acid and that it is likely that k_2' is greater than k_2''.

After inspection of the graph of $k_{cat}/K_{M\,app}$ plotted against $1/K_{M\,app}$ for BAA hydrolysis by papain (Whitaker and Bender, 1965) which was treated as a straight line, it was felt that a recalculation of k_3 and K_s from that portion of the data which better fit a straight line might be instructive. It was found that a plot of their data from pH 8.05 to 8.93 was in a reasonably straight-line relationship. The new value for K_s is 5.63 ± 0.85 × 10^{-2}, which is in excellent agreement with K_s found for BAEE, and the new value for k_3(lim) was 6.58 ± 2.92. The similarity of K_s for BAA and for BAEE confirms the conclusions reached by Kirsch and Ingelstrom (1966) who found that the nature of the leading group is not of great importance as a determinant of K_s for substrates of papain. New values were calculated for k_2 employing eq 4 and for k_2(lim) employing eq 8. The rate constant of deacylation was estimated by use of eq 3 or 6, assuming that K_s is constant. The results are plotted in Figure 8. The solid line is the theoretical curve calculated from eq 9 assumming k_3' = 6.58 ± 2.92, k_3'' = 21.33 ± 5.19, pK_2 = 7.0.

Sluyterman (1964) and Kirsch and Katchalski (1965) have studied the effect of pH on the Michaelis–Menten constants of the papain-catalyzed hydrolysis of benzoylglycine ethyl ester. They found that k_{cat} was constant over a pH range from 4.6 to 8.4; thus, no evidence for an ionizable group with a pK_a of 7 affecting deacylation could be observed. On the other hand, the dependence of $K_{M\,app}$ on (H^+) may indicate that K_s is similarly dependent over this pH range.

If we compare the pH dependency of the four substrates of papain (BCME, BAA, BAEE, and BGEE), we find that the k_3 for each of the first two shows an inflection at pH 7 which can be explained by the presence of two active forms of the acyl-enzyme whose concentrations are regulated by the ionization of a single ionizing group. BAEE may also be exhibiting the same properties but with the pK_a of this group shifted to a higher pH; finally, BGEE, which does not exhibit this pH dependence, may also be found

to be similar if the investigations are carried out at more alkaline pH values. The dependence of k_3 on an ionized carboxyl group is observed with BAEE but cannot be established for the others with the data presently available. Acylation, on the other hand, seems to be controlled by an acidic and basic group resulting in a bell-shaped pH-dependence curve for all except BCME which is more complicated in acidic media. It is clear that the presence of a positive charge on a papain substrate confers no increased specificity or enhancement of the hydrolytic rate.

Acknowledgment

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