The pH Dependencies of Individual Rate Constants in Papain-Catalyzed Reactions*

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ABSTRACT: This report describes measurements of acylation rate constants (k_2) and apparent binding constants ($K_S = (k_{-1} + k_2)/k_1$) for substrates of papain over a range of pH. The Michaelis–Menten parameters (k_m and k_{cat}) for the papain-catalyzed hydrolysis of isopropyl and methyl hippurates and isopropyl, benzyl, and *p*-methoxyphenyl *N*-methanesulfonylglycinates have been determined over a range of pH at 35°. Assuming a three-step mechanism:

$$E + RCOX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot RCOX \xrightarrow{k_2} RCOR \xrightarrow{k_3} E + RCO_2H$$
$$+ XH$$

k_2 and K_S may be calculated for the isopropyl esters because k_{cat} for these substrates lies well below k_3 for the common acyl-enzyme. This calculation requires a *bona fide* value for k_3 (supplied by k_{cat} for the other esters where deacylation is shown to be rate determining) and the assumptions that the rate-determining step is acylation for the isopropyl esters and that the three-step mechanism is valid for all substrates. These assumptions are more directly validated by experiment than those used in previous attempts to dissect composite Michaelis–Menten parameters. The pH dependence of k_2 is found to be bell shaped with $pK_1 \sim 4.5$ and $pK_2 \sim 8.5$. K_S is probably a true binding constant ($k_{-1} \gg k_2$) since it is found to be invariant in the pH range where k_2 varies significantly, and this conclusion is strengthened by the observation that uncharged reversible inhibitors with substrate-like structures have pH-independent competitive inhibition constants. A new mechanism is proposed for papain-catalyzed hydrolyses where an imidazole gives rise to the acid pK_a in acylation and deacylation by acting as a general base abstracting a proton from thiol and nucleophile, respectively; the abnormally low pK_a is caused by hydrogen bonding with an asparagine. The base pK_a in acylation is due to the ionization of a thiol which reacts in its un-ionized form.

It is now generally accepted that papain-catalyzed hydrolyses of esters can be depicted minimally by the kinetic scheme (1)

$$E + RCOX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot RCOX \xrightarrow{k_2} ECOR \xrightarrow{k_3}$$
$$+ XH$$
$$E + RCO_2H \quad (1)$$

where E·RCOX is an enzyme–substrate complex and ECOR represents acylated papain. Acyl-papains have been observed spectroscopically using *N*-cinnamoylimidazole (Brubacher and Bender, 1966) and methyl thionohippurate (Lowe and Williams, 1965) as substrates. The constancy of k_{cat} for a series of ester substrates of a common acid with leaving groups of widely differing lability is kinetic evidence for an acylated papain and moreover for a kinetic scheme with at least three steps (Lowe and Williams, 1965); k_{cat} is then identified as k_3 and k_2 exceeds k_3 in the given series (Lowe and Williams, 1965; Kirsch and Igelström, 1966; Bender and Brubacher, 1966). It can be argued that the constant k_{cat} represents a rate-determining conformational change of the enzyme–substrate complex (Lake and Lowe, 1966), but this identity can be discounted partly because of the observation of acyl-papains, but largely because "titration" kinetics have been observed using a number of labile ester substrates (Bender *et al.*, 1966; results quoted in Lake and Lowe, 1966; de Jersey *et al.*, 1966).

The observed Michaelis–Menten parameters can be derived for eq 1 using the steady-state treatment and are given in eq 2 and 3.

$$K_m = (k_3/k_1)(k_{-1} + k_2)/(k_2 + k_3)$$

$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (2)$$

$$k_{cat}/K_m = k_1k_2/(k_{-1} + k_2) = k_2/K_S \quad (3)$$

Previous attempts to measure k_2 and K_S for papain and its substrates (using the three-step kinetic model) have been based on pH–rate studies and involved the use of eq 4 derived from eq 2 and 3.

$$k_{cat} = k_3 - k_3K_m/K_S \quad (4)$$

If k_3 and K_S are independent of pH in the range studied then k_{cat} should be linear in K_m with an intercept on the k_{cat} axis of k_3 and of K_S on the K_m axis; k_2 can then be calculated using eq 3 at each pH value.

Whitaker and Bender (1965) measured the Michaelis–Menten parameters over a pH range for α-*N*-Bz-L-ArgEt and α-*N*-Bz-L-Arg-NH_2 and found that k_{cat} was linear in K_m at pH >5.5; assuming an invariant k_3 (arguing from the pH dependence for the deacylation of cinnamoyl-papain) it was concluded that K_S was also invariant in order that this linearity be preserved. Equation 4 could not be applied in the lower pH range (<5.5) because k_3 was not constant. It was argued that the acid pK_a observed in k_{cat}/K_m was caused by a

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TABLE I: Analytical and Physical Properties of Substrates and Inhibitors.[a]

Compound	Mp (°C)	Comments
Methyl hippurate	80–81.5	80–81.5 (Hammond and Gutfreund, 1959)
Isopropyl hippurate	85–87	85.5–86.5 (Epand and Wilson, 1963)
Isopropyl mesylglycinate	56–59	[Recrystallized from benzene–petroleum ether (bp 40–60°)] *Anal.* Calcd: C, 36.9; H, 6.67; N, 7.18. Found: C, 36.7; H, 6.69; N, 7.14
Benzyl mesylglycinate	78–79	(Recrystallized from benzene–petroleum ether) *Anal.* Calcd: C, 49.4; H, 5.35; N, 5.76. Found: C, 49.1; H, 5.33; N, 5.90
p-Methoxyphenyl mesylglycinate	112–113	(Recrystallized from water) *Anal.* Calcd: C, 46.3; H, 5.02; N, 5.41. Found: C, 46.5; H, 4.95; N, 5.54
Benzamidoacetonitrile	139–141	144 (Klages and Haack, 1903)
Acetamidoacetonitrile	75–77	77 (Johnson and Gatewood, 1929)
2-Benzamidoethanol	61–62	58 (Knorr and Rössler, 1903)
Methyl acetylglycinate	55–57	57–59 (Curtius and Goebel, 1888)
Benyzl acetylglycinate	47–49	46–47.5 (Sheehan and Corey, 1952)
p-Nitrophenyl acetylglycinate	123–124	123–125 (Bryce *et al.*, 1965)

[a] Microanalysis by Miss F. Duckworth, University of Kent.

variation in k_2 rather than K_S. The main criticisms of these arguments are: that the assumption of a constant k_3 is not directly tested for the common acid employed, that the justification of a constant K_S in the high pH range is only indirectly verified by the resultant linear plot, and that K_S cannot be calculated directly for each pH value. A further series of substrates was examined by Bender and Brubacher (1966) using a similar approach (*p*-nitrophenyl, benzyl, and methyl esters of α-*N*-carbobenzoxy-L-lysine) and plots of k_{cat} *vs.* K_m were reasonably linear. The same criticisms can be leveled at this dissection but in this case the values of k_{cat} must be very close to k_3 for the benzyl and *p*-nitrophenyl esters and it is evident that k_3 will be determined accurately for the pH range where it is constant by plotting k_{cat} *vs.* K_m. Determination of the K_S intercept will, however, involve gross errors (since $k_{cat} \approx k_3$) and the results obtained may be fortuitous for this pair of esters. When $k_{cat} \approx k_3$ it is likely that any variations in k_{cat} predicted from eq 4 will be swamped by experimental error, and differentiation of the Michaelis–Menten equation indicates that errors in k_{cat} should be proportional to those in K_m which could result in a good but meaningless linearity in eq 4. It was not possible for these authors to demonstrate that K_S was constant in the acid region (pH <5.5).

Stopped-flow measurements have been employed to measure k_2 and K_S (Hubbard and Kirsch, 1968) but were unsatisfactory owing to large values of K_S (substrates were nitrophenyl esters of carbobenzoxyglycine) and because papain is not sufficiently soluble in the solvents employed.

Sluyterman (1966) found that α-*N*-Bz-L-ArgEt prevented the activation of papain by cysteine and proposed that this substrate bound to the inactive enzyme. Binding constants were calculated and shown to be close to the corresponding K_m values for the active enzyme measured under similar conditions. It is difficult, however, to interpret these results since although the binding constants are meaningful they apply only to the inactive enzyme. Further work of this author (Sluyterman, 1968), using chloroacetic acid as an irreversible inhibitor of papain in the presence of various substrates, showed that $k_2 \ll k_3$ for α-*N*-Bz-L-ArgEt at pH 6 (*i.e.*, $K_m = K_S$). At this pH, Whitaker and Bender (1965) found $k_2 \approx 3k_3$. It is likely that the former result is the better estimate in view of the arguments presented above but Sluyterman's work does rest on some assumptions, notably that the rate of inactivation of free papain equals that for the papain–α-*N*-Bz-L-ArgEt complex. We report here values of k_2 and K_S for two substrates of papain over a pH range determined by a method involving assumptions more directly validated by experimental evidence than those used previously. Acylation (k_2) for an ester with k_{cat} significantly lower than k_3 must be comparable or less than k_3 and may be calculated using k_{cat}, k_3, and eq 2. Substituting k_2 and k_{cat}/K_m in eq 3 yields K_S. The deacylation rate constant (k_3) may be obtained in a number of ways—the simplest being to measure k_{cat} for a number of labile ester substrates of a common acid. The experimental observations, namely that k_2 has a bell-shaped pH dependency and that K_S is independent of pH, are in agreement with previous extrapolations and, moreover, extend these results to a low pH region.

We have assumed that all the ester substrates of papain hydrolyze *via* the same mechanism. Supporting evidence for this assumption is that all the esters show bell-shaped pH profiles for k_{cat}/K_m and esters of hippuric acid fit a linear free energy relation between k_0/K_m and the rate constant for reaction of hydroxide ion with the corresponding acetate ester (Bender and Kézdy, 1965). Other more complicated schemes are possible and even probable in the light of future experimentation on, for example, conformational changes. The aim of this investigation is the calculation of individual rate constants according to eq 1 and in a more complicated

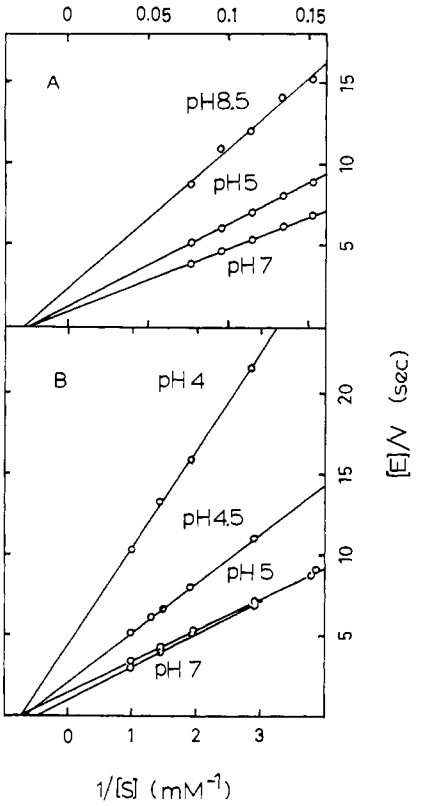


FIGURE 1: Typical Lineweaver–Burk plots for isopropyl esters (refer to Tables V and VII for conditions): (A) isopropyl hippurate; (B) isopropyl mesylglycinate.

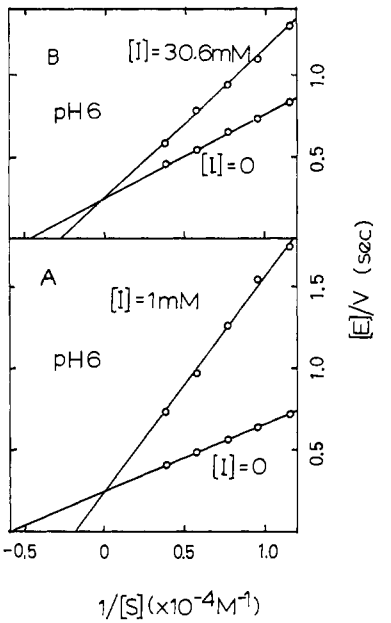


FIGURE 2: Typical Lineweaver–Burk plots for acylaminoacetonitrile inhibitors using methyl hippurate as substrate: (A) hippuronitrile; (B) acetylaminoacetonitrile (refer to Table VIII for conditions).

model these parameters will represent composite rate consants whose dissection is a future problem.

Experimental Section

Materials. Papain (EC 3.4.4.10) was prepared from the solidified papaya sap (we are grateful to the Wallerstein Corp. for a generous gift) *via* the method of Kimmel and Smith (1954) and was recrystallized twice. The hippurates and mesylglycinate[1] esters were prepared from the acid chloride; derivatives of aminoacetonitrile and ethanolamine and esters of acetylglycine were prepared according to published procedures (Table I). α-*N*-CBz-L-Tyr-*p*-Np was purchased from the Sigma Chemical Co. Acetonitrile was purified according to the method of Lewis and Smyth (1939) and other materials were of analytical reagent standard; deionized water was used throughout the investigation. Analytical and physical properties of the substrates and inhibitors are given in Table I and structures were confirmed by infrared spectra.

Methods. Papain was activated by a modification of the method of Soejima and Shimura (1961). The crystalline suspension of papain was dissolved in sodium chloride solution at the appropriate ionic strength and swirled with a crystal of *p*-thiocresol for about 40 min and then with a little toluene. The suspension of toluene and papain solution was filtered through a moist filter paper which retained the organic phase. The papain solution was assayed roughly by the absorption at 280 mμ using ϵ_{280} 51,100 and mol wt 20,700 (Bender *et al.*, 1966)[2] but a rate assay using the easily available methyl hippurate was routinely employed. Since the absolute kinetic parameters of methyl hippurate have not previously been reported titration experiments with α-*N*-CBz-L-Tyr-*p*-Np (Bender *et al.*, 1966) were used to determine the normality of a standard papain solution. The molarity of enzyme used in this work was normally about 10^{-6} M but, in the case of isopropyl hippurate hydrolyses, was about 10^{-5} M.

Kinetic Measurements. Titration experiments with α-*N*-CBz-L-Tyr-*p*-Np were performed using a Unicam SP800 spectrophotometer according to the method of Bender *et al.* (1966). The hydrolysis of the other esters was measured with a pH-stat (Radiometer SBR2c coupled with a titrator II and a pH meter 25) and corrections at low pH values for the incomplete ionization of the liberated acid were made using pK_a's determined under identical conditions. Rates of hydrolysis often decreased slowly with time at low pH values where product inhibition by the liberated acid was observed and in these cases the progress curves were fitted to a third-order polynomial (eq 5).

$$[\mathrm{NaOH}] = a + bt + ct^2 + et^3 \qquad (5)$$

$$\frac{d[\mathrm{NaOH}]}{dt} = b + 2ct + 3et^2$$

A computer calculated the polynomial which best fitted the experimental date using the least-squares procedure of Booman and Niemann (1956) and then computed the initial rate of uptake of sodium hydroxide. In this way progressive

[1] Mesylglycine = *N*-methanesulfonylglycine.

[2] Recent studies (Drenth *et al.*, 1968) suggest a molecular weight in excess of 20,700 but this does not affect the accurate assays.

TABLE II: Solvent Effect.[a]

% CH_3CN	k_{cat} (sec^{-1})	K_m(app) (mM)
0	4.02 ± 0.51	31.9 ± 9.3
10	4.02 ± 0.21	23.9 ± 3.2
15	3.62 ± 0.59	35.6 ± 8.5
20	3.47 ± 0.25	36.8 ± 4.4

[a] The conditions were: 35°, 0.35 M ionic strength, 0.001 M EDTA, pH 6, methyl hippurate as substrate, [S] = 0.01–0.03 M, [E] *ca.* 10^{-6} M.

denaturation and product inhibition effects were eliminated. Michaelis–Menten parameters were obtained using initial rates of hydrolysis and were computed using a program based on the method of Wilkinson (1961).

Representative Lineweaver–Burk plots are shown (Figures 1 and 2) for isopropyl ester hydrolysis and for inhibition by acylaminoacetonitrile derivatives. Each set of data from the same papain stock solution gave little standard error but the errors increased when different stocks were employed and the comparison of two sets of data depended on the accuracy of the assay. The fitting of kinetic parameters to theoretical functions of the hydrogen ion concentration was carried out using a computer program based on the semiobjective method of Hansen (1962).

Results

Titration Studies. Papain was titrated with α-*N*-CBz-L-Tyr-*p*-Np according to the method of Bender *et al.* (1966); using the corrections and nomenclature described in the above work a plot of $1/\sqrt{\pi}$ *vs.* $1/[S_0]$ was constructed and the papain solution standardized. The K_m for the titrating substrate ($9.1 \pm 1.1 \times 10^{-7}$ M) agreed well with that previously determined (6.3×10^{-7} M) and the papain was found to have a normality 65.5% of that calculated from the concentration measured using the extinction at 280 mμ. An assay with methyl hippurate (0.3 M ionic strength, 35°, pH 6) on the same papain stock showed k_{cat} = 4.02 ± 0.06 sec^{-1} and the standard error in measuring the normality was ± 2.5%. The previous value for k_{cat} for methyl hippurate under the same conditions was 2.72 sec^{-1} based on a rate assay *vs.* α-*N*-Bz-L-ArgEt (Lowe and Williams, 1965) and we regard the present as the most accurate since the original rate assay depended on a protein concentration measurement. Kinetic results (k_{cat}, k_3, and k_2) for substrates of papain previously described (Lowe and Williams 1965) should thus be multiplied by the factor 4.02/2.72 in order to obtain absolute values.

TABLE III: Ionic Strength.[a]

Ionic Strength (M)	0.01	0.06	0.11	0.21	0.31
Relative Rate	0.93	0.99	1.0	1.0	1.0

[a] The conditions were: 35°, pH 6, 0.001 M EDTA, methyl hippurate as substrate, [S] = 2.37×10^{-2} M, [E] *ca.* 10^{-6} M.

TABLE IV: Acetylglycine Substrates.[a]

pH	K_m (M × 10^3)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})
	Benzyl ester ([S] = 0.005–0.01 M)		
4.0[b]	27.2 ± 10.1	1.14 ± 0.32	
4.5[b]	14.8 ± 1.3	1.95 ± 0.10	
5.0[b]	13.0 ± 1.2	2.43 ± 0.13	
6.0	12.6 ± 0.9	2.69 ± 0.12	
7.0	15.9 ± 1.1	2.51 ± 0.13	
8.0	16.7 ± 2.0	2.72 ± 0.24	
8.5	42.7 ± 15.7	2.53 ± 0.79	
	Methyl ester ([S] = 7.6×10^{-2} M)[c]		
4.0[b]			0.493
4.5[b]			1.04
5.0[b]			1.61
6.0			2.00
7.0			1.94
7.5			1.75
	p-Nitrophenyl ester		
6.0	1.23 ± 0.30	2.93 ± 0.42	2390 ± 680

[a] Conditions were: 35°, 10% CH_3CH, 0.3 M ionic strength. [b] Results corrected using pK_a = 3.75 for acetylglycine under above conditions. [c] [S] < K_m throughout the pH range.

Organic Solvents. A study of the effect of acetonitrile on the Michaelis–Menten parameters was made because isopropyl hippurate is not sufficiently soluble in water to obtain accurate kinetics. The results of previous studies (Kirsch and Igelström, 1966) are in accord with our conclusions, and Table II shows that up to 20% v/v acetonitrile–water there is little change in either k_{cat} or K_m; it is concluded that acetonitrile does not act as an inhibitor and that results from media containing up to 20% acetonitrile can be compared with those from aqueous solution, although this extrapolation is not necessary for the comparisons of this work.

Ionic Strength. The effect of changing the ionic strength on the catalytic ability has been determined by Stockell and Smith (1957) using a charged substrate (α-*N*-Bz-L-ArgEt). The results of this investigation using methyl hippurate (Table III) agree with the previous investigation and show little effect on the hydrolysis rate from 0.06 to 0.3 M. It is therefore possible to compare kinetic parameters measured within this range of ionic strength. This is important since previous investigations (Lowe and Williams, 1965; Bender and Brubacher, 1966) have favored 0.3 M ionic strength, whereas at lower strengths papain (and isopropyl hippurate) is more soluble and hence more amenable to kinetic study.

Acetylglycinate Esters. Table IV shows the Michaelis–Menten parameters for acetylglycinate esters. The identity of k_{cat} for the benzyl and *p*-nitrophenyl esters and the pH profile of k_{cat} for the benzyl ester indicate that for these esters $k_3 = k_{cat}$. The methyl ester was interesting in that rate of hydrolysis was proportional to substrate concentration up

TABLE V: Hippuric Acid Substrates.[a]

pH	k_{cat} (sec^{-1})	k_2 (sec^{-1})	K_m (M × 10^3)	K_S (M × 10^3)
Isopropyl ester ([S] = 0.006–0.014 M)				
5.0[b]	0.763 ± 0.098	0.945 ± 0.15	39.8 ± 6.3	49.2 ± 9.8
6.0	0.930 ± 0.174	1.22 ± 0.30	35.5 ± 8.5	46.6 ± 18
7.0	1.02 ± 0.016	1.37 ± 0.03	38.8 ± 6.8	52.8 ± 4.7
8.0	0.636 ± 0.12	0.760 ± 0.16	37.9 ± 8.8	44.3 ± 16
8.5	0.400 ± 0.084	0.444 ± 0.104	34.9 ± 9.6	38.8 ± 15
Methyl ester [(S] = 0.01–0.03 M)				
3.6[b]	3.72 ± 0.37		110 ± 11	
4.0[b]	4.37 ± 0.45		66 ± 6.6	
5.0[b]	4.00 ± 0.21		21.4 ± 2.0	
6.0	4.00 ± 0.40		15.9 ± 0.7	
7.0	4.25 ± 0.27		19.3 ± 2.8	
8.0	4.00 ± 0.18		23.8 ± 2.0	
8.5	4.00 ± 0.45		38.3 ± 0.8	

[a] Conditions were: 10% CH_3CN, 35°, isopropyl ester at 0.1 M ionic strength, methyl ester at 0.3 M ionic strength, 0.001 M EDTA. [b] Results corrected using $pK_a = 3.6$ under above conditions.

TABLE VI: Limiting Values of Kinetic Parameters.[a]

Esters	k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-3})	k_3 (sec^{-1})	k_2 (sec^{-1})
Mesylglycinate			
p-Methoxyphenyl	1.91 (4.45, 8.60)	13.8 (4.25)	
Benzyl	1.84 (4.47, 8.64)	13.3 (4.26)	
Isopropyl	0.00503 (4.4, 8.4)	13.3 (4.26)	1.11 (4.55, 8.60)
Hippurate			
Methyl	246 (4.43, 8.33)	4.02 (pH independent)	
Isopropyl	0.0271 (4.60, 8.34)	4.02 (pH independent)	1.34 (4.60, 8.20)
Acetylglycinate			
Benzyl	207 (4.48, 8.30)	2.76 (4.14)	
Methyl	0.00206 (4.50, 8.30)		

[a] Figures in parentheses are pK_a values.

to the highest concentration used ($<10^{-1}$ M), indicating that, provided an enzyme substrate complex existed, $K_m > 0.1$ M. Division of these pseudo-first-order rate constants by enzyme concentration yields k_{cat}/K_m for this substrate.

Hippuric Acid Esters. Lowe and Williams (1965) indicated that k_{cat} for methyl hippurate closely approximates k_3 and titration work with *p*-nitrophenyl hippurate has confirmed this (quoted in Lake and Lowe, 1966). A separate set of Michaelis–Menten parameters for methyl hippurate was determined at 10% acetonitrile concentration over a pH range and combined with values of k_{cat} and K_m for isopropyl hippurate to give k_2 and K_S (Table V). Studies below pH 5.0 were difficult for the isopropyl ester because the errors involved in the determination of k_{cat} and K_m were of the same order of magnitude as these parameters. The factors giving rise to these large errors are: poor substrate solubility, inhibition of the reaction by products at low pH, and incomplete ionization of the product acid at low pH values. Deacylation (k_3) was essentially pH independent for the methyl ester and a value of 4.02 sec^{-1} was used as k_3 at all pH values.

The independence of k_3 for methyl hippurate in the acid pH range deserves some comment as it differs from the pH dependence for k_3 for most other substrates (Bender and Brubacher, 1966; Williams and Whitaker, 1967). Bender and Brubacher (1966) point out the hazards of pH-stat work at low pH values as opposed to spectrophotometry, but this investigation clearly shows that acid pH profiles for k_3 may be observed in this manner. A possible explanation of the phenomenon is that the amide oxygen of the hippuryl-papain participates in deacylation (competing with the normal process involving a group with an acid pK_a) to form an oxazolone intermediate by expelling the thiol group of papain. It would

TABLE VII: Mesylglycinate Substrates.[a]

pH	k_{cat}/K_m (M^{-1} sec^{-1})[b]	k_{cat} (sec^{-1})	k_2 (sec^{-1})	K_m (M × 10^3)	K_S (M × 10^3)[c]	K_S (M × 10^3)
			Isopropyl ester ([S] = 0.025–0.1 M)			
3.4[d]		0.064 ± 0.005	0.067 ± 0.004	165 ± 19		172 ± 26
4.0[d]	1.04	0.232 ± 0.12	0.244 ± 0.012	137 ± 11	235	144 ± 15
4.5[d]	2.24	0.499 ± 0.007	0.512 ± 0.007	155 ± 4	228	159 ± 9
5.0	3.38	0.711 ± 0.028	0.742 ± 0.03	140 ± 8	212	147 ± 12
6.0	4.21	1.09 ± 0.14	1.18 ± 0.17	214 ± 16	281	233 ± 2o
7.0	4.27	1.07 ± 0.064	1.14 ± 0.07	226 ± 18	267	241 ± 28
8.0	3.43	0.854 ± 0.039	0.92 ± 0.02	237 ± 14	268	256 ± 19
			Benzyl ester ([S] = 0.004–0.018 M)			
3.4[d]		1.85 ± 0.07		9.94 ± 0.84		
4.0[d]		4.93 ± 0.07		10.6 ± 0.3		
4.5[d]		8.95 ± 0.12		8.42 ± 0.25		
5.0		10.9 ± 0.31		9.32 ± 0.56		
6.0		11.8 ± 0.24		6.07 ± 0.32		
7.0		14.3 ± 0.15		7.96 ± 0.18		
8.0		14.1 ± 0.50		10.1 ± 0.7		
8.6		13.1 ± 0.06		13.3 ± 0.1		
8.95		12.1 ± 0.07		20.1 ± 0.2		
			p-Methoxyphenyl ester ([S] = 0.003–0.013 M)			
3.4[d]		1.73 ± 0.09		12.8 ± 1.3		
4.0[d]		5.06 ± 0.41		10.0 ± 1.4		
4.5[d]		9.92 ± 0.71		9.58 ± 1.25		
5.0		10.8 ± 0.42		7.91 ± 0.59		
6.0		13.7 ± 0.22		7.48 ± 0.23		
7.0		13.0 ± 0.78		6.48 ± 0.81		
8.0		14.7 ± 2.1		9.18 ± 2.46		

[a] Conditions were: 35°, 0.3 M ionic strength, 10% CH_3OH. [b] Determined at [S] < K_m. [c] Calculated from k_{cat}/K_m from first-order conditions. [d] Results corrected using pK_a = 3.30 under the above conditions.

be expected that neither mesylglycyl- nor carbobenzoxyglycyl-papain would form an intermediate, and in fact these thio esters hydrolyze normally (Table VI; Williams and Whitaker, 1967). Acetylglycyl-papain, however, should behave similarly to hippuryl-papain and Tables IV and VI show this not to be the case. The cause of the anomalous pH profile for k_{cat} for methyl hippurate, although still lacking a satisfactory explanation, does not affect the validity of the calculations presented here.

Mesylglycinate Esters. There is now little reason to doubt that the observation of a constant k_{cat} for substrates of a common acid reflects a rate-limiting deacylation step in papain-catalyzed hydrolyses. Mesylglycine esters have almost identical values of k_{cat} except for the isopropyl ester; phenyl, *p*-chlorophenyl, *m*-nitrophenyl, and *p*-nitrophenyl esters have k_{cat} values, respectively: 10.8, 11.0, 12.5, and 11.0 sec^{-1} at pH 6 (E. C. Lucas and A. Williams, 1968, unpublished data). To show that k_3 was rate limiting over the whole pH range two labile ester substrates (benzyl and *p*-methoxyphenyl) were investigated and found to have identical values of k_{cat} (within experimental error) over the entire pH range. The k_{cat} values used in Table VII to estimate k_2 and K_S were calculated from the limiting data of Table VI for the benzyl ester. An alternative calculation of K_m (and hence of K_S) was *via* k_{cat}/K_m from pseudo-first-order kinetics at [S] < K_m (see Table VII) and k_{cat} derived from the Michaelis–Menten analysis; the resulting K_S is plotted *vs.* pH in Figure 2. A more accurate pH dependence for K_m was thus obtained as the same enzyme stock was used throughout the pH range. In order to check the ionization of the sulfonamide NH in the pH range employed for these substrates isopropyl mesylglycinate was titrated with sodium hydroxide up to the higher pH limit, but no ionization was observed.

Inhibitors. The inhibitors tested here (Table VIII) were shown to be competitive *vs.* the substrate methyl hippurate; two inhibitor concentrations were employed and the variation of the observed K_m for methyl hippurate was used to calculate K_I.

Binding. Since the composite parameter (K_S) is essentially independent of pH while k_2 varies significantly (Tables IV and VII; Figures 3 and 4) either k_2, k_1 and k_{-1} have identical bell-shaped pH profiles or k_2 is much less than k_{-1} (eq 3). Of these possibilities the latter is the most attractive since k_{-1} is a physical process involving the diffusion of substrate from the enzyme surface and possibly the fission of hydrogen bonds, whereas k_2 involves the chemical fission of an ester linkage.

TABLE VIII: Competitive Inhibition Constants.[a]

	pH	K_I (M × 10^3)
Acetamidoacetonitrile[b]	3.6	33.3 ± 5.0
	4.0	34.1 ± 2.6
	5.0	39.6 ± 4.7
	6.0	40.8 ± 4.1
	7.0	30.7 ± 2.3
	8.0	34.2 ± 5.0
	8.5	35.3 ± 4.6
Benzamidoacetonitrile[c]		
	4.0	0.379 ± 0.036
	5.0	0.427 ± 0.021
	6.0	0.382 ± 0.034
	7.0	0.386 ± 0.057
	8.0	0.419 ± 0.026
	8.5	0.357 ± 0.032
2-Benzamidoethanol		
	6.0	>1000
2-Acetamidoethanol[d]		
	6.0	>1000

[a] Conditions were: 35°, 0.3 M ionic strength, 0.5% CH_3CN, [S] = 0.01–0.03 M. [b] Inhibitor concentration, 0.0408 and 0.0206 M. [c] Inhibitor concentration, 0.001 and 0.0005 M. [d] Inhibitor had poor analysis but a good infrared spectrum.

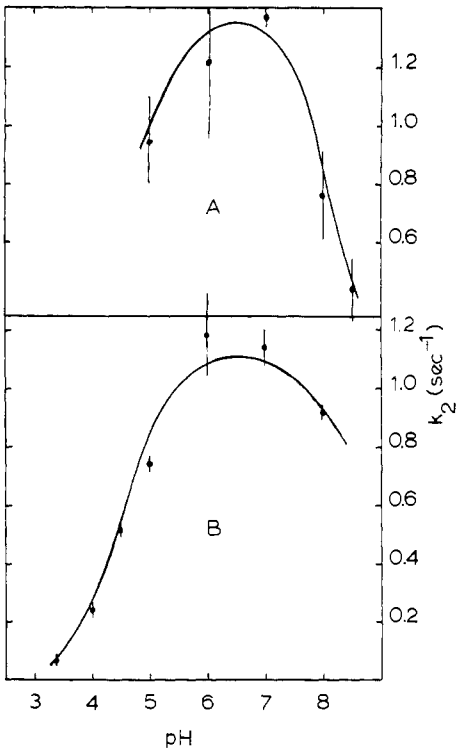


FIGURE 3: Variation of k_2 with pH for isopropyl hippurate (A) and isopropyl mesylglycinate (B); conditions as in Table IV and VII; lines calculated from values in Table VI.

The value of K_S is thus equivalent to a true binding constant k_{-1}/k_1 which is therefore pH independent. The small trend observed in K_S for each isopropyl ester studied here might be caused by a small incursion of k_2; however, in the absence of more positive evidence we ascribe this trend to a microscopic medium effect, but it should be pointed out that the variation is close to the experimental error in determining K_S.

Discussion

The pH dependencies for k_{cat}/K_m (= k_2/K_S) for the substrates studied here (Table VI) are in agreement with those for all other substrates of papain and the assignment of the acid and base pK_a's (Table VI) to either k_2 or K_S has been the subject of much discussion. Previous authors have concluded that K_S is pH independent while k_2 has a bell-shaped dependency (Lowe and Williams, 1965; Bender and Brubacher, 1966; Whitaker and Bender, 1965). The results of this communication demonstrate this conclusion directly (Figures 3 and 4 and Table VI).

The conclusion that K_S is pH independent is supported by studies with *N*-carbobenzoxyglycine amide (Sun and Chou, 1963); k_{cat} has a bell-shaped pH profile, while K_m is invarient from pH 4 to 7; recent work has shown k_3 to be almost certainly sigmoid (Williams and Whitaker, 1967) and larger than k_{cat} for the amide, indicating that k_2 probably has a bell-shaped pH profile and that K_S is pH independent.

Results from different laboratories can be combined to yield a K_S and k_2 pH profile for the papain-catalyzed hydrolysis of carbobenzoxyglycylglycine. Williams and Whitaker (1967) found that k_{cat} (limiting value = 5.2 sec^{-1}) for *p*-nitrophenylcarbobenzoxyglycine had a sigmoid pH profile. Smith *et al.* (1958) measured the hydrolysis of the corresponding glycine peptide; k_{cat} (0.4–0.9 sec^{-1}) was always much less than that for the *p*-nitrophenyl ester; k_2 and K_S are therefore

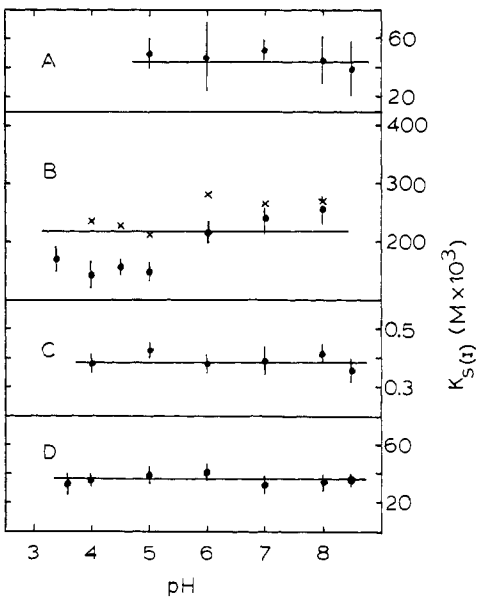


FIGURE 4: Variation of K_S (K_I) with pH for isopropyl hippurate (A), isopropyl mesylglycinate (B), from Michaelis–Menten analysis (X, from pseudo-first-order rate constant), benzamidoacetonitrile (C), and acetamidoacetonitrile (D); conditions as in Tables V, VII, and VIII.

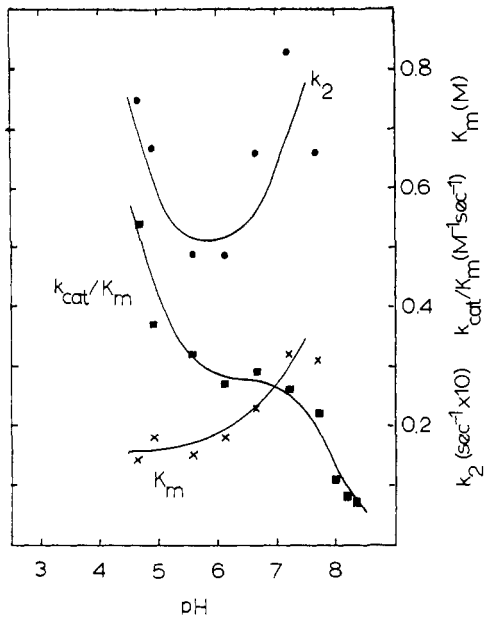


FIGURE 5: Effect of pH on carbobenzoxyglycylglycine as a substrate of papain. Parameters calculated from Smith *et al.* (1958) and Williams and Whitaker (1967).

calculable according to eq 2 and 3. The increase in K_S at pH values above 7, although at variance with results for other substrates, is reminiscent of the pH dependencies of inhibition constants for acylamino acid anions and chymotrypsin (Johnson and Knowles, 1967); as the base group ionizes the overall charge at the active site becomes negative and hence has less attraction for the negatively charged substrate. An alternative explanation is that a "distant" group on ionization causes a geometry change at the binding site. The results of this investigation favor the former conclusion since uncharged substrates and inhibitors have pH-independent binding constants and it is therefore probable that the group giving rise to the base ionization (expressed in k_2) is close to the active site and is probably identifiable as the thiol of cysteine-25.[3] The increase in k_{cat}/K_m for the hydrolysis of carbobenzoxyglycylglycine as the pH decreases has been attributed (Smith *et al.*, 1958) to an interaction of the negatively charged substrate with the active site which is poor at base pH values but good at acid pH values where a base at the active site has become neutralized. However, an examination of Figure 5 shows that the increase reflects a rise in k_2 rather than in binding (essentially K_S).

Support for the identification of K_S for the isopropyl esters with a true binding constant is derived from the observation that benzamidoacetonitrile and acetamidoacetonitrile have pH-independent competitive inhibition constants (Table VIII). These reversible inhibitors are similar in structure to substrates in possessing an amide and a polarized multiple bond. It is significant that 2-acetamidoethanol and 2-benzamidoethanol which also resemble substrates are not inhibitors at all ($k_{-1}/k_1 > 1$ M); we conclude that two binding interactions are necessary for the association of a compound with papain (neither acetonitrile nor benzamide alone inhibit the enzyme). One binding locus on the enzyme attracts the amide moiety while the other locus attracts the polarized multiple bond; the hydroxyl in the ethanolamine derivatives does not act as a binding group. The existence of a third locus on the enzyme usually identified as a negative group which attracts the ϵ-ammonium group of lysine derivatives (possibly aspartate-160~; Drenth, *et al.*, 1968) has little effect on the magnitude of K_S (compare Tables V and VII with $K_S = 10.7.10^{-3}$ M for methyl α-*N*-carbobenzoxylysinate; Bender and Brubacher, 1966); this third locus probably helps to orientate the α-carbonyl group.

Any mechanism for papain must predict the pH independence of the binding constant (k_{-1}/k_1) and the groups constituting the binding loci on the enzyme therefore do not ionize in the pH range studied here.

A comparison of the binding ability of the acetonitrile inhibitors (Table VIII) shows that the acetyl derivative is a poorer inhibitor than the benzamidoacetonitrile (possibly owing to hydrophobic attraction in the latter case). This relationship is reflected exactly in the comparison between k_{cat}/K_m for the substrates methyl acetylglycinate and methyl hippurate (Table VI). We ascribe the difference in k_{cat}/K_m for these substrates to a difference in K_S and a constant k_2. The ratio of k_{cat}/K_m for the *p*-nitrophenyl esters of acetylglycinate (Table IV) and hippurate (Lowe and Williams, 1965) is also the same as for the K_i values of the acetonitrile derivatives. The view that binding and acylation are mutually exclusive is supported by the observations that, although K_S differs for the two isopropyl esters studied here, the k_2 values are almost identical (Table VI).

Relationship of the Results to Possible Mechanisms. The results of this investigation show that any mechanism proposed for papain must predict that acylation (k_2) has a bell-shaped pH profile with pK_a's ~ 4.5 and ~ 8.5 and that deacylation (k_3) has a sigmoid pH dependency with pK_a ~ 4. It is our view, and that of most authors (Bender and Brubacher, 1966; Kirsch and Igelström, 1966), that the acid pK_a in both acylation and deacylation is due to the same ionizing group, and that deacylation is the microscopic reverse of acylation.

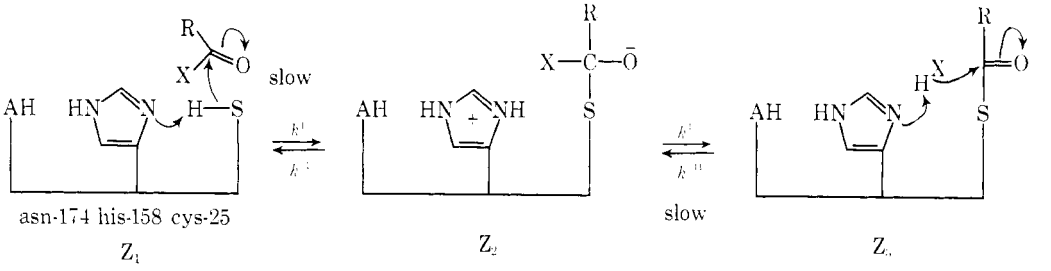
Other evidence that must be considered when evaluating proposed mechanisms is as follows. Papain-catalyzed hydrolyses proceed *via* an acyl-enzyme in which the thiol group of cysteine-25 is acylated (Lowe and Williams, 1965; Husain and Lowe, 1965); the work of Husain and Lowe (1968) and the X-ray structure (Drenth *et al.*, 1968) show that the imidazole side chain of histidine-158 is only about 4 Å from cysteine-25. Other groups which the X-ray structure shows to be around the active site are aspartic-157 and -64 (*ca.* 10 Å from cysteine-25), asparagine-174 which is hydrogen bonded to the imidazole of histidine-158 (Drenth, 1968), and tryptophan-176.

The easiest explanation for the absence, in deacylation, of the base pK_a (present in acylation) is that the ionization of the thiol is effectively masked by the acyl group (Bender and Brubacher, 1966; Bender and Kézdy, 1965). This is supported by independent observations of a pK_a of ~ 8.5 for a thiol group using fluorescence emission measurements (Barel and Glazer, 1969) and using irreversible inhibitors (Bender and Brubacher, 1966).

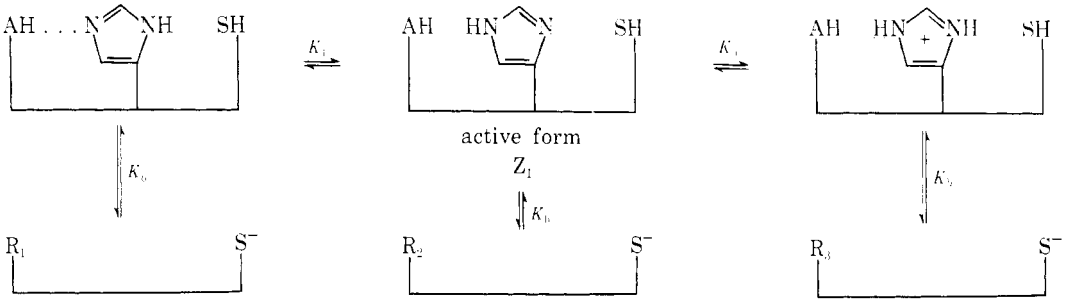
The pH dependence for k_2 implies that the thiolate anion

[3] The numbering of amino acids residues in papain is that from the X-ray structure (Drenth *et al.*, 1968).

SCHEME I: Proposed Mechanism

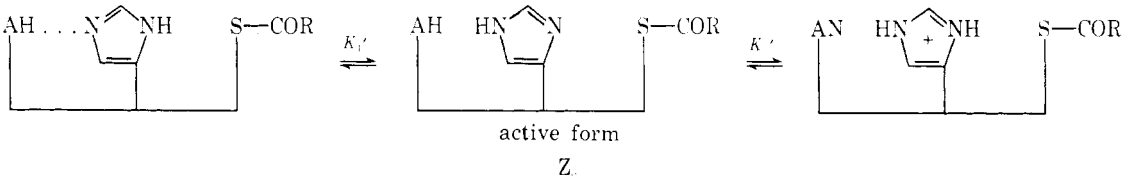


Ionization of Z_1 in Acylation



$$[Z_1] = (1/K_1)[\text{Total E·RCOX}]/(1 + [H]/K_1K_a)(1 + K_b/[H])$$

Ionization of Z_3 in Deacylation



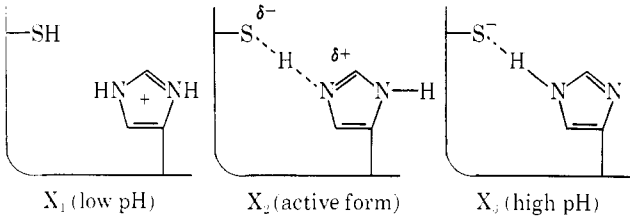
$$[Z_3] = (1/K_1')[\text{Total ECOR}]/(1 + [H]/K_1'K_a')$$

is less nucleophilic than the thiol group but this anomaly can be explained in two ways. (a) Acylation is due to a concerted process involving an acid and thiolate anion where the concentration of reactive species would give the correct pH dependency; this explanation is not satisfying because the maximum proportion of active enzyme (in the "zwitterionic" form) would be 1/10,000th of the total available protein when the two pK_a's are separated by about 4 pK_a units. A more definite exclusion of the action of the "zwitterionic" form is possible; the *p*-nitrophenyl ester of α-*N*-carbobenzoxylysine has a rate constant of $2.6.10^7$ M^{-1} sec^{-1} for the *overall* formation of acyl-papain from free enzyme and substrate (k_{cat}/K_m). If the active enzyme was 10^{-4} of the total available protein the active enzyme and substrate would have to come together at a rate of $2.6.10^{11}$ M^{-1} sec^{-1} to achieve the above rate constant. This figure is in excess of the diffusion rate constant normally found for such reactions (Eigen and Hammes, 1963). (b) The formation of a negative thiolate anion could cause an unfavorable change in geometry of the active site. The possibility that the alkaline pK_a is due to an unfavorable geometry at the active site caused indirectly by the ionization of a group ($pK_a \sim 8.5$) far from the active site (similarly to chymotrypsin; Bender *et al.*, 1966b; Sigler *et al.*, 1968) cannot be completely ruled out but there is as yet no positive evidence.

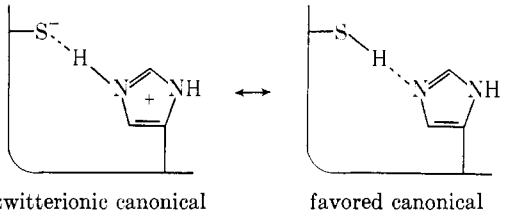
The acid pK_a is also anomalous because it lies in the region attributed to the ionization of carboxylic acids, and there is no evidence available for the existence of such a group close to the thiol cysteine-25 (Drenth *et al.*, 1968). Two explanations seem possible: (a) if the group is involved covalently, for example as a general acid or base, then the pK_a of one of the available groups close to the thiol must be altered (*e.g.*, the imidazole of histidine-158 changes its pK_a from 6 to 4.5); (b) the active-site geometry could be controlled by a distant carboxylate anion.

We propose a new mechanism for papain-catalyzed hydrolyses (see Scheme I) which involves a free imidazole acting as a general base attacking a thiol proton in acylation (k^1) or a water (or nucleophile) proton in deacylation (k^{-11}). We postulate that the apparent pK_a (in acylation and deacylation) of the imidazole is lowered by the donation of a hydrogen bond from the amide nitrogen of the adjacent asparagine-174 to the tertiary nitrogen of the imidazole ring. Such a hydrogen-bonded imidazole could not act as a general base but it is the *free* imidazole which is active. The equations in Scheme I show how the apparent pK_a is related to the normal pK_a for imidazole and the equilibrium constant for hydrogen bonding. To reduce the pK_a from 6 to 4 requires an equilibrium constant (K_1 and K_1') between free and hydrogen-bonded species of about 10^2; this would give rise to a free energy

SCHEME II: Mechanism of Husain and Lowe (1968)



SCHEME III: Resonance Interpretation of Hydrogen Bond between Thiol and Imidazole.



difference of about 2–3 kcal/mole which can easily be supplied by a hydrogen bond of the type N–H---N (see, *e.g.*, Pauling, 1960).

The shift in pK_a of imidazole of histidine-15 from 6 $\sim$ 6.5 to about 5 in lysozyme (Piszkiewicz and Bruice, 1968) is good supporting evidence for the type of mechanism proposed here; in lysozyme the imidazole is made more acidic by hydrogen bonding with the proton of threonine-89. It can be calculated that the proportion of active papain (Z_1 or Z_3) is $1/K_1$ of the total available enzyme. That is, one-hundredth of the total papain is utilized. The thiol group is not hydrogen bonded and gives rise to the normal pK_a of 8.5. The effect of pH on the concentration of the intermediate species (Z_2) is immaterial to the observed pH dependencies of acylation or deacylation, because steps k^{-1} and k^{11} (see Scheme I) have

$$\text{forward reaction (acylation)} \qquad k_f = \frac{k^1 k^{11}}{(k^{-1} + k^{11})} \tag{6}$$

$$\text{reverse reaction (deacylation)} \qquad k_r = \frac{k^{-11} k^{-1}}{(k^{-1} + k^{11})} \tag{7}$$

identical pH profiles since these reactions are essentially the same; eq 6 and 7 show that all pH variations cancel except those due to k^{-11} and k^1 which are sigmoid and bell shaped, respectively.

The mechanism proposed by Husain and Lowe (1968) deserves some comment as it predicts a bell-shaped pH dependency for k_2 with pK_a's of the correct order. The reactive species (X_2 in Scheme II) was proposed to be a thiol hydrogen bonded to an imidazole and the pH dependence of its concentration is bell shaped. The thiol–imidazole hydrogen bond effectively lowers the pK_a of the imidazole by about two units and raises the pK_a of deprotonation of the *system* above that of the imidazolium (normally $\sim$ 6.5). A disadvantage of the scheme is that it requires an extra mechanism to explain the acid pK_a in deacylation since the thiol is now masked by the acyl group. A further criticism concerns the base pK_a of 8.5 observed in acylation; Husain states that the hydrogen-bonded reactive species deprotonates with a pK_a intermediate between thiol and imidazolium (*ca.* 8.5 and 6.5, respectively) but the observed pK_a is closer to that of a thiol. Their argument implies that the important canonical in the resonance structure for the hydrogen bond is the zwitterion structure (Scheme III) and these are known not to contribute much to the structure of the hydrogen bond especially where the bond is weak (Murrell, 1969; Bellamy, 1958).

The favored canonical of the resonance structure is one where there is no charge separation (Scheme III) and here the pK_a for deprotonation should be intermediate between that of a thiol ($\sim$8.5) and that of an imidazole ($\sim$13), but this is not the observed result. We do not believe that mechanisms involving thiol as hydrogen-bond donors are valid for the above reasons and because there is little evidence for the existence of such hydrogen bonds in the literature (Bellamy, 1958) and also in the X-ray model (Drenth, 1968). Hydrogen bonds between the thiol and an acceptor where they have been observed (Bellamy, 1958) are very weak and in order to obtain a decrease in pK_a an equilibrium constant between free and hydrogen-bonded thiol must be favorable to the bonded state (to the extent of 100-fold for a shift of two pK_a units). Such strong hydrogen bonding is conceivable between the amide nitrogen of asparagine and imidazole but not between a thiol and imidazole.

Concluding Remarks

Papain resembles the corresponding serine proteinase chymotrypsin in that both enzymes have similar molecular weights, catalyze reactions *via* an acyl-enzyme, and have a similar structure at the active site. We have shown that the more detailed mechanisms are analogous: apart from the quantitative difference in pK_a, binding of substrate and inhibitor is pH independent in papain but depends on a pK_a of about 8–9 in chymotrypsin, but a conformational change has not yet been observed in papain. Differences in proposed mechanisms may be resolved with time but at the present the major difference is the absence of hydrogen bonding of imidazole to the nucleophile (thiol) in papain and the presence of such hydrogen bonding (to serine) in chymotrypsin.

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