Kinetics and Thermodynamics of the Reactions of Acyl-Papains. Effects of pH, Temperature, Solvents, Ionic Strength, and Added Nucleophiles*

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ABSTRACT: The rates of hydrolysis, alcoholysis, and aminolysis of 3-(2-furyl)acryloyl-papain depend on the basic form of an enzyme group with a pK_a of approximately 4.7. The heat of ionization of this residue is very close to zero, suggesting that it is a carboxyl group. Effects of variations in ionic strength and solvent composition of the medium are by contrast more like those expected from the ionization of an amine. Thus the usual classical methods for assigning kinetic pK_a's give conflicting conclusions with respect to the assignment of the kinetically important acidic pK_a of papain. The possibility that the valley in the high pH region of the pH *vs.* rate profile for the hydrolysis of acyl-papains may represent general acid catalysis by a protonated histidine was investigated through a study of the rates of reaction of furylacryloyl-papain with amines and alcohols as a function of pH. Decreases in rates were observed for all of the acyl-transfer reactions between pH 8 and 11, but the pH *vs.* rate profiles were in no case characteristic of a single ionizing group. These observations are difficult to reconcile with a proposed general acid catalysis mechanism. The thermodynamic stability of hippuryl-papain at pH 6.0 is about 3 kcal greater than that of ethyl hippurate, and is estimated to be about 8 kcal greater than a model thiol ester. These calculations suggest that the enzyme would be acylated to a significant degree by carboxylic acid products at low pH.

It is well established that the papain-catalyzed hydrolysis of esters proceeds *via* the formation of a covalent thiol ester intermediate formed from the acyl moiety of the substrate and the sulfhydryl group of papain. The deacylation reactions of acyl-papains have been studied both in the steady state with substrates for which deacylation is rate determining and with isolated, metastable acylenzymes. In all cases the deacylation reactions have been found to depend on the basic form of an enzyme group with a pK_a of 3–5. These findings have recently been reviewed by Lowe (1970). It has been suggested that the enzyme group ionizing in the range pH 3–5 is either a carboxyl (Smith and Kimmel, 1960) or an imidazole (Husain and Lowe, 1968) required in basic form as a general base catalyst, or that it is a carboxylate group required for the proper conformation of the enzyme (Sluyterman and Wolthers, 1969). Hydrolysis of the acyl-papain intermediate is independent of pH from pH 5.5 to 9, but at pH values above 9 the pH dependence appears to vary with the acyl group. Williams and Whitaker (1967) have reported that the hydrolysis of benzyloxycarbonylglycyl-papain is independent of pH from pH 5.5 to 9.5, while some decrease in rates at pH values above 9 has been noted with α-*N*-benzyloxycarbonyl-L-lysyl- (Bender and Brubacher, 1966), *trans*-cinnamoyl- (Brubacher and Bender, 1966), 3-(2-furyl)acryloyl- (Hinkle and Kirsch, 1970), and 3-(3-indolyl)acryloyl- (Hinkle, 1970) papains. Sluyterman and Wolthers (1969) have suggested that the pH dependence exhibited by these latter substrates results from the deprotonation of an imidazole residue (pK_a = 9.5–10) which is required in acidic form to act as a general acid catalyst in the deacylation reaction. It is known from the structure of crystalline papain, determined by X-ray crystallography at 2.8-Å resolution, that an imidazole is located 3.4 Å and an aspartate 7.5 Å from the active thiol group (Drenth *et al.*, 1970). Any of the mechanisms mentioned above, therefore, is plausible in view of the structure of papain.

This communication reports the results of studies of the pH dependence of the deacylation reactions of stable acyl-papains. On the basis of results obtained with other enzymes and with model compounds it is expected that the responses of a carboxyl group to changes in temperature, ionic strength, and dielectric constant will be quite different from the responses of an imidazole group. In an attempt to identify the essential amino acid with a pK_a of 3–5, the effect of these variables on the pK_a of FA-papain[1] hydrolysis has been investigated. The reactions of FA-papain were also studied at high pH in water and in the presence of added nucleophilic amines and alcohols. The experiments were designed to determine whether deacylation of the acyl-papain intermediate requires the acidic form of an enzyme group which acts as a general acid catalyst.

Experimental Section

Materials. FA- and IA-papain were prepared as previously described (Hinkle and Kirsch, 1970). Glycinamide·HCl (Aldrich) was recrystallized from ethanol–water, and methoxyamine·HCl (Eastman) was sublimed. $TrpNH_2$·HCl was purchased from Miles Laboratories or from Sigma and benzyl alcohol was from Matheson Coleman & Bell.

Methods. Glycinamide and methoxyamine hydrochlorides were stored in concentrated solution at 4°. $TrpNH_2$·HCl was stored in solution under nitrogen and protected from light.

Immediately before use amine solutions were adjusted to

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[1] The abbreviations used are: FA, 3-(2-furyl)acryloyl; IA, 3-(3-indolyl)acryloyl.

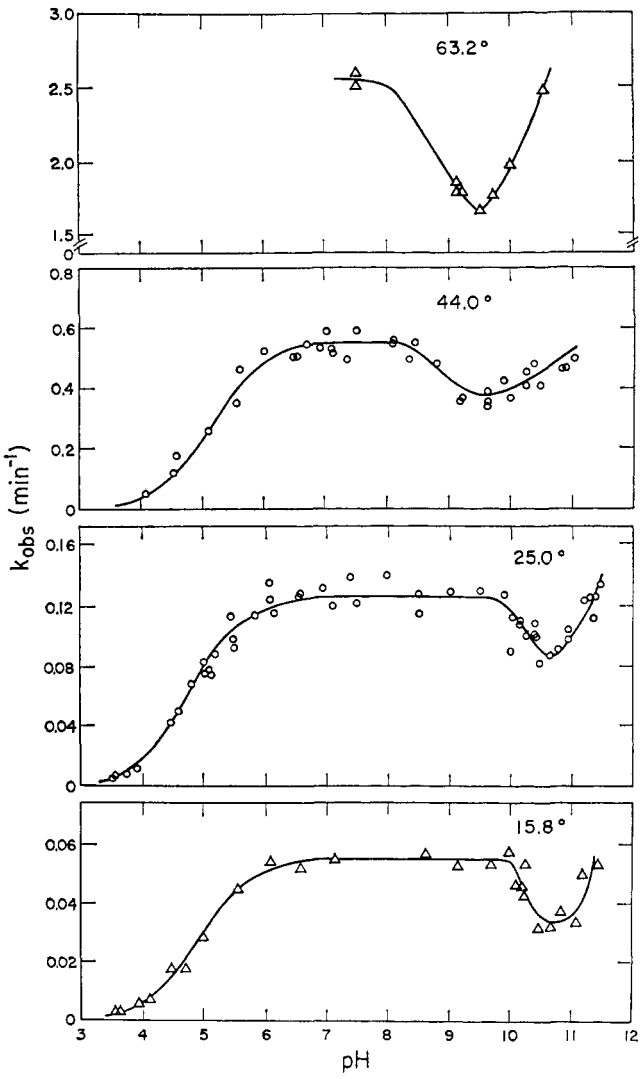


FIGURE 1: The pH dependence of the rates of reaction of FA-papain at various temperatures. The conditions were 50 mM buffers, 0.5 mM EDTA, ionic strength 0.50, and the temperatures indicated. The acid limbs of the pH–rate curves are theoretical for the values of pK_a and k_{lim} given in Table I.

the pH of a kinetic run with concentrated KOH and the amine was added to the appropriate buffer solution before the addition of acyl-enzyme. Buffers and the methods of following the rates of the reactions of acyl-papains have been described (Hinkle and Kirsch, 1970). The pK_a values of the amines were determined by titration at the ionic strength of the experiment in which they were used. Values of pK_a and k_{lim} for the reactions of FA-papain were calculated using program HYPERB (Hanson *et al.*, 1967).

Results

Temperature Dependence of the Reactions of FA-Papain. Since the heats of ionization of carboxyl groups are typically in the range −0.6 to +2.1 kcal per mole while those of imidazole groups are in the range +6.9 to +7.5 kcal per mole (Greenstein and Winitz, 1961), a study of the effect of temperature on the pH dependence of the hydrolysis of the isolated FA-papain could be of aid in assigning the group responsible for this pK_a. The results shown in Figure 1 indicate that varying the temperature from 15.8 to 44° has very little effect on

TABLE I: Values of the Acidic pK_a Observed in the Hydrolysis of FA-Papain under Various Conditions.

Temp (°C)	pK_a	90% Confidence Limits for pK_a	k_{lim} (min^{-1})	90% Confidence Limits for k_{lim} (min^{-1})
15.8[a]	4.93	4.86–4.99	0.0548	0.045–0.062
44.0[a]	5.15	4.99–5.27	0.556	0.310–0.690
25.0[a]	4.81	4.76–4.88	0.126	0.107–0.141
25.0[b]	4.63	4.58–4.72	0.132	0.097–0.157
25.0[b,c]	3.91	3.86–3.94	0.804	0.705–0.887

[a] Values of pK_a and k_{lim} were obtained from the data in Figure 1, ionic strength 0.5. [b] Hinkle and Kirsch (1970), ionic strength 0.05. [c] In 30% (v/v) dioxane.

the acid limb of the pH–rate profiles. Computed values for the acidic pK_a and for the rate constant on the plateau regions of the pH–rate curves (k_{lim}) at various temperatures are given in Table I, which also includes the results of earlier experiments carried out at 25° and ionic strength 0.050, and at 25° in 30% dioxane–water mixtures.

The heat of ionization, ΔH_i, is defined by eq 1. In Figure 2

$$\Delta H_i = RT^2 \frac{(\mathrm{d} \ln K_a)}{\mathrm{d}T} = 2.303R \frac{(\partial \mathrm{p}K_a)}{\partial 1/T} \qquad (1)$$

the pK_a values for FA-papain hydrolysis are plotted against $1/T$. The dashed line is drawn for a hypothetical histidine with ΔH_i = +7.5 kcal/mole and the dotted line is for acetic acid,

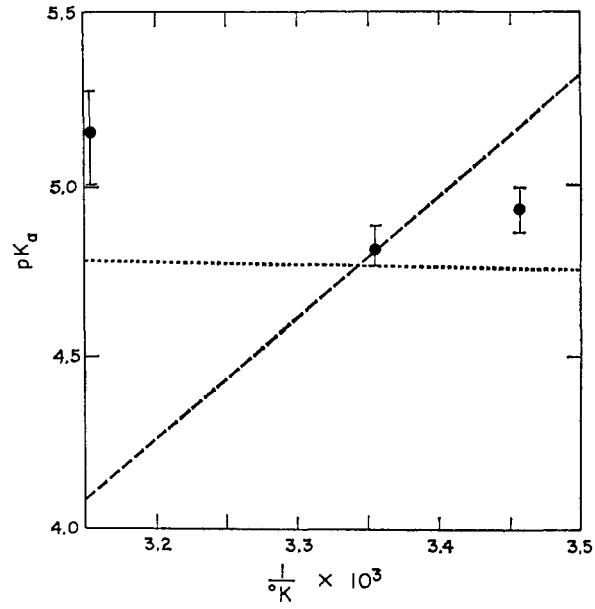
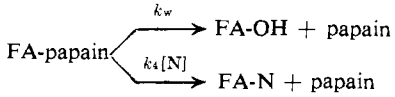


FIGURE 2: The pK_a as a function of temperature for the acidic group involved in FA-papain hydrolysis. (●) The pK_a values for FA-papain hydrolysis, from Table I. The error flags represent the 90% confidence levels of the determinations. (- - -) The temperature dependence of the ionization of acetic acid based on ΔH_i = −0.092 kcal/mole and a pK_a = 4.76 at 25° (Harned and Owen, 1950). (— — —) The temperature dependence of the ionization of a hypothetical histidine with ΔH_i = +7.5 kcal/mole and an arbitrarily chosen pK_a = 4.805 at 25°.

SCHEME I



chosen to represent a typical carboxylic acid. The heat of ionization associated with the acidic limb of the pH–rate curve for FA-papain hydrolysis is near zero and not that expected for a normal histidine residue. Smith and his coworkers studied the temperature dependence of reactions of papain with a number of substrates under steady-state conditions, and also found the heat of ionization of the acidic enzyme group to be near zero (Smith and Parker, 1958; Smith *et al.*, 1958; Stockell and Smith, 1957). The measured rate constants were complex parameters for the previously studied substrates reflecting either in part or primarily the rates of acylation. Therefore it appears that the same enzyme group with a heat of ionization close to zero is required for both acylation and deacylation. On the basis of the temperature dependence of the pH–rate curves alone it might be concluded that the ionizing group is either a glutamic or aspartic acid. However, as shown in Table I and as previously discussed (Hinkle and Kirsch, 1970; Lowe, 1970) the effect of 30% dioxane is to decrease the acidic pK_a for deacylation, a result expected for the ionization of an amine. This point will be discussed further below.

The pH–rate profiles for the reactions of FA-papain in water are characterized by a valley at higher pH values at all temperatures (Figure 1). Similar pH dependence has been observed for the hydrolysis of *trans*-cinnamoyl- and IA-papain (Brubacher and Bender, 1966; Hinkle, 1970). As previously discussed, the minimum in the pH–rate profile for the reactions of stable acyl-papains probably occurs because pH-dependent deacylation and denaturation reactions both proceed at appreciable rates between pH 9 and 12 (Brubacher and Bender, 1966; Hinkle and Kirsch, 1970). The observed rate constants, obtained from the decrease in absorbance at λ_{max} of the acyl-enzymes, measure the total rate of disappearance of native acyl-papain and may therefore be complex rate constants including terms for both hydrolysis and denaturation. As shown in Figure 1, the shape and minimum value of the valley in the pH–rate curves is strongly temperature dependent. As the temperature is raised the observed rates of deacylation begin to decrease at lower pH values and continue to decrease over a wider range of pH. In our previous study at 25° and low ionic strength (0.050) the valley in the pH curves for FA-papain deacylation was spread over a considerably broader range of pH than that now observed at the same temperature and an ionic strength of 0.500 (Figure 1), but in both cases the magnitude of the dip is about 30% below the plateau. However, it is difficult to interpret pH–rate data for the reactions of acyl-papains in water because both denaturation and hydrolysis take place at high pH. For this reason the reactions of FA-papain were studied in the presence of added amines and alcohols.

Reactions of Acyl-Papains with Added Nucleophiles. In the presence of added nucleophilic compounds the total rates of deacylation of acyl-papains are increased due to direct attack of the nucleophile on the acyl-enzyme (Brubacher and Bender, 1966, 1967). In Scheme I k_w is the observed rate constant in water; k_w will equal the rates of hydrolysis at moderately acidic or neutral pH but will include both the rates of hydrolysis and denaturation in base. It is expected that in the presence of amines or alcohols the *total* rate of deacylation will be greatly increased while the rates of hydrolysis and denaturation should not change very much.[2] Under such conditions the pH dependence of the reaction between acyl-enzyme and nucleophile may be determined from the difference between the rates of acyl-papain disappearance in the presence and absence of nucleophile

$$k_{obsd} = k_w + k_4[N] \quad (2)$$

so that at any pH value:

$$k_4 = \frac{k_{obsd} - k_w}{[N]} \quad (3)$$

In this manner the pH dependence of the rates of *deacylation* (k_4) can be obtained despite the existence of concurrent denaturation. Accordingly, the reactions of FA-papain with added nucleophiles have been studied over the entire range of pH.

The rates of reaction of FA-papain in the presence of added nucleophilic compounds obeyed first-order kinetics under all conditions used, and the values of k_{obsd} were shown to be linearly dependent on the concentrations of amines and alcohols, indicating that the reactions are adequately described by Scheme I and eq 3. However, there is evidence that in the steady-state reactions of papain with ester substrates, nucleophiles do bind to the acyl-enzyme (Fink and Bender, 1969; Hinkle and Kirsch, 1971).

In an attempt to observe saturation of the rates of deacylation by a nucleophile the reactions of IA-papain were measured in the presence of increasing concentrations of $TrpNH_2$, the most selective of the nucleophiles thus far examined in its reactions with acyl-papains (Brubacher and Bender, 1966, 1967). Since the rates of IA-papain hydrolysis are 20- to 40-fold slower than those of other acyl-papains (Hinkle and Kirsch, 1970) it was possible to measure the reactions with $TrpNH_2$ over a very wide range of amine concentration with this acyl-enzyme. As shown in Figure 3, k_{obsd} is linearly dependent on $TrpNH_2$ concentration over the entire range of amine concentration examined (0–0.04 M). At a $TrpNH_2$ concentration of 0.04 M the total rate of deacylation of IA-papain was 1120 times the rate of hydrolysis and the half-time for deacylation was less than 5 sec. It may be concluded from these results that if $TrpNH_2$ does bind to IA-papain then the dissociation constant of the acyl-enzyme–nucleophile complex must be considerably greater than 0.04 M.

The pH dependence of k_{obsd} for the reactions of FA-papain in the presence of 73 mM methoxyamine is shown by the solid line in Figure 4. Values of k_4 were calculated assuming that only the free base form of methoxyamine ($pK_a = 4.7$) reacts with FA-papain, *i.e.*

$$k_4 = \frac{(k_{obsd} - k_w)}{[N]_{total}} \left(\frac{[H^+]}{K_a} + 1 \right) \quad (4)$$

[2] Rossi and Bernhard (1971) have shown that the nucleophile, hydroxylamine, effects parallel increases in the rates of deacylation and of denaturation of acyl-chymotrypsins near neutral pH in the presence of the denaturating agents urea, guanidine·HCl, or sodium dodecyl sulfate. These results have been interpreted in terms of the partitioning of a common intermediate, formed from acyl-enzyme and nucleophile, between denatured and deacylated products. The present experiments, performed at high pH in the absence of added denaturants, are unlikely to be subject to an analogous explanation because the mechanism(s) of protein denaturation at high pH are different from those mediated by the denaturing agents listed above (Tanford, 1968).

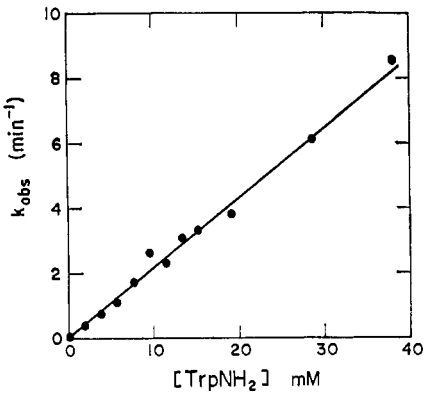


FIGURE 3: The reactions of IA-papain with $TrpNH_2$. The conditions were: 25°, 45 mM sodium pyrophosphate buffer (pH 8.25), 0.45 mM EDTA, and 4.75% acetonitrile, and ionic strength 0.45.

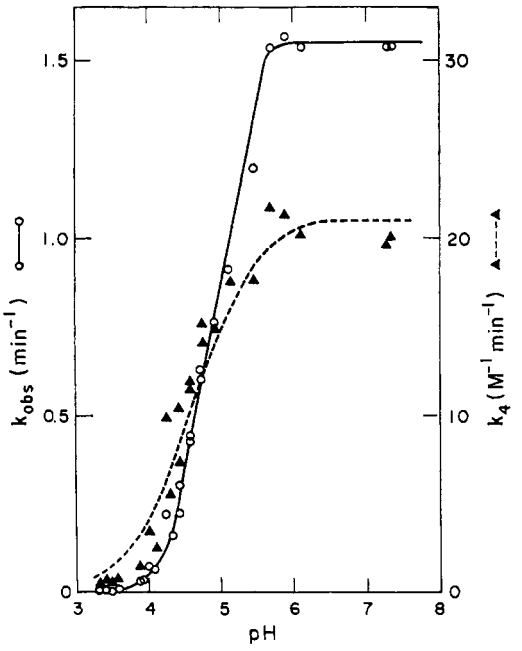


FIGURE 4: The reaction of FA-papain with methoxyamine. The conditions were: 25° in 0.05 M buffers containing 0.5 mM EDTA, 0.073 M methoxyamine, and an ionic strength of 0.500. The value of k_4 was calculated from the concentration of the free base form of the amine based on a pK_a value for methoxyamine of 4.70. The dashed line is theoretical for an enzyme pK_a = 4.6 and k_{lim} = 21.0 M^{-1} min^{-1}.

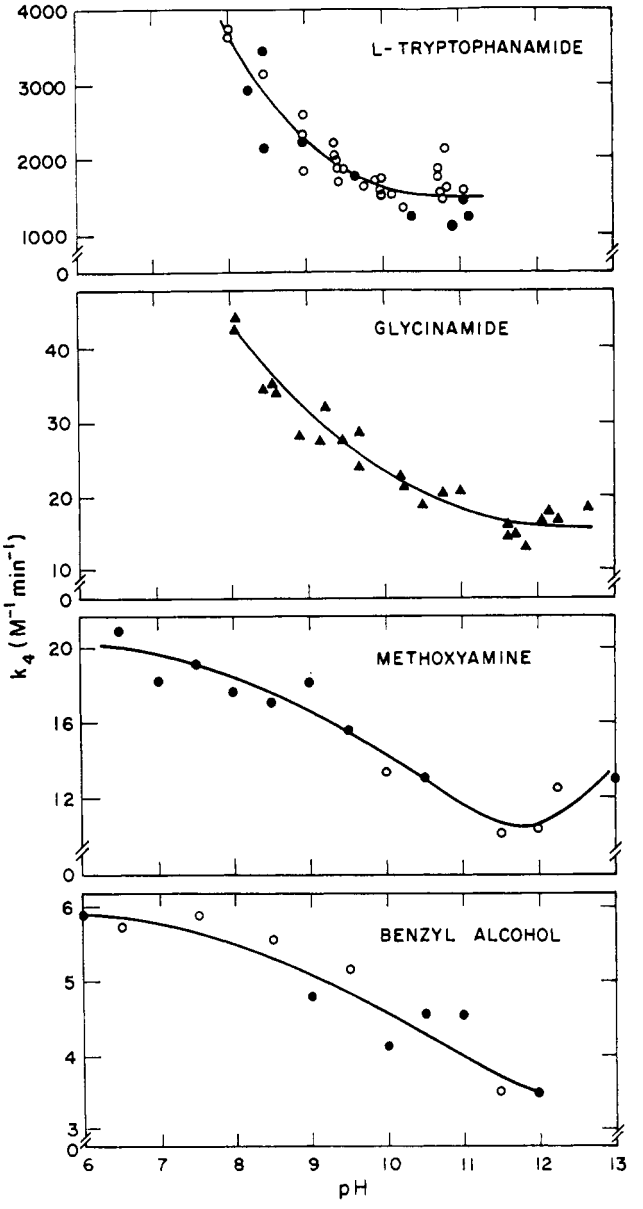


FIGURE 5: The reactions of FA-papain with nucleophiles. The conditions were: 25°, 50 mM buffers, 0.5 mM EDTA, and ionic strength 0.50. $TrpNH_2$ was (●) 0.196 or (○) 0.392 mM; glycinamide was 83.3 mM; methoxyamine was from 8 to 38.1 mM; and benzyl alcohol was 0.158 M. The filled symbols for methoxyamine and benzyl alcohol represent the average of two to six determinations and the open symbols are single determinations. The values of k_4 were calculated based on a pK_a = 7.65 for $TrpNH_2$ and 8.1 for glycinamide.

The experimentally determined values of k_4 agree well with a theoretical curve for a reaction dependent on the free base form of an enzyme group with a pK_a of 4.6 (dashed line in Figure 4). When tryptamine (pK_a = 10.25) was used as a nucleophile it was again found that only the free base form of the amine participates in the deacylation reaction. These results indicate first, that the reaction of acyl-papain with an amine depends on the same deprotonated enzyme group as the reaction with water, and second, that protonated amines have little or no effect on the rates of deacylation. The latter conclusion is interesting since Fink and Bender (1969) obtained evidence from steady-state studies which suggested that the protonated form of $TrpNH_2$ may affect the reactions of papain with *p*-nitrophenyl-*N*-acetyl-L-tryptophanate. In addition, the reactions of benzyl alcohol with FA-papain were studied as a function of pH and k_4 was again shown to depend on the basic form of an enzyme group with a pK_a of 4.6. Therefore, the enzyme group required in basic form for the hydrolysis of the acyl-papain intermediate is also required for alcoholysis and aminolysis.

The pH dependence of the rate constants for the reactions of several amines and benzyl alcohol with FA-papain in basic solution is shown in Figure 5. Values of k_4 were calculated as described in eq 2 and 4. These experiments were carried out at nucleophile concentrations where the observed rates of FA-papain deacylation were 2.5–15 times greater

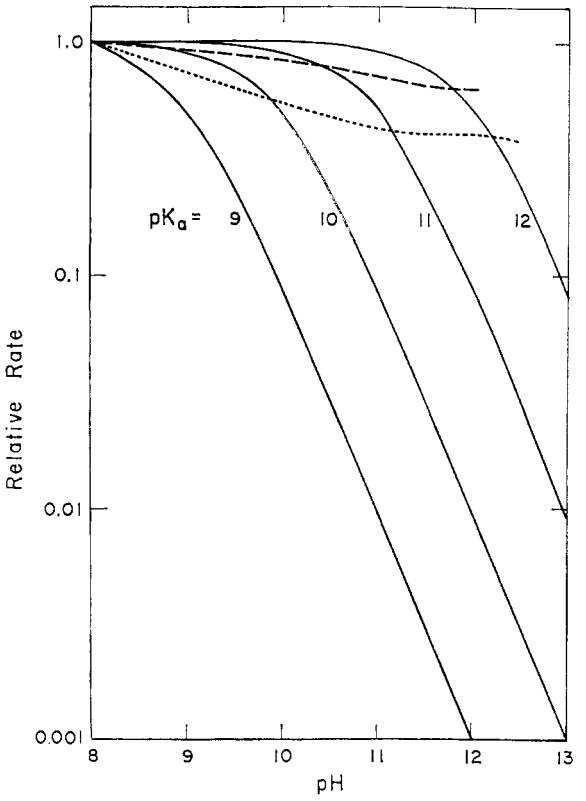


FIGURE 6: Theoretical and observed pH dependence of the reactions of FA-papain with nucleophiles. The relative rate, plotted as the ordinate, is the ratio of k_4 at the indicated pH to k_4 at pH 8 for (— — —) benzyl alcohol or (- - -) glycinamide. The relative rates were determined from the lines drawn in Figure 5. (———) Theoretical curves for the pH dependence of a reaction requiring the acidic form of an enzyme group with the pK_a value indicated in the figure. The relative rate = $[H^+]/([H^+] + K_a)$.

than the rates observed at the same pH values in water. The calculated values of k_4 should, therefore, be insensitive to any small changes in the rates of hydrolysis or denaturation which might be effected by nucleophiles. If a protonated imidazole group acts as a general acid catalyst in the deacylation reaction as proposed by Sluyterman and Wolthers (1969), then the rate constants for the reaction of FA-papain with nucleophiles should be dependent on the acidic form of an enzyme group with a pK_a of 9.5–10. As shown in Figure 5, the rate constants for the aminolysis and alcoholysis of FA-papain (k_4) decrease by 40–60% between pH 8 and 12. However, the shapes of the curves are not theoretical for a reaction requiring the acidic form of a single titrable enzyme group. This point is shown more clearly in Figure 6, where the observed pH–rate profiles for the reaction of FA-papain with glycinamide and benzyl alcohol are compared with theoretical curves for a reaction completely dependent on the ionization of an hypothetical enzyme group with various pK_a values. The data for glycinamide and benzyl alcohol are presented because the values of k_4 for these nucleophiles displayed the largest and smallest pH effects, respectively. Similar results were obtained for the reactions of IA-papain with glycinamide and methoxyamine (Hinkle, 1970). The same conclusion was also reached in an earlier study in which the apparent rate constants for the hydrolysis of FA-papain, corrected for the rate of denaturation, did not appear to depend on the acidic form of a titrable enzyme group with a pK_a of 9.5–10 (Hinkle and Kirsch, 1970; Hinkle, 1970).

Discussion

Acidic Portion of the pH–Rate Profiles. The reactions of acyl-papain intermediates with water, amines, and alcohols depend on the basic form of a dissociable enzyme group which is probably a carboxyl or an imidazole. That the pK_a is independent of the acyl acceptor argues that it represents a true dissociation constant and not a change in rate-determining step involving an intermediate product in deacylation (Vishnu and Caplow, 1969). The kinetically determined pK_a of this residue varies from 3.33 with α-*N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester as substrate (Bender and Brubacher, 1966) to 4.8 for FA-papain (Table I). The heat of ionization of the essential acidic group of papain was found to be near zero for the hydrolysis of FA-papain, typical of a carboxyl ionization but not of an imidazole. The heats of ionization of imidazoles in proteins can however vary considerably. For example, two histidines in ribonuclease with pK_a values of 6.2 and 6.7 have heats of ionization of, respectively, 4.0 and 8.2 kcal per mole (Roberts *et al.*, 1969). However a heat of ionization of zero is well below any reported value for a protein histidine, as is the apparent pK_a value of 3–5 (*e.g.*, Cohen, 1969).

The pK_a of the acidic group of papain undergoes a large negative shift in the presence of organic solvents. The pK_a for FA-papain hydrolysis decreases from 4.6 in water to 3.9 in 30% dioxane–water (Table I), and the pK_a for *trans*-cinnamoyl-papain hydrolysis shifts from 4.65 in water to 4.15 in 20% dioxane–water (Lowe, 1970). Organic solvents usually cause an increase in the pK_a of carboxyl groups and have only a small effect on the pK_a of imidazoles, for which protonation involves the formation of no new net charge. The pK_a values of aliphatic carboxyl groups are 0.6–1.0 pH unit higher in 30% dioxane–water than in water (based on interpolation of data at 20% and 45% dioxane–water reported in Robinson and Stokes, 1959). On the other hand, the pK_a of histidine methyl ester decreases from 5.23 in water to 5.07 in 30% dioxane–water (Piszkiewicz and Bruice, 1968), and that of 4-(5)bromoimidazole from 3.65 in water to 3.32 in 20% dioxane–water (Lowe, 1970). Several enzyme histidines have been found to undergo smaller shifts in pK_a in organic solvent–water mixtures. In lysozyme a kinetically assigned histidine pK_a shifts from 5.2 in water to 5.3 in 30% dioxane–water (Piszkiewicz and Bruice, 1968). In trypsin a kinetically important residue, presumably histidine, has a pK_a of 6.06 in water and 5.80 in 50% dioxane–water, although the pK_a values become identical when the ionic strength is raised (Inagami and Sturtevant, 1960). The pK_a of the acidic group of papain decreases as the concentration of dioxane is raised, suggesting that the ionizing group is an imidazole, but the observed shifts in pK_a are considerably larger than expected from studies with either model compounds or other enzyme histidines.

As shown in Table I, an increase from 0.050 to 0.500 in the ionic strength resulted in a shift in the apparent pK_a of FA-papain hydrolysis from 4.63 to 4.81. The pK_a values of carboxylic acids usually decrease while those of amino groups increase when the ionic strength is raised (Edsall and Wyman, 1958). Although this result again suggests that the ionizing residue is His-159, the effect of salt on the pK_a values of carboxyl groups can vary so that this is not a strong criterion for distinguishing between the ionization of a carboxyl or an imidazole group.

Clearly it is not possible to unambiguously assign the pK_a of *ca.* 4.8 to any amino acid on the basis of the effects of

temperature, solvents, and ionic strength on the pH–rate curves for the reactions of FA-papain. It is known from X-ray crystallographic studies that His-159 is in an ideal position to participate in the deacylation reaction (Drenth *et al.*, 1970). The problem with this interpretation is simply that a pK_a of 3–5 and $\Delta H_i \sim 0$ are abnormally low for a protein histidine residue. The pK_a of a histidine can be lowered by either hydrogen-bond formation or the presence of a positively charged environment (Meadows *et al.*, 1967). Hydrogen bonding to either the sulfhydryl group of Cys 25 (Lowe, 1970) or to the amide nitrogen of Asn-175 (Lucas and Williams, 1969) has been suggested to account for the low pK_a of His-159 in papain. Since the residue hydrogen bonded to the second nitrogen atom of the imidazole in question is asparagine and not aspartic acid (Drenth *et al.*, 1970; Husain and Lowe, 1970), a mechanism analogous to the "charge relay system" proposed for chymotrypsin (Blow *et al.*, 1969) cannot be operative. A direct measurement of the pK_a of His-159 will probably be necessary before the identity of the essential acidic group of papain can be established with certainty.

Basic Portion of the pH–Rate Profiles. The rates of hydrolysis of three acyl-papains and of α-*N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester measured in the steady-state decrease at pH values above 9 (Brubacher and Bender, 1966; Hinkle and Kirsch, 1970; Hinkle, 1970; Bender and Brubacher, 1966). The pH dependence of the reactions between FA-papain and various nucleophiles was studied in order to learn whether deacylation requires the acidic form of an enzyme group with a pK_a above 9. The results presented in Figure 6 show that at pH values above neutrality, the alcoholysis and aminolysis of FA-papain do not depend on the ionization of a single enzyme group. Since similar pH–rate curves were explained for the reaction of the acyl-enzyme with water, alcohols, and amines, it appears that the reactions between the acyl-enzyme and alcohol and amine nucleophiles proceed by the same mechanism as the reaction with water. It is unlikely, therefore, that the slight dip in the pH *vs.* rate profiles can be explained by the simple participation of His-159 as a general acid catalyst as proposed by Sluyterman and Wolthers (1969). Several explanations which might account for the pH dependence of the reactions of FA-papain at high pH have already been presented (Hinkle and Kirsch, 1970).

Limitations of the Catalytic Role of the Active-Site Thiol. The acyl-papains which have been stable enough to isolate have so far only been prepared through the use of the corresponding acyl-imidazoles (Bender and Brubacher, 1966; Hinkle and Kirsch, 1970) or oxazolinones (de Jersey and Zerner, 1969). The absorption spectra of the native acyl-enzymes are not characteristic of thiol esters although those of the denatured acyl-papains are. The steady-state hydrolysis of methyl thionohippurate is characterized by a transient absorption spectrum indicative of the expected thiono ester (Lowe and Williams, 1965). Thus, while the evidence that the papain-catalyzed hydrolysis of many substrates does indeed proceed through the intermediacy of an acyl-enzyme is now conclusive (see above), the strongest support for its being a thiol ester comes from the thionohippurate spectra and the kinetic preference of the acyl-enzymes for amine *vs.* oxygen nucleophiles (Brubacher and Bender, 1966) in accord with expectations from studies of model thiol esters (Connors and Bender, 1961). Other data, such as that demonstrating that the enzyme is inactivated by alkylating or otherwise blocking the thiol group (Smith and Kimmel, 1960; Wallenfels and Eisele, 1968; Sluyterman, 1967, 1968; Chaiken and Smith, 1969; Klein and Kirsch, 1969) and the rapid rates of reaction of this particular moiety with alkylating agents (Wallenfels and Eisele, 1968) do not prove that the thiol acts as a nucleophile toward acyl carbon in the catalyzed reaction.

While thiols such as mercaptoethanol react readily with active esters such as *p*-nitrophenyl or phenyl carbobenzoxyglycinate to form thiol esters (Kirsch and Igelström, 1966), they do not react to an appreciable extent with esters having relatively poor leaving groups, such as methanol or ethanol, or with amides, because of the 2.8- and 7-kcal thermodynamic barrier against this reaction at pH 7, corresponding to 1 and 0.001% extent of reaction at equilibrium for the esters and amide, respectively.[3] Nor, because the rates of alkaline hydrolysis of esters and thiol esters are essentially equal (Rylander and Tarbell, 1950), can thiolate ions act as nucleophilic catalysts for the hydrolysis of the more stable acyl derivatives. Therefore, any proposed mechanism for papain catalysis embodying *solely* general base catalyzed activation of the thiol cannot account for the observed rates of reaction since the *maximum* activation that can be so obtained would be in the complete removal of the proton to form the anion, corresponding to a Brønsted coefficient of $\beta = 1.0$.[4]

Thermodynamic Stability of Acyl-Papains. It is possible to calculate the free energy of formation and hydrolysis of hippuryl-papain from data obtained earlier in this laboratory (Henry and Kirsch, 1967). Under conditions of $[S] \ll K_s$, the reaction of ethyl hippurate with papain can be described according to eq 5, where AE, P_1, and P_2 represent hippuryl-

$$E + S \underset{k_{-2}}{\overset{k_2/K_s}{\rightleftharpoons}} AE \xrightarrow{k_3[H_2O]} E + P_2 \qquad (5)$$
$$\qquad\qquad + \\ \qquad\qquad P_1$$

papain, ethanol, and hippuric acid, respectively, and K_s is the dissociation constant. The values of the three rate constants and the equilibrium constant for the formation of the acyl-enzyme are given in Table II. An estimate of the free energy of hydrolysis of ethyl hippurate can be obtained from that of ethyl acetate with the assumption that the equilibrium constant for eq 6 is equal to 1, *i.e.*, that the free energy of transfer of the hippuryl group from ethanol to water is the same as that for the acetyl group. A similar assumption was

$$\text{ethyl hippurate} + \text{acetic acid} \rightleftharpoons \text{ethyl acetate} + \text{hippuric acid} \qquad (6)$$

made by Inward and Jencks (1965) in evaluating the free energy of hydrolysis of furoyl-chymotrypsin. The free energy of hydrolysis of ethyl acetate at 39° is −1800 cal/mole in the pH-independent convention of Jencks *et al.* (1960). This value together with that for the pK_a of hippuric acid at 40° = 3.59 (Sluyterman, 1964) gives $\Delta F = -5250$ cal/mole for the free energy of hydrolysis of ethyl hippurate at pH 6.

The free energy *vs.* reaction coordinate diagram for the reaction of papain with ethyl hippurate is shown in Figure 7. Hippuryl-papain is approximately 3 kcal/mole more stable

[3] Calculated from the data of Gerstein and Jencks (1964).

[4] The fact that the deacylation reaction is also catalyzed by the enzyme does not nullify this argument because this latter step is rate limiting in reactions of ethyl esters (Henry and Kirsch, 1967), and at least partly so in the reactions of amides (Whitaker and Bender, 1965); therefore, the rate of acylation must be faster than the overall catalyzed reaction.

TABLE II: Rates, Equilibria, and Free Energies for the Reaction of Ethyl Hippurate with Papain at pH 6.0 and 40°.[a]

Parameter	Value	$\Delta F^{\ddagger}$ [b] (kcal/mole)	ΔF (kcal/mole)	
k_2/K_s [c]	161 M^{-1} sec^{-1} [d]	15.2		
k_2/K_s [c]	82 M^{-1} sec^{-1} [e]	15.6		
k_{-2}	1.38 M^{-1} sec^{-1} [e]	18.2		
k_3	0.0917 M^{-1} sec^{-1} [d,e]	19.8		
K_{eq} [f]	60,[e] 116[d]		2.54[e]	2.95[d]

[a] Calculations are based on the scheme shown in eq 5 and the data of Henry and Kirsch (1967). [b] Calculated from the relationship $\Delta F^{\ddagger} = RT \ln kh/k_B T$. [c] Calculated value of k_{cat}/K_m. [d] Measured in 6.7% (v/v) acetonitrile. [e] Measured in 6.7% acetonitrile plus 1.72 M [^{14}C]ethanol. [f] Equilibrium constant for acyl-enzyme formation.

than ethyl hippurate and is calculated to be 4.9 kcal/mole more stable than *O*-hippuryl-*N*-acetylserinamide, since esters of *N*-acetylserinamide are 1860 kcal/mole less stable than ethyl esters (Inward and Jencks, 1965). Since thiol esters are typically *ca.* 3 kcal/mole *less* stable than oxygen esters, (Jencks *et al.*, 1960) the particular structure of native papain must contribute approximately 8 kcal/mole toward stabilizing acyl-papain *if it is a thiol ester*. Some of this energy could reside in favorable bonding interactions between the hippuryl moiety and the protein, but the unfavorable dissociation constants (K_s) for such substrates as isopropyl hippurate (Lucas and Williams, 1969) suggest that only about one-quarter of the required ΔF can be expected from this source. Furoyl-chymotrypsin has been calculated to be about 3 kcal more stable than the model compound *O*-furoyl-*N*-acetylserinamide (Inward and Jencks, 1965).

The thermodynamic stability of the acyl-enzyme is only 2.2 kcal less than that of the product acid at pH 6.0, and is calculated to be equal to it at pH 4.4. This calculation lends quantitative support to the suggestion that enzyme–carboxylic acid inhibitor complexes are acyl-enzymes at low pH (Whitaker and Bender, 1965).

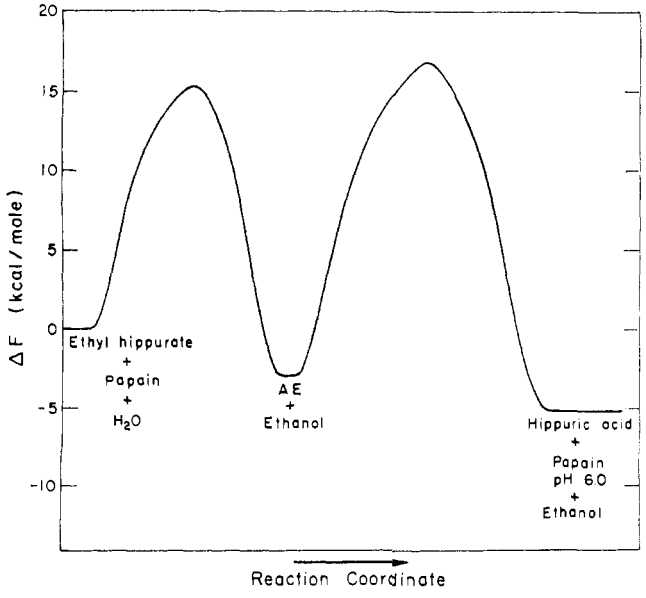


FIGURE 7: Reaction coordinate diagram for the reaction of ethyl hippurate with papain at pH 6.0 and 40°. See Table II and text.

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Interaction of Acetic Acid with Poly-L-glutamic Acid and Serum Albumin*

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ABSTRACT: The combined application of electrophoresis, ultracentrifugation, optical rotatory dispersion, and circular dichroism to the interaction of acetic acid with poly-L-glutamic acid and bovine serum albumin has provided fresh insight into the mechanisms underlying the effect of acetate and other carboxylic acid buffers on the electrophoretic behavior of proteins in acidic media, Thus, for example, the characteristic increase in the net positive charge on the protein molecule due to interaction with the undissociated buffer acid is now understood in terms of binding of the acid to side-chain carboxyl groups with concomitant increase in their p*K*. Moreover, binding need not be cooperative for resolution of bimodal reaction boundaries to occur, since the forementioned mode of interaction generates coupled gradients of acid concentration and pH in the electrophoresis column. Binding of acetic acid favors the helical conformation of poly-L-glutamic acid and causes subtle alteration in the tertiary structure of bovine serum albumin.

Our interest in the interaction of acetic acid with proteins stems from observations on the nonenantiographic, bimodal electrophoretic patterns displayed by a variety of highly purified proteins in acidic media containing acetate or other carboxylic acid buffers (Cann and Phelps, 1957, 1959; Cann, 1958–1961). Experimental evidence has been advanced to support interpretation of the patterns in terms of reversible complexing of the protein with undissociated buffer acid, with concomitant increase in the net positive charge on the protein. Although complex formation does not change the frictional coefficient of the protein molecule significantly, it does cause subtle structural alterations. A theoretical basis for this interpretation was provided (Cann and Goad, 1965) by a theory of electrophoresis of reversibly interacting systems of the type

$$P + nHA \rightleftharpoons P(HA)_n \qquad (1)$$

where P represents a protein molecule or other macromolecular ion in solution and $P(HA)_n$ its complex formed by binding *n* moles of a small, uncharged constituent, HA, of the solvent medium, *e.g.*, undissociated buffer acid. It is assumed that P and $P(HA)_n$ possess different electrophoretic mobilities and that equilibrium is established instantaneously. These computations account for the essential features of the electrophoretic behavior of proteins in acidic media containing varying concentrations of carboxylic acid buffer. Resolution of the patterns into two peaks occurs because of changes in the concentration of HA accompanying reequilibration during differential transport of P and $P(HA)_n$ and maintenance of the resulting concentration gradients of the electrically neutral molecule. Thus, the two peaks correspond to different equilibrium compositions and not to separated P and $P(HA)_n$. Subsequently, Bull and Breese (1967a) demonstrated by equilibrium dialysis that ovalbumin binds undissociated acetic acid and its normal short-chain homologs. It remains to elucidate the mechanism of interaction. The questions posed are: "What are the binding sites on the protein?" "How does binding increase the positive charge on the protein molecule?" "What is the nature and mechanism of the conformational changes wrought by acetic acid?" Answers to these questions have been sought by the combined application of electrophoresis, ultracentrifugation, optical rotatory dispersion, circular dichroism, and measurement of biological activity to the interaction of acetic acid and its homologs with poly-L-glutamic acid, bovine

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