Effects of Substituents on the Rates of Deacylation of Substituted Benzoyl Papains. Role of a Carboxylate Residue in the Catalytic Mechanism†

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ABSTRACT: The effect of ring substituents on the rates of deacylation of 8 meta- and para-substituted benzoyl papains was evaluated. The rate constants were found to depend upon a single ionizing group of pK_a = 4.2–4.3, and to decrease by a factor of approximately 2.2 when measured in 94% D_2O/H_2O. The rates of deacylation are increased greatly by electron-withdrawing groups on the benzene ring. The Hammett ρ value is 2.74 ± 0.32. A plot of the rate constants for deacylation of the benzoyl papains against the corresponding constants for substituted benzoyl chymotrypsins generates a straight line of slope 1.0. This result suggests a very similar distribution of charge on the benzoyl moiety in the transition state for the two enzymes, which is interpreted in terms of the net charge of the transition state for the deacylation of nonspecific acyl papains being equal to −1, with the general base catalyzed assistance to the attack of water on the acyl enzyme being provided by the negatively charged Asp-158 rather than by the neutral Asn-175-His-159 hydrogen bond network. This result together with a survey of literature data suggests that the role of Asp-158 in papain catalysis has been underestimated. The evidence advanced to date in support of the proposition that an imidazolium-159-cysteine-25 thiolate ion pair exists in native papain is evaluated and considered to be insufficient to decide the issue.

The proteolytic enzyme papain (EC 3.4.22.2) poses a dilemma for considerations of the detailed mechanism of action in that the proximity of His-159 to Cys-25 at the active site (Husain & Lowe, 1968; Drenth et al., 1970) suggests a general base catalyzed activation of the thiol by imidazole similar to the charge relay system operative in the serine protease family (Blow et al., 1969), while the pH vs. rate profile and numerous other properties of the enzyme (see Discussion) are most readily interpreted in terms of a carboxylate ion occupying this role. A possibility for gaining further insight into this question arises from the fact that the magnitudes of Hammett ρ values for acyl transfer reactions of acyl-substituted benzoate esters have been found to be highly sensitive to the charge on the attacking nucleophile (Caplow & Jencks, 1962; Williams & Salvadori, 1971; Hubbard & Kirsch, 1972). Indeed, the ρ values for the rates of deacylation of substituted benzoyl chymotrypsins are very close to those observed for alkaline hydrolysis (Caplow & Jencks, 1962; Williams & Salvadori, 1971; Wang & Shaw, 1972; Amshey et al., 1975; Kirsch et al., 1968; Kirsch, 1972). These observations support the conclusion that the net charge of the charge relay system is −1 in deacylation.

Since His-159 is hydrogen bonded to the neutral Asn-175 in papain (Drenth et al., 1970) rather than to a carboxylate ion, the net charge of the analogous triad in papain is 0, and the Hammett ρ value for the rates of deacylation of substituted benzoyl papains should be significantly less than those observed for chymotrypsin (Hubbard & Kirsch, 1972). Conversely, similar dependencies on electron withdrawal of the rates of reaction for the two substituted benzoyl enzymes would be expected if a negatively charged carboxyl group played the role of the general base catalyst for the deacylation reaction in papain. We report here a determination of the Hammett ρ value for the rates of deacylation of substituted benzoyl papains.

Materials and Methods

Materials

Papain. Tetrathionate inactivated papain was prepared, activated, and assayed as described earlier (Hinkle & Kirsch, 1970; Kirsch & Igelström, 1966). The specific activity was 15 units/μg, where 1 unit represents the amount of papain that catalyzes the hydrolysis of 1 nmol of Z-Gly-*p*-NPE[1] per min under the assay conditions.

Enzyme concentrations were determined from the absorbance at 278 nm based on $\epsilon_{1\%}^{1cm}$ = 25 (Glazer & Smith, 1961) and a molecular weight of 23 000 (Drenth et al., 1968).

Benzoylimidazoles. Benzoylimidazoles were purchased from Aldrich Chemical Co. or synthesized as described below. *p*-Cl, *p*-CH_3, and *p*-CH_3O benzoylimidazoles were prepared by the method of Gerngross (1913) modified as follows: A solution of 10 mmol of sublimed imidazole (Aldrich Chemical Co.) dissolved in ca. 10 mL of distilled dry acetonitrile was diluted to ca. 150 mL with dry ether. The appropriate acyl chloride was added dropwise with stirring at 4 °C. The reaction was stirred 2–3 h at room temperature, filtered to remove imidazolium chloride, and evaporated to dryness in a rotary evaporator. *p*-CH_3O benzoylimidazole was recrystallized from dry cyclohexane, mp 66–68 °C. *p*-CH_3 and *p*-Cl benzoylimidazoles were recrystallized from a mixture of dry hexane–heptane–cyclohexane–petroleum ether (5:3:1:1), mp 70–72 and 84–86 °C, respectively. The literature mp for the *p*-CH_3O, *p*-CH_3, and *p*-Cl compounds are 69–71, 69–71, and 85–86.5 °C, respectively (Caplow & Jencks, 1962). *p*-H, *m*-Cl, *m*-F, and *p*-F benzoylimidazoles were prepared as above except that no ether was added to the acetonitrile solution of the imidazole

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[1] Abbreviation used: Z-Gly-*p*-NPE, *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester.

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and the acylimidazole solutions were used without further purification to prepare the acyl papains.

Other Materials. Z-Gly-*p*-NPE was available from previous studies (Kirsch & Igelstrom, 1966). Acetonitrile was refluxed over K_2CO_3, distilled, and stored over molecular sieves. Tetrahydrofuran was dried for 3 h over $LiAlH_4$ powder, 2–4 g/L, refluxed, distilled, and stored under N_2 (Fieser & Fieser, 1967). D_2O (99.87%) was purchased from Bio-Rad. All other materials used were reagent grade.

Methods

Preparation and Isolation of Substituted Benzoyl Papains. Typically 0.25 mL of a ca. 1.43 mM papain suspension was mixed with 0.05 mL of 0.2 M potassium phosphate buffer, pH 6.8, containing 0.1 M cysteine and 0.5 mM EDTA. Activation proceeded at room temperature for 15–20 min.

Active papain solution (0.3 mL), prepared as described above, was acidified with 0.05 mL of 2 M sodium formate buffer, pH 3.18, containing 0.5 mM EDTA. The acidification was carried out with 2 mM formate buffer (pH 3.0) for the *m*-F and *m*-Cl derivatives. Active papain is stable above pH 3, but inactivates rapidly below this pH (Hinkle, 1970). The acidification was followed immediately by addition of 0.05 mL of the appropriate solution of acylimidazole in acetonitrile (50–75 mM). After 3 min at room temperature, the solution was applied to a Bio-Gel P-2 (100–200 mesh) column (1.1 × 18 cm) equilibrated with pH 3.06 buffer (0.01 M potassium formate, 0.5 mM EDTA, I_c = 0.12, 4 °C). The column was eluted at a flow rate of 0.4–0.6 mL/min. Fractions of 1 mL were collected and assayed for protein at 278 nm and for SH content according to Ellman (1959). Acyl papains appeared in fractions 8 and 9 and were separated by two fractions from cysteine, unreacted acylimidazole, and free acids. The concentrations of the recovered acyl papains were 80–100 μM. These fractions were kept at 0 °C and were used as soon as possible.

Kinetics and Other Methods. All kinetic measurements were carried out at 25 ± 0.1 °C. The temperature was checked before and after each run with a Thermistor telethermometer (Yellow Spring Instruments Co.). The pH of the solutions of each run were read on a Radiometer 25 SE or PHM4C pH meter fitted with a combined type B electrode and standardized at two pH values in the range of the pH of the measurement.

The buffers used for the kinetic determinations were: pH 3–3.7, 0.1 M potassium formate; pH 3.7–5.7, 0.1 M potassium acetate; and pH 5.7–7.2, 0.04 M potassium phosphate. The ionic strength of the buffer solutions was adjusted to 0.12 with KCl. All buffers were 0.5 mM in EDTA. Organic solvent solutions are expressed as volume percent. The D_2O experiments were carried out in 94% D_2O by adding 0.06 mL of acyl enzyme sample in H_2O to 0.94 mL of the appropriate buffer in D_2O. Deionized water was used throughout.

The rates of deacylation of the acyl papains were monitored as follows: an aliquot of between 0.05 and 0.20 mL of the acyl enzyme was rapidly warmed to 25 °C and added to a cuvette containing sufficient buffer equilibrated at the temperature of the experiment to give a final volume of 1.0 mL. Kinetics were generally monitored by the decrease in absorbance at 277 nm using the expanded scale of either a Unicam SP800A or a Gilford Model 220 recording spectrophotometer. The deacylation of *p*-CH_3O benzoyl papain was followed at 320 nm. The concentration of the acyl enzyme in each run was ca. 5–20 μM. A change from acetate to phosphate buffer at a fixed pH did not effect the rates of hydrolysis. At pH above 6.2 and enzyme concentrations greater than 5 μM, nonlinear plots of $\log(A_t - A_\infty)$ vs. time were obtained; and the apparent rate constants were observed to increase with enzyme concentration. Similar observations of nonlinear kinetics at high enzyme concentrations and pH >7 were made in following the hydrolysis of furylacryloyl- and indolylacryloyl papain (Hinkle & Kirsch, 1970) and transcinnamoyl papain (Brubacher & Bender, 1966) and were attributed to the reaction of nonactive site nucleophilic groups of papain with the acyl enzyme (Hinkle & Kirsch, 1970). In order to overcome this difficulty, the experiments conducted at pH values greater than 6.1 were performed either at low enzyme concentrations (5–8 μM) or the rate constants were measured at several enzyme concentrations and extrapolated to zero. pH vs. rate profiles for the deacylation of five acyl papains were obtained from 12 to 20 determinations over the pH range 3 to 7.

TABLE I: Limiting Rate Constants and pK_a Values Controlling the Deacylation of Substituted Benzoyl Papains.

Substituent	k_3^{lim} (min^{-1})[b]	pK_a[b]
p-CH_3O	0.0321 (0.0006)	4.29 (0.06)
p-CH_3	0.0807 (0.0020)	4.21 (0.06)
m-CH_3	0.244 (0.005)	4.31 (0.05)
p-H	0.271 (0.004)	4.30 (0.04)
p-F	0.384 (0.013)[a]	
p-Cl	0.531 (0.012)	4.32 (0.05)
m-F	2.49 (0.12)[a]	
m-Cl	2.90 (0.11)[a]	

[a] Parameters were obtained from several determinations at the plateau of the pH vs. rate profile. All other constants reported were obtained from the least-squares fit to eq. 1. [b] SE in parentheses.

The values of k_3^{lim} and of the pK_as controlling deacylation were calculated from eq 1

$$k_3 = \frac{k_3^{lim} K_a}{K_a + H} \quad (1)$$

with the aid of the computer program HYPERB (Hanson et al., 1967). The Hammett plot was drawn from the k_3^{lim} values except for the *p*-F, *m*-Cl, and *m*-F derivatives, for which the average k_3 of five to seven determinations at pH 6.18 was used. The slope of the Hammett plot (ρ value) was determined by a weighted least-squares computer program.

Results

Papain immediately subsequent to treatment with a substituted benzoylimidazole under the conditions described exhibits no activity with Z-Gly-*p*-NPE as substrate. Reactivity toward this substrate returns with time at rates equal to the spectrophotometrically determined rates of deacylation.

Kinetics of Deacylation and pH vs. k_3 Profile. The rates of deacylation of substituted benzoyl papains obey first-order kinetics at pH <6 as explained in the Experimental Section. The full pH vs. rate profiles for deacylation of five of the substituted benzoyl papains have been determined (Figure 1). The limiting rate constants for deacylation and the apparent controlling ionization constants are collected in Table I. The rates of deacylation show a sigmoidal dependence on pH with apparent pK_as of 4.2–4.3, which are within the range of other studies of the rates of deacylation of acyl papains (Hinkle & Kirsch, 1970, 1971; Lowe, 1970a; Glazer & Smith, 1961). A Hammett plot of the k_3^{lim} values is given in Figure 2.

Effect of Deuterium Oxide and of Organic Solvents on the Rates of Deacylation. The rates of deacylation of several of the substituted benzoyl papains studied are more than twofold slower in 94% D_2O/H_2O (Table II) in agreement with previ-

TABLE II: Effect of D_2O and Organic Solvents on the Limiting Rate Constant for the Deacylation of Substituted Benzoyl Papains.[a]

Substituent	k_3 (D_2O) (min^{-1})	k_3 (H_2O)/ k_3 (D_2O)	k_3 (10% THF) (min^{-1})	k_3 (10% THF)/ k_3 (H_2O)	k_3 (6.7% CH_3CN) (min^{-1})	k_3 (6.7% CH_3CN)/ k_3 (H_2O)
p-CH_3O			0.11	3.43	0.0343 0.039[b]	1.14
p-CH_3			0.4358	5.40	0.0936 0.0900[b]	1.14
m-CH_3	0.114	2.14	1.012	4.15		
p-H	0.122	2.22				
p-F	0.173	2.22	2.17	5.64		
p-Cl	0.237	2.24	2.57	4.83		

[a] Values of k_3 (H_2O) are given in Table I. The rate constants were determined at pH (pD) 6.2 and 25 °C. [b] Rate constants determined by recovery of activity of papain toward Z-Gly-*p*-NPE. All others were determined spectrophotometrically.

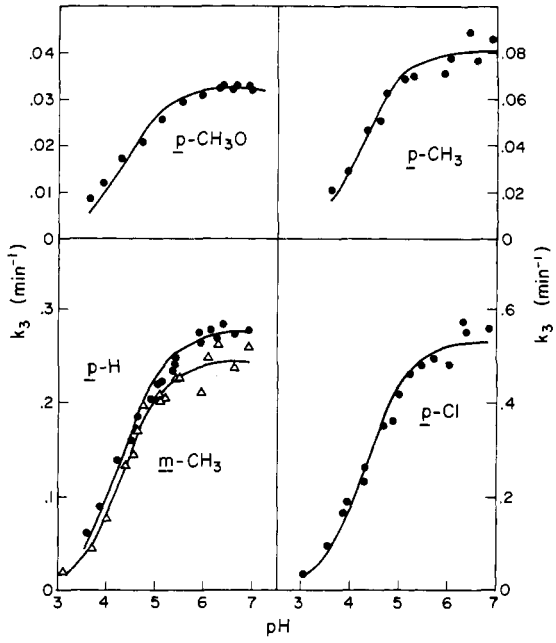


FIGURE 1: The pH dependence of the rates of deacylation of substituted benzoyl papains at 25 °C and ionic strength 0.12. Details are given in the Experimental Section. The solid lines were calculated to give the least-squares fit to eq 1.

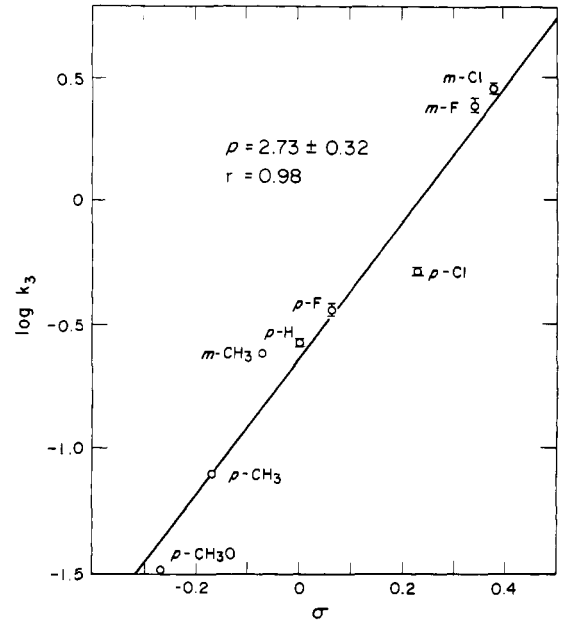


FIGURE 2: Hammett plot of the limiting values of the rates of deacylation of substituted benzoyl papains at 25 °C, I_c = 0.12. The values for the *p*-F, *m*-F, and *m*-Cl derivatives are based on an average of five to eight determinations at pH 6.18. The slope of the line was calculated by a weighted least-squares procedure. The error flags are ± the standard error of the k_3^{lim} values (Table I) and are within the diameter of the circle representing the points for the *p*-H, *p*-CH_3, *p*-CH_3O, and *m*-CH_3-benzoyl derivatives. The σ values were obtained from the compilation of McDaniel & Brown (1958).

ously observed solvent D_2O isotope effects on deacylation (Whitaker & Bender, 1965; Brubacher & Bender, 1966). Dramatically large increases in the rates of hydrolysis of indolylacryloyl, furylacryloyl, and transcinnamoyl papains are effected by certain nonreacting organic solvents (Hinkle & Kirsch, 1970; Kirsch, 1972). Furylacryloyl papain, for example, deacylates 12-fold faster in a 30% (v/v) solution of tetrahydrofuran/water than in water alone at pH 7.5. Similar increases in the rates of deacylation of the substituted benzoyl papains are brought about by the addition of tetrahydrofuran. Acetonitrile, a smaller organic molecule, has a much smaller accelerating effect as has been noted previously (Table II).

Discussion

Interpretation of the ρ Values. The absolute magnitude of ρ is a measure of the sensitivity of the reaction rate to electron withdrawal at the reaction center (e.g., Exner, 1972). Reactions of anionic nucleophiles on substituted benzoyl esters are characterized by ρ values ≥ 2, while reactions of neutral nucleophiles with these derivatives exhibit ρ values ≤ 1.4 (Kirsch, 1972; Hubbard & Kirsch, 1972).

It is by now well established that the mechanism for deacylation of acyl chymotrypsins involves general base assisted attack of water upon the acyl enzyme mediated by the "charge relay system" (Blow et al., 1969; Henderson, 1970; Birktoft et al., 1976; Kraut, 1977).

COO^-	$H\cdots Im$	$H—O$
Asp	His	Ser
102	57	195

The charge relay system has a net charge of −1, and this is manifested in the observed ρ value of 2.4 ± 0.2 compiled for the deacylation rates of *p*-MeO, *p*-Me, *p*-H, *p*-Cl, *p*-F, *m*-Cl, and *m*-F benzoyl chymotrypsins from the data depicted in Figure 3. This figure is in good agreement with the observations on nonenzymatic systems discussed above. The virtually identical sensitivity to electron withdrawal of the rates of

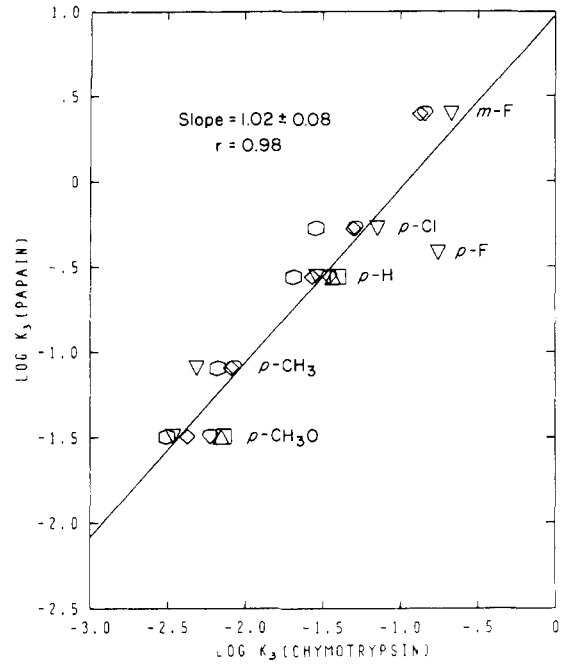


FIGURE 3: The rate constants for deacylation of substituted benzoyl papains as a function of the corresponding rate constants for benzoyl chymotrypsins. The papain values are from Table I. The chymotrypsin rate constants plotted have all been normalized where necessary to k_3^{lim} values and 25 °C using pK_a(app) values of 7.42 and 7.33, for the *p*-H and *p*-CH_3O substituted derivatives respectively (Bernhard et al., 1966; Vishnu & Caplow, 1969). An average pK_a(app) of 7.25 based on the work of the above authors and of Amshey et al. (1975) was used to normalize the remaining rate constants. A typical enthalpy of activation for the deacylation reaction of 15.4 kcal/mol (Marshall & Chen, 1973) was used to make the small temperature correction in the data of Wang & Shaw (1972) taken at 22 °C. Chymotrypsin data of: (△) Caplow & Jencks (1962); (◇) Vishnu and Caplow (1969); (○) Wang and Shaw (1972); (○) Williams & Salvadori (1971); (▽) Amshey et al. (1975); (□) Bernhard et al. (1966).

deacylation of substituted benzoyl chymotrypsins and of benzoyl papains (Figure 3) argues that the net charge in the transition state for deacylation is very similar for the two enzymes (i.e., −1). The rate constants for deacylation of the substituted acyl papains are about an order of magnitude greater than the average of those reported for the corresponding derivatives of chymotrypsin. The important problem is to assign the group of apparent pK_a near 4 having an apparent net charge of −1 which appears to control the rates of deacylation of acyl papains.

Histidine-159 and Aspartate-158. It is generally argued that the equivalent role of the catalytic triad of the charge relay system of chymotrypsin is played by Asn-175-His-159-Cys-25 in papain (e.g., Lowe, 1976; Bendall & Lowe, 1976a,b; Drenth et al., 1976). This point of view is supported by the location of the imidazole ring which is about 3.5 Å from the cysteine sulfur atom, and by the parallel effects of added dioxane upon the pK_a(app) controlling the deacylation rates and upon the pK_as of histidine and other imidazole derivatives (Lowe, 1970a). On the other hand the nearest carboxylate moiety (Asp-158) is ca. 7 Å from the cysteine sulfur atom. While this is too great a distance to permit a direct interaction without a significant conformational change, Asp-158 mediated general base catalysis of proton transfer from a water molecule attacking the acyl enzyme (Kirsch & Igelström, 1966) might be possible in view of the recently demonstrated conformational mobility of the active site (Sluyterman et al., 1977). Moreover, this hypothesis readily accommodates the experimental facts that the pK_a(app) of the critical catalytic residue is near 4 and the ΔH_i is near 0 (Hinkle & Kirsch, 1971).[2] It is further in accord with the conclusions of Lewis & Shafer (1974) that the upward shift in pK_a and the spectrum of a nitrophenol reporter group bound covalently to the active site cysteine suggests a negatively charged environment. The role of a carboxylate moiety bearing a charge of −1 acting as a general base catalyst in deacylation would clearly be well accommodated by the Hammett ρ values recorded in this study.

There are a number of recent additional observations in the literature which do not appear to have been summarized elsewhere which lend further support to the proposition that the role of the carboxylate has been underplayed, while that of His-159 should at least be open to debate. Although only the negative experiment is definitive in chemical modification studies, i.e., the demonstration that the modification of a particular residue has no effect on activity proves that it is not involved in the reaction mechanism, it is significant to note that two groups have specifically modified between 1.5 and 6 carboxyl residues by the water soluble carbodiimide–ethyl glycinate coupling reaction concomitant with a complete loss in activity (Löffler & Schneider, 1974; Perfetti et al., 1976). It was further reported by the latter workers that the competitive inhibitor, benzamidoacetonitrile, protects the enzyme against inactivation and prevents the reaction of two of the six accessible carboxyl groups.

In addition to the carboxyl derivatization experiments a number of efforts have been made to modify His-159 chemically. Okumara & Murachi (1975) report that the rates of inactivation by methylene blue induced photooxidation depend upon a pK_a of 6.6. The interpretation of this experiment is complicated by the fact that the rate of loss of activity is greater than the rate of derivatization of the histidine. Clark & Lowe (1976) have criticized the conclusion of Okumara & Murachi that His-159 has a normal pK_a by pointing out that photooxidation would rapidly convert the thiol of Cys-25 to a negatively charged sulfenic acid which would raise the pK_a of the histidine. Indeed, other negatively charged derivatives of cysteine do raise the pK_a of the group that quenches the fluorescence of Trp-177 (Sluyterman & deGraaf, 1970; Clark & Lowe, 1976). It is assumed (e.g., Lowe, 1976; Lowe & Whitworth, 1974) but not proven that it is His-159 that is responsible for the fluorescence quenching. Insofar as we are aware there is nothing in the literature that precludes the assignment of the role of the quenching residue to another group such as Asp-158.[3] Bendall & Lowe (1976a) have recently found evidence that in certain circumstances two ionic groups interact in

[2] There is a marked general tendency for ΔH_i values to decrease with decreasing pK_a for all acids, particularly in highly acidic media (e.g., Arnett et al., 1974). Liotta et al. (1973) have shown that the ΔH_i for a series of meta- and para-substituted anilinium ions, cationic nitrogen acids which may be close analogues of imidazolium ion for the purpose of comparison of ionization thermodynamics, decrease approximately 1 kcal/mol for each unit decrease in pK_a. The application of this factor to the imidazolium ion, assuming a normal pK_a of 7 and ΔH_i of 8.8 kcal/mol (Izatt & Christensen, 1970), gives a predicted ΔH_i of ca. 5.8 kcal/mol for a hypothetical imidazolium ion of p$K_a \approx 4$. This is far larger than that observed for the group of p$K_a \approx 4$ which controls the rate of deacylation. Perhaps an even stronger case against ΔH_i being a strong function of pK_a (app) for imidazole derives from the work of Flogel & Biltonen (1975) who find that the pK_a (app) of three of the four histidine residues of ribonuclease A shift upward by as much as 2.2 units upon complexation of the enzyme with 3′-CMP without a corresponding change in the ΔH_i values.

[3] This interpretation is supported by the recent observation of Johnson & Shafer (personal communication) that the ΔH_i for the residue quenching the fluorescence is near zero. There is some evidence that RCOOH is a more effective quenching agent than RCO_2^- (Cowgill, 1963).

controlling the fluorescence of this residue (see below). Finally, Löffler & Schneider (1975) report that only one of the two histidines of papain is modified by diazo-1*H*-tetrazole with a pH dependence suggesting that the histidine has a normal pK_a. However, the fact that this reagent reacts with most of the tryptophans and tyrosines of the protein makes the detailed interpretation of this result difficult. On the balance of the evidence so far available, we conclude that *it is most likely that the deacylation of nonspecific acyl enzymes is catalyzed by a carboxylate group (probably Asp-158) acting as a Brønsted base.* It is not clear to what extent this conclusion applies to the deacylation of specific acyl papains or to the acylation reaction.

It has recently become apparent that a cooperative ionization process involving at least two groups is manifest in the acidic limb for a number of spectral and catalytic properties of papain. (This does not include the rates of the deacylation reactions described here and earlier (Hinkle & Kirsch, 1970, 1971) which depend cleanly on a single ionizing group.) Bendall & Lowe (1976a) report positive cooperativity in the pH dependence of the fluorescent properties of 2-hydroxyethylthiopapain and attribute this to two groups each having pK_as near 4 which they assign to His-159 and Asp-158. By contrast negative cooperativity is observed in the pH dependence of the ^{19}F chemical shift of 2,2,2-trifluoro-1,1-dideuterioethylthiopapain presumably as a result of a different interaction of the same two ionizing groups. Interestingly, one of these ionizing residues was determined to have a large positive heat of ionization (His-159?) and the other a moderately negative ΔH_i (Asp-158?) (Bendall & Lowe, 1976b). In addition the k_{cat}/K_m values for a number of specific substrates exhibit pH dependences indicative of positive cooperativity (Shafer et al., 1977). Interaction of these two residues for specific substrates but not for nonspecific ones may help to explain their large differences in rates of reaction (Hinkle & Kirsch, 1970).

The Ion Pair in Papain. Lowe (1970b) pointed out that an ion pair formed from His-159 and Cys-25 might account for the high reactivity of papain toward an alkylating reagent.

$$-S^- \ ^+HIm$$

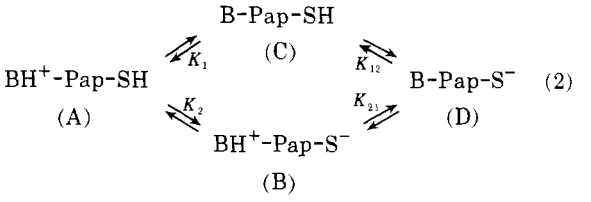
This suggestion has since secured the favor of a number of authors (Polgar, 1973, 1974; Drenth et al., 1975; Lewis et al., 1976; Sluyterman & Wijdenes, 1976). We, along with Lowe (1976) have some reservations. Because of the importance of this concept for the papain mechanism, it is worthwhile to discuss the objections at this point. Polgar offers three pieces of evidence in support of this hypothesis:

(1) The rate of reaction of chloroacetamide and 2-bromopropionamide with the enzyme thiol is influenced by two ionizable groups—one of p$K_a \approx 8$ and the other of p$K_a \approx 4$ (Polgar, 1973). The monoprotonated species, presumably the ion pair, reacts only at about 2% of the rate of the high pH form of the enzyme. This observation shows clearly that two forms of the enzyme differing by a full positive charge are capable of undergoing alkylation on sulfur, but gives no information about the location of the proton. It does not require an ion pair.

(2) A very small solvent deuterium isotope effect is observed on the rate of the alkylation reaction reported at the single pH (PD) value of 5.5. This is cited as evidence against general base catalysis by another group on the enzyme (Polgar, 1973). However, to our knowledge alkyl transfer reactions from alkyl halides to sulfur have not reported to be general base catalyzed in any model system. Studies of the pH dependence of the rates of reaction show that they depend cleanly upon the concentration of RS^- (e.g., Lindley, 1960). Nonetheless, a reduction in rate might be expected due to the influence of the solvent upon the ionization constant of the thiol for a non-ion pair mechanism. Solvent isotope effects on the pK_as of thiol acids are, however, much smaller than on oxygen or nitrogen acids (Jencks & Salvesen, 1971). The absence of a solvent isotope effect on alkylation is, therefore, an important argument in favor of the ion pair mechanism. It would be worthwhile to examine the rate ratio as a function of pH.

(3) Some evidence has been offered (Polgar, 1974) for spectroscopic detection of thiol anion absorbance at pH values below 8, which has been criticized by Lowe (1976).

By measuring the pH dependence of the difference in proton content between papain-S-SMe and papain, Lewis et al. (1976) have shown that the ionization of the active site thiol is linked to at least one another ionizing group assuming the thiomethyl group can be taken as a model for a proton at the sulfur atom. The stepwise ionization proceeds according to eq 2.



(2)

As discussed by these authors the four microscopic ionization constants cannot be unambiguously assigned from the observed macroscopic ionization constants without making an arbitrary assumption. One possibility considered is that pK_1 is identical with the pK_a of the same group in papain-S-SMe. With this assumption they calculate that pK_2 = 3.34, pK_{12} = 7.55, pK_{21} = 8.47, and that papain exists ca. 90% as the ion pair at physiological pH. This procedure, however, leads to the uncomfortable result that the dissociation of a proton from the thiol from species C (eq 2) has a high ΔH_i while dissociation from the same atom in species A (pK_2) has ΔH_i near 0. Conversely the dissociation of the proton from BH^+ from species B (pK_{21}) has a large ΔH_i but is near 0 when the proton dissociates from the same atom in species A (pK_1). (See Table I of Lewis et al., 1976.) An alternate arbitrary assumption is that K_{12} represents the dissociation constant of the group controlling the alkaline limb of the papain pH vs. k_{cat}/K_m profile (e.g., 8.5, Whitaker & Bender, 1965), and that the pK_a of BH^+ is not the same in papain SH and papain S-SMe. This assumption can give rise to values of K_2 and K_1 which do not differ very significantly from K_{12} and K_{21} respectively with the concomitant resolution of the ΔH_i dilemma.[4] We conclude that the present evidence is insufficient to decide the existence of an ion pair. More precise physical measurements which focus on single residues and do not require chemical perturbants will be required to resolve this issue.

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[4] The experimental data of Lewis et al. (1976) suggest an even more complicated interaction of ionizing groups, particularly in the pH 4-6 region where the steepness of the profile suggests a positive cooperative interaction between two groups of similar pK_a. These authors also considered an alternative to the ion pair in which papain exists predominantly as a species in which the thiol proton is hydrogen bonded to the base B as consistent with their data.

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