# Evidence for Conformational Restrictions within the Active Site of Acyl Papains Which Influence the Rates of Hydrolysis*

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ABSTRACT: The rates of hydrolysis of three isolated, relatively stable β-acryloyl papains, furylacryloyl (FA-), indolylacryloyl, and *trans*-cinnamoyl, are markedly accelerated by nonreacting organic solvents. For example, the rate of hydrolysis of FA-papain is 30-fold greater in 50% dioxane–water than in water alone. Dioxane effects only small increases in the rates of deacylation of acyl-enzymes formed from α-*N*-carbobenzoxyglycyl- and -L-lysyl-*p*-nitrophenyl esters. The results are interpreted in terms of a mechanism in which inert organic solvent molecules bind to the active site region of papain in a manner which forces the acyl moiety into a more reactive orientation *vis à vis* the enzyme catalytic groups, thereby increasing the rates of acyl transfer to water. The orientation of more reactive acyl-enzymes such as carbobenzoxyglycyl- and L-lysyl- is more nearly optimal and the acceleration by organic solvents accordingly much less. The entropies of activation for the hydrolysis of FA-papain in water and in 30% dioxane are in accord with this interpretation. The rates of deacylation of FA-papain depend on the basic form of an enzyme group with $pK_a$ = 4.63 (water) or 3.91 (30% dioxane) and are independent of pH from pH 6 to 9. Between pH 9 and pH 11.5 the rates of hydrolysis decrease by approximately 30% (water) or 15% (30% dioxane). In acidic solution the rates of denaturation of FA-papain depend on $[H^+]^4$ (water) or $[H^+]^2$ (30% dioxane). The behavior of native and previously denatured FA-papain has also been examined in strongly basic solution.

All currently available evidence indicates that the papain-catalyzed hydrolysis of esters proceeds *via* the formation of an acyl-enzyme intermediate between the thiol group of the enzyme and the acyl moiety of the substrate (*e.g.*, Lowe and Williams, 1965a,b; Kirsch and Igelström, 1966; Henry and Kirsch, 1967; Bender and Brubacher, 1966; Brubacher and Bender, 1966). However, although the three-dimensional structure of papain is now known at 2.8-Å resolution (Drenth *et al.*, 1968), it is not yet clear which enzyme groups in addition to the sulfhydryl moiety are involved in the catalytic process.

The isolation of stable acyl-enzyme intermediates permits examination of a single step in the catalytic mechanism, the deacylation reaction. Two such acyl-enzymes, thionohippuryl (Lowe and Williams, 1965a) and *trans*-cinnamoyl (Brubacher and Bender, 1966) papain, have been prepared. The rate of deacylation of *trans*-cinnamoyl papain depends on the basic form of an enzyme group of $pK_a \sim 4.7$, consistent with the pH dependence observed for the deacylation of all papain substrates under steady-state conditions. Both an imidazole (Husain and Lowe, 1968) and a carboxyl side chain are near the active cysteine of papain (Drenth *et al.*, 1968) but it is not known if the $pK_a$ of 3–5 represents the ionization of one of these groups participating as a general base catalyst or the ionization of some distant carboxyl group required to maintain the active conformation of the enzyme. It has also been suggested (Sluyterman and Wolthers, 1969) that the pH dependence observed at high pH (>9) in the deacylation of *trans*-cinnamoyl (Brubacher and Bender, 1966) and α-*N*-carbobenzoxy-L-lysyl (Bender and Brubacher, 1966) papain is due to the ionization of an imidazole which is required in protonated form to act as a general acid catalyst.

In order to obtain more information on the nature of the enzyme groups involved in papain catalysis it was of interest to examine the effect of organic solvents on the pH dependence of the hydrolysis of stable acyl-enzyme intermediates. A number of investigators have studied the effects of added nucleophiles on the rates of deacylation of papain-catalyzed reactions and have provided strong evidence suggesting that amines and alcohols are specifically bound to the active center (Brubacher and Bender, 1966, 1967; Henry and Kirsch, 1967; Schechter and Berger, 1967; Fink and Bender, 1969; Berger and Schechter, 1970; Lucas and Williams, 1969). Only limited information is available, however, on bulk solvent effects on papain-catalyzed reactions (Henry and Kirsch, 1967; Sluyterman, 1967; Fink and Bender, 1969; Lucas and Williams, 1969). The present communication reports the isolation of two new acyl papains. The kinetics of hydrolysis of these acyl-enzymes has been examined under a variety of conditions in water and in inert organic solvent–water mixtures. Evidence is presented to suggest that nonreacting organic solvent molecules share the binding site of papain with the acyl group in a manner which improves the orientation of the acyl moiety and increases the overall rates of hydrolysis.

## Experimental Procedure

*Materials.* PAPAIN. Three-times-crystallized papain was prepared from dried papaya latex (We are grateful to the Wallerstein Co., New York, N. Y., for the gift of this material) in the absence of cysteine (Masuda, 1959) by a slight modification of

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the procedure of Kimmel and Smith (1954). The enzyme was inactivated with a slight molar excess of sodium tetrathionate (Eastman Chemical Corp.) (Sanner and Pihl, 1963) before the last recrystallization and stored at 4° as a 28-mg/ml suspension at pH 4 in 0.01 M sodium acetate buffer containing 0.5 mM EDTA. The tetrathionate-treated papain exhibited no activity with ZGlyNP[1] as substrate before activation and a specific activity of 1.28 after activation with cysteine (activation and assay as described by Klein and Kirsch, 1969). The specific activity of the preparation remained unchanged throughout the course of these experiments.

TLCK-inactivated papain was prepared by treating a solution of 0.8 mM cysteine-activated papain with 1.0 mM TLCK (Cyclo Chemical Corp.) at pH 4.5 in 0.2 M sodium acetate buffer containing 0.5 mM EDTA. After 15 min at room temperature the residual enzymatic activity was less than 1% of the initial activity as measured in the ZGlyNP assay.

Enzyme concentrations were determined from the absorbance at 278 mμ based on $E_{1\%}^{1cm}$ 25 (Glazer and Smith, 1961) and a molecular weight of 23,000 (Drenth *et al.*, 1968). The number of free sulfhydryl groups was determined by the method of Ellman (1959).

*Acylimidazoles. N-trans*-Cinnamoylimidazole was purchased from Aldrich Chemical Co. and recrystallized twice from *n*-hexane. 3-(2-Furyl)acryloylimidazole was prepared from 3-(2-furyl)acrylic acid (Aldrich Chemical Co.) by the mixed anhydride method (Bernhard *et al.*, 1965), and recrystallized from carbon tetrachloride; mp 114–114.5°, mp (Bernhard *et al.*, 1965) 113–114°. 3-(3-Indolyl)acryloylimidazole was also prepared by the mixed anhydride method; 20 mmoles of isobutyl chloroformate (Eastman White Label) and 20 mmoles of triethylamine were added to a solution of 20 mmoles of 3-(3-indolyl)acrylic acid (Aldrich Chemical Co.) in 50 ml of dioxane. The mixture was allowed to stand for 20 min at room temperature, cooled in ice, and filtered to remove triethylammonium chloride; 20 mmoles of sublimed imidazole was then added to the filtrate. After 6 hr at room temperature the mixture was evaporated to dryness at 40° in a rotary evaporator and the residue crystallized from benzene: mp 181–182° (uncor) after recrystallization from benzene; mp (lit.) 190°.

*Solvents.* Acetonitrile and dimethylformamide were distilled and stored over molecular sieves. Pyridine was distilled from calcium hydride and stored over sieves. Dioxane (Matheson Coleman and Bell) was refluxed and distilled from freshly ground lithium aluminum hydride under nitrogen atmosphere, and stored under nitrogen protected from light. All other solvents were reagent grade and used without further purification.

*Other Materials.* ZGlyNP was available from previous studies (Kirsch and Igelström, 1966). ZLysNP was purchased from Cyclo Chemical Corp. Sodium dodecyl sulfate was obtained from Dupont and recrystallized twice from ethanol. Bio-Gel P2 was purchased from Bio-Rad Laboratories and equilibrated for at least 12 hr in the appropriate buffer before use; 30% hydrogen peroxide (Superoxol) was obtained from Mallinckrodt and diluted immediately before use. Either L-cysteine·HCl (Nutritional Biochemical Corp.) or DL-cysteine·HCl (Calbiochem) was used for the activation of papain. *N*-Acetyl-L-cysteine was obtained from Calbiochem and 5,5′-dithiobis(2-nitrobenzoic acid) from Aldrich Chemical Co. All inorganic salts and buffers were reagent grade and used without further purification.

## Methods

*Preparation of Acyl Papains.* A suspension of papain (0.25 μmole in 0.25 ml) was activated with 0.05 ml of 0.1 M cysteine in 0.2 M potassium phosphate buffer containing 0.5 mM EDTA at pH 7 for 15–40 min at room temperature; 15 min is adequate time for complete activation of tetrathionate-treated papain under these conditions. The active enzyme solution was acidified by the addition of 0.05 ml of 2.0 M sodium acetate buffer, pH 4.0, containing 0.5 mM EDTA, and 0.05 ml of a solution of acylimidazole in acetonitrile was added immediately; TC-imidazole was 0.05 M, FA-imidazole, 0.1 M, and IA-imidazole, 0.02 M. After approximately 5 min at room temperature the solution was applied to a 1 × 20 cm Bio-Gel P2 column equilibrated with 0.01 M sodium formate or sodium citrate buffer at pH 3.7 containing 0.5 mM EDTA and a trace of thymol. The elution buffers were made to the ionic strength of the experiment with KCl, or in experiments involving sodium dodecyl sulfate, with NaCl. The columns were eluted at a flow rate of 0.2–0.5 ml/min and approximately 1-ml fractions were collected and scanned immediately in a spectrophotometer. Acyl papains appeared in fractions 5–7 and were separated by at least two fractions from cysteine, unreacted acylimidazoles, and free acids. The fractions containing acyl-enzyme were stored in the elution buffer in an ice bath and used before significant deacylation had occurred. Typical concentrations of the stock solutions were 20–100 μM acyl papain.

*Deacylation Kinetics.* An aliquot (20–50 μl) of the acyl-enzyme stock solution was warmed to approximately room temperature and added to a cuvet containing either 1.0 or 2.5 ml of the appropriate buffer equilibrated at the temperature of the experiment. At temperatures other than 25° the temperature of the reaction solution was checked immediately before and after the kinetic run with a Yellow Springs Instrument Co. thermistor. In all cases the temperature remained constant within ±0.1°. Kinetics were monitored by the decrease in absorbance at $\lambda_{max}$ of the acyl papain (360 mμ for FA-papain, 330 mμ for TC-papain, 400 mμ for IA-papain) on the 0–0.1 *A* scale of either a Unicam SP800A or Gilford Model 220 recording spectrophotometer. Measurements of the rate of deacylation of previously denatured FA-papain were carried out at 330 mμ. After completion of kinetic runs the pH values of the solutions were read immediately on a Radiometer 25SE or PHM4c pH meter fitted with a combined Type B electrode. At temperatures other than 25° the solutions were maintained at the temperature of the kinetic run during pH measurement.

Kinetics studies were performed at either ionic strength 0.05 or 0.5. The following experiments were carried out at a final ionic strength of 0.5 in 0.05 M buffers: the reactions of sodium dodecyl sulfate denatured papain; the temperature dependence of FA-papain hydrolysis in water; and the rates of hydrolysis of FA-papain at pH values above 12. Ionic strength in these reactions was maintained with KCl except in experiments in the presence of sodium dodecyl sulfate, in which case

[1] The abbreviations used are: ZGlyNP, benzyloxycarbonylglycine *p*-nitrophenyl ester; ZLysNP, α-*N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester; TLCK, α-*N*-tosyl-L-lysine chloromethyl ketone; FA-, 3-(2-furyl)acryloyl-; IA-, 3-(3-indolyl)acryloyl-; TC-, *trans*-cinnamoyl-.

only sodium salts were used. All other experiments were done at an ionic strength of 0.05. All buffers contained 0.5 mM EDTA; no buffer effects were noted when rates were followed as a function of buffer concentration. In the pH range specified the following buffers were used: 1.0–2.8, HCl–NaCl; 2.8–4.5, sodium formate; 4.0–6.0, sodium acetate; 6.0–8.0, sodium or potassium phosphate; 8.5–10.0, sodium borate; 10.5–12.5, sodium or potassium phosphate; 9.5–11.0, sodium or potassium carbonate; above 12.5, KOH–KCl or NaOH–NaCl. In 30% dioxane the ranges of the various buffers were approximately 0.5 pH unit higher. Organic solvent solutions were made volume per cent and the concentration of water in these solutions was calculated by weight. Unless otherwise noted, all experiments were carried out at 25.0 ± 0.1°. For measurements of the rate of deacylation of denatured FA-papain a stock solution of FA-papain was brought to 1% sodium dodecyl sulfate at pH 3.7 and aliquots added to the appropriate buffers, which also contained 1% sodium docecyl sulfate.

*Other Methods.* Michaelis–Menten parameters were determined for the hydrolysis of ZGlyNP in 27.6% dioxane essentially by the procedure of Kirsch and Igelström (1966). Kinetic runs were carried out at 25° in potassium phosphate buffers containing 0.5 mM EDTA, pH 7.0, and a final ionic strength of 0.05. Final concentrations were: 5–110 μM ZGlyNP; 60 nM papain; 7.3% acetonitrile; and 27.6% dioxane. Values of $k_{cat}$ and $K_m$ for the hydrolysis of ZLysNP were determined in the same buffer at a pH of 6.8 and final concentrations: 5.2–208 μM ZLysNP; 22 nM papain; 1.5% acetonitrile; and 27.6% dioxane. Reactions were started by the addition of enzyme. Blank rates of hydrolysis were determined at each concentration of substrate and were negligible in the case of ZGlyNP and less than 20% of the enzyme-catalyzed rates in the case of ZLysNP. Values of $k_{cat}$ in aqueous solution for this batch of enzyme were determined from the rate of hydrolysis of substrate at concentrations greater than 12 times $K_m$. The values of $K_m$ and $V_m$ in 27.6% dioxane were calculated by program HYPERB (Hanson *et al.*, 1967).

The concentration of peroxides in 50% dioxane was determined by oxidation of potassium iodide by standard methods (Day and Underwood, 1958) except that the iodine produced was measured spectrophotometrically at 410 mμ. Standard solutions of dioxane to which 0.04–0.4 mM hydrogen peroxide had been added were titrated to determine the sensitivity of the assay and an upper limit of 0.035 mM oxidizing material was established.

The rate of release of *p*-nitrophenol from *p*-nitrophenyl acetate was monitored at pH 10.48 at 400 mμ in potassium carbonate buffers at 25°. Rates were determined in 50% dioxane solutions containing 0 to 0.4 mM *added* hydrogen peroxide. An upper limit of 0.04 mM peroxide contamination was calculated assuming that the observed rate in 50% dioxane solution was entirely due to hydrogen peroxide.

All spectra were obtained on a Unicam SP800A recording spectrophotometer. The spectra of FA- and IA-*N*-acetylcysteine were obtained by adding FA- or IA-imidazole to 1 ml of a solution containing 0.1 M *N*-acetylcysteine in potassium carbonate buffer at pH 10.0 to give final concentrations of approximately 20 μM *S*-acyl-*N*-acetylcysteines and scanning immediately. The spectra of protonated FA- and IA-imidazole were measured by adding 1 M HCl to solutions of the neutral acylimidazoles to give a final concentration of 0.01 M HCl, and scanning immediately. Extinction coefficients were determined by comparison with those of the unprotonated acylimidazoles based on the values reported by Bernhard *et al.* (1965).

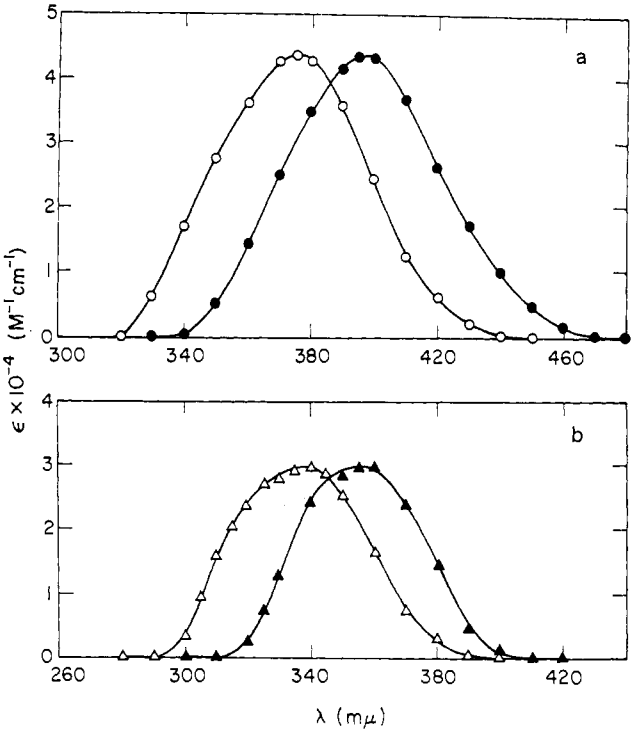



FIGURE 1: (a) (●) The spectrum of IA-papain *vs.* papain; (○) the spectrum of sodium dodecyl sulfate denatured IA-papain *vs.* papain. (b) (▲) The spectrum of FA-papain *vs.* papain; (△) the spectrum of sodium dodecyl sulfate denatured FA-papain *vs.* papain. The conditions for the native acyl-enzymes were: 25°, pH 5.4, 0.05 M sodium acetate buffer containing 0.5 mM EDTA, at an ionic strength of 0.5. The conditions for the denatured acyl-enzymes were: 25°, pH 4.0, 1% sodium dodecyl sulfate, sodium formate buffer containing 0.5 mM EDTA, at an ionic strength of 0.05.

## Results

*Preparation and Properties of Acylpapains.* Good yields of either FA- or IA-papain were obtained when acylation was carried out at pH values near 4.0. When papain previously inactivated with either TLCK or sodium tetrathionate was treated with IA- or FA-imidazole under conditions identical with those in these experiments, the spectrum of the enzyme after isolation on Bio-Gel P2 was indistinguishable from that of untreated papain, indicating that the single sulfhydryl group blocked by these reagents is the only site of acylation.

The absorption spectra of native and denatured FA- and IA-papain are shown in Figure 1. IA-papain is moderately stable at pH 5.5 and its spectrum was determined directly. The spectrum of FA-papain was determined by repeatedly scanning the sample and extrapolating the absorbance values taken at short wavelength intervals to zero time. Extinction coefficients as a function of wavelength were obtained by permitting samples to deacylate completely and comparing the total absorbance change of the acyl-enzyme (AE) to that of the

TABLE I: Absorption Spectra of RC(O)X.

| X | $\lambda_{max}$ (m$\mu$) ($\epsilon \times 10^{-4}$) for R = | | |
|---|---|---|---|
| | *trans*-Cinnamoyl | Furylacryloyl | Indolylacryloyl |
| Imidazole | 307 (2.5)[a] | 340 (2.7)[a] | 378 (3.0)[a] |
| Imidazolium | | 353 (2.7)[b] | 395 (3.0)[b] |
| *S*-Acyl-*N*-acetylcysteine | | 338[b] | 363[b] |
| *S*-Acylcysteine | 306 (2.3)[c] | | |
| *S*-Acyl-*N*,*N*-dimethylcysteamine | | | 365 (3.0)[d] |
| Papain | 326 (2.6)[c] | 360 (3.0)[b] | 398 (4.3)[b] |
| Denatured papain | 301–309[c] | 337 (3.0)[b] | 373 (4.3)[b] |
| Glyceraldehyde 3-phosphate dehydrogenase | | 344 (3.0)[e] | |
| Denatured glyceraldehyde 3-phosphate dehydrogenase | | 337 (3.0)[e] | |
| *N*-Acetylserinamide | 281 (2.4)[f] | 309[a] | 335[a] |
| Chymotrypsin | 292 (1.8)[g] | 320 (2.0)[g] | 360 (1.8)[g] |
| Denatured chymotrypsin | 281 (1.8)[g] | 310 (2.0)[g] | 335 (1.9)[g] |
| Novo subtilisin | 289 (1.9)[h] | | 349 (2.5)[h] |
| Denatured novo subtilisin | 284 (2.0)[h] | | 340 (2.1)[h] |

[a] Bernhard *et al.* (1965). Somewhat lower extinction coefficients for these compounds have been reported by Oliver *et al.* (1967). [b] Conditions are given in the text. [c] Bender and Brubacher (1964). [d] Dunn and Bernhard (1969). [e] Malhotra and Bernhard (1968). [f] Bender *et al.* (1962a). [g] Oliver *et al.* (1969). [h] Johansen *et al.* (1969).

product acid (A), based on the extinction coefficients for the free acids reported by Bernhard *et al.* (1965).

$$\epsilon_{\lambda}^{AE} = \frac{\Delta OD_{\infty,\lambda}^{AE}}{\Delta OD_{\infty,\lambda_{max}}^{A}} \times \epsilon_{\lambda_{max}}^{A}$$

Denatured FA- and IA-papain are stable and the spectra were obtained by adding sodium dodecyl sulfate to known amounts of the native acyl-enzymes; extinction coefficients were determined from the relationship

$$\epsilon_{\lambda}^{AE,denat} = \frac{OD_{\lambda}^{AE,denat}}{OD_{\lambda_{max}}^{AE}} \times \epsilon_{\lambda_{max}}^{AE}$$

The spectra of denatured FA- and IA-papain were not altered by chromatography on Bio-Gel P2 and the position of the absorption maxima did not change over the pH range 0.8–12.0.

The spectral characteristics of FA- and IA-papain are compared with those of other acyl-enzymes and with various model compounds in Table I. Denatured acyl papains exhibit spectra similar to the corresponding *S*-acyl-*N*-acetylcysteines. The fact that the absorption maxima do not change in acid is further evidence that the acyl groups are on the sulfhydryl residue of papain and not on an imidazole group. Protonation shifts the absorption maxima of FA- and IA-imidazoles about 20 m$\mu$ to the red. Under denaturing conditions the p$K_a$ of an enzyme histidine should be near normal and certainly above 0.8.

The preparations of acyl-enzyme used in these experiments contained between 0.3 and 0.65 mole of acylated enzyme per mole of total papain. The degree of acylation reflects the sulfhydryl content of the papain, which was found to be 65% by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

*Deacylation in Water.* The observed rates of deacylation were strictly first order and independent of concentration in the range 0.3–3 $\mu$M acyl-enzyme. When the rates of deacylation were followed by the decrease in absorbance at $\lambda_{max}$ of the acyl-enzyme, the increase in absorbance at $\lambda_{max}$ of the free acid, and the increase in enzymatic activity toward ZGlyNP, first-order and identical rate constants were obtained. Deacylating samples were scanned repeatedly and tight isosbestic points were obtained, indicating that no intermediate accumulates in the reaction.

At high pH and at high (>5 $\mu$M) concentrations of acyl-enzyme slightly nonlinear plots of log ($A_t - A_\infty$) *vs.* time were obtained and the apparent rate constants increased with increasing enzyme concentration. This effect was negligible below pH 7 and increasingly severe at higher pH values. At high concentrations the infinity absorbance at $\lambda_{max}$ of the acyl enzyme did not reach zero, as expected from the spectrum of free acids. This latter observation was also made by Brubacher and Bender (1966) for the hydrolysis of TC-papain. It seemed likely that these results were due to the reaction of the acyl-enzymes with a nucleophilic group on the papain molecule itself, probably an amino group. In order to test this possibility, FA-papain was allowed to deacylate at pH 9 for more than 20 half-lives at an initial acyl-enzyme concentration of 30 $\mu$M and the products were isolated on Bio-Gel P2. The spectrum of the isolated protein was significantly different from that of native papain. The difference spectrum of the isolated product *vs.* papain showed an absorption maximum between 305 and 310 m$\mu$ and was unchanged in the pH range 2–7, indicating that the spectrum was not due to furylacrylic acid. This spectrum is consistent with FA-NHR or FA-OR (Bernhard *et al.*, 1965) but not with that of denatured FA-papain, which absorbs at 330 m$\mu$ (Table I). The addition of 20 $\mu$M tetrathionate-treated papain to low concentrations of FA-papain also caused an increase in apparent rate constant and a non-

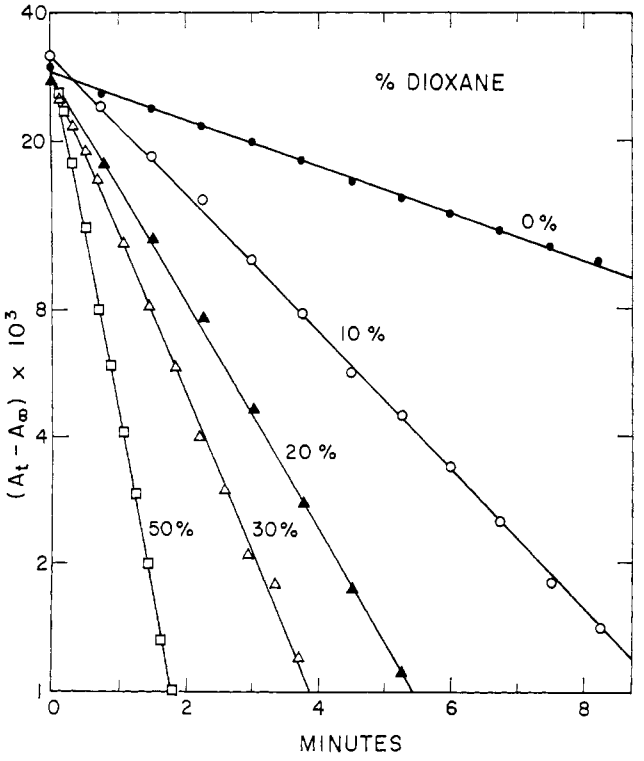



FIGURE 2: Typical first-order plots of the rates of deacylation of FA-papain in dioxane–water mixtures. Rates were determined from the change in absorbance at 360 mμ at 25° at pH 7.5 in potassium phosphate buffers containing 0.5 mM EDTA and an ionic strength of 0.05.

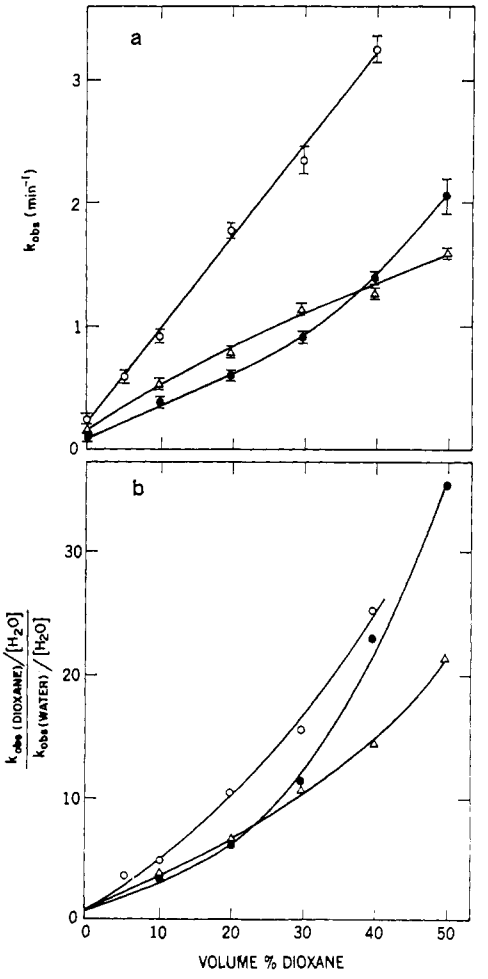



FIGURE 3: (a) Observed first-order rate constants for the hydrolysis of (●) FA-, (△) IA-, and (○) TC-papain as a function of dioxane concentration. The ordinate for IA-papain is times 20. Conditions were those given in Figure 2. The error flags are ± the standard deviations of the determinations. (b) The ratio of the second-order rate constants for the hydrolysis of FA-papain in dioxane solutions to those in water for: (●) FA-, (△) IA-, and (○) TC-papain. The second-order rate constants were determined from the data in part A, based on the following rate constants in water: FA, 0.116 min$^{-1}$; IA, 0.0076 min$^{-1}$; TC, 0.220 min$^{-1}$.

linear first-order plot, demonstrating that this effect is at least in part due to an intermolecular reaction and that it is not due to autolysis, which would require a free sulfhydryl group on papain.

All further experiments reported in this communication were carried out at enzyme concentrations dilute enough to be within the range of concentration independence.

*Effect of Organic Solvents.* The rates of hydrolysis of acyl papains are dramatically increased in the presence of organic solvents. Typical first-order plots of the hydrolysis of FA-papain in water and dioxane solutions are shown in Figure 2. The observed rates of hydrolysis of FA-, IA-, and TC-papain in the presence of increasing concentrations of dioxane are shown in Figure 3a; the rate constants, after correction for decreasing water concentration, are compared to the aqueous rates in Figure 3b. In 30% dioxane solution the observed rates of hydrolysis of FA-papain at pH 7.5 were strictly first order and identical over a 13-fold range of protein concentration. The products of the reaction were isolated on a Bio-Gel P2 column and found to be spectrally identical with native papain and furylacrylic acid both of which were present in the expected amounts. The absorption spectra of FA-papain in 24% dioxane and IA-papain in 45% dioxane, pH 7.5, were not significantly different from those in water alone.

The following experiments indicate that the effect of dioxane is reversible. First, FA-papain (final concentration 13 μM) was brought to 40% dioxane at pH 7.0. At times equivalent to 30, 64, and 80% deacylation, aliquots were withdrawn and rapidly diluted to a final concentration of 3% dioxane and the rates of deacylation of the remaining acyl-enzyme were monitored. The rates obtained were those expected for 3% dioxane, demonstrating that no time-dependent irreversible change in papain took place at the higher dioxane concentration. Second, FA-papain was permitted to deacylate for exactly one half-life in 49% dioxane at pH 7.0 and rapidly diluted to give final concentrations of dioxane equal to 5, 14, 23, and 31% and the rates of deacylation were followed. Again the resultant rates were those predicted from the data shown in Figure 3. It was shown qualitatively that deacylation in 30% dioxane is accompanied by the formation of free enzyme as measured by the increase in enzymatic activity toward ZGlyNP.

The activity of papain toward ZGlyNP and ZLysNP was determined in 27.6% dioxane to see whether the enhancement effect of organic solvents on the rates of deacylation is also operative under steady-state conditions. The results of these

TABLE II: Effect of Dioxane on the Kinetics of Papain-Catalyzed Hydrolysis of Esters.[a]

| Substrate | Solvent | $K_m$ (M × $10^6$) | $k_{cat}$ ($sec^{-1}$) | $k_{cat}/K_m$ ($M^{-1}$ $sec^{-1}$ × $10^{-6}$) | $\frac{k_{cat}\ (dioxane)}{k_{cat}\ (H_2O)}$[b] | $\frac{K_m\ (dioxane)}{K_m\ (H_2O)}$ |
|---|---|---|---|---|---|---|
| ZGlyNP | $H_2O$ | 9.3[c] | 4.83 | 0.52 | | |
| ZGlyNP | 27.6% dioxane | 192 ± 11 | 11.5 ± 0.5 | 0.06 | 3.3 | 21 |
| ZLysNP | $H_2O$ | 3.16[d] | 36.4 | 11.5 | | |
| ZLysNP | 27.6% dioxane | 63.3 ± 6.3 | 42.0 ± 1.6 | 0.66 | 1.6 | 20 |

[a] Conditions are given in the Experimental Section. [b] Rate constants are corrected for the decreased concentration of water in dioxane solutions. [c] Kirsch and Igelström (1966). [d] Fink and Bender (1969).

experiments are given in Table II; $k_{cat}$ for ZGlyNP and ZLysNP reflects the rate of deacylation, which is rate limiting with these substrates (Kirsch and Igelström, 1966; Bender and Brubacher, 1966). It is clear from Table II that the primary effect of the solvent is to increase $K_m$ and that the acceleration of the rate of deacylation is less prominent with good substrates than with the relatively stable acyl-enzymes.

It is extremely unlikely that the increased rates of deacylation in dioxane were due to nucleophilic contaminants in the solvents. The dioxane was carefully prepared and an upper limit of 40 μM established for the concentration of peroxides, the most probable nucleophilic contaminants (see Experimental Section for details). Neither the addition of 400 μM $H_2O_2$ nor the use of undistilled or old dioxane had any effect on the rate of deacylation of FA-papain in 30% dioxane at pH 7.5. Finally, as shown in Table III, the rate of hydrolysis of FA-papain at neutral pH is increased by a large number of organic solvents differing widely in structure.

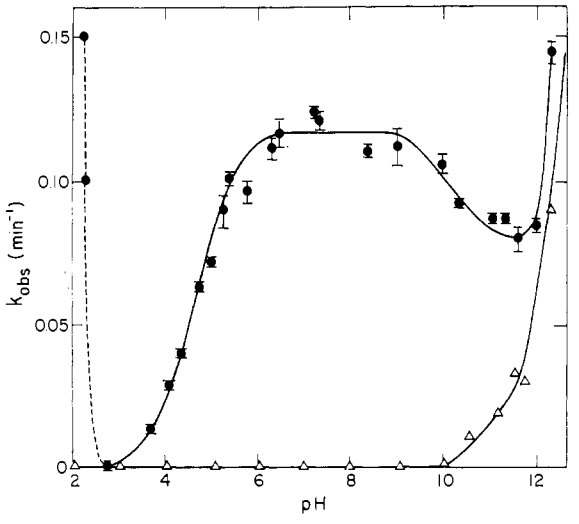



FIGURE 4: The pH dependence of the rates of reaction of FA-papain in aqueous solution. (●—●) The rate of hydrolysis of FA-papain. The solid line is theoretical from pH 3 through pH 9 for a $pK_a$ = 4.63 and $k_{lim}$ = 0.116 $min^{-1}$. (●- - -●) The rates of denaturation of FA-papain. (△) The rate of hydrolysis of sodium dodecyl sulfate denatured FA-papain in buffers containing 1% sodium dodecyl sulfate. The conditions are given in the text. The error flags are ± the standard deviation of three or more determinations.

*pH Dependence of the Rates of Reaction of FA-Papain in Aqueous Solution.* The pH dependence of the deacylation of FA-papain in aqueous solution is shown by the solid lines in Figure 4. The acidic $pK_a$ for hydrolysis is 4.63 ± 0.03 and the limiting value of $k_{H_2O}$ is 0.116 ± 0.008 $min^{-1}$. The $pK_a$ of 4.63, determined at an ionic strength of 0.05, is similar to that of 4.7 reported by Brubacher and Bender (1966) for the hydrolysis of TC-papain at an ionic strength of 0.3. The dip in the pH–rate profile at high pH values was also observed in the hydrolysis of TC-papain and is found for IA-papain (P. M. Hinkle and J. F. Kirsch, unpublished observations). We have found, as did Brubacher and Bender (1966), that this is neither an ionic strength nor a buffer effect. The logarithms of the rates of reaction of FA-papain at high pH are plotted against pH in Figure 5a. The slope of the line drawn through the points is 1.4. The products of the reaction at high pH were shown both spectrally and by product isolation to be 3-(2-furyl)acrylic acid and papain.

TABLE III: Effect of Organic Solvents on the Rates of Deacylation of FA-Papain.

| Solvent[a] | $k_{obsd}$ ($min^{-1}$)[b] | $\frac{k_{obsd}\ (30\%\ solvent)}{k_{obsd}\ (H_2O)}$[c] |
|---|---|---|
| $H_2O$ | 0.12 | |
| Acetone | 0.49 | 5.9 |
| Acetonitrile | 0.21 | 2.6 |
| Dimethoxyethane | 0.67 | 8.2 |
| Dimethylformamide | 0.47 | 5.7 |
| Dimethyl sulfoxide | 0.39 | 4.7 |
| Dioxane | 0.80 | 10 |
| Pyridine[d] | 0.85 | 10 |
| Tetrahydrofuran | 1.0 | 12 |

[a] Reactions carried out at pH 7.5 in potassium phosphate buffers containing 0.5 mM EDTA and a final ionic strength of 0.05 at 25°. Organic solvents, where used, were 30% (v/v). [b] The observed rate constants represent an average of two or more determinations. [c] The rates are corrected for the decreased concentration of water in solutions containing organic solvent. [d] The rate constant is not corrected for possible nucleophilic attack of pyridine on the acyl-enzyme.

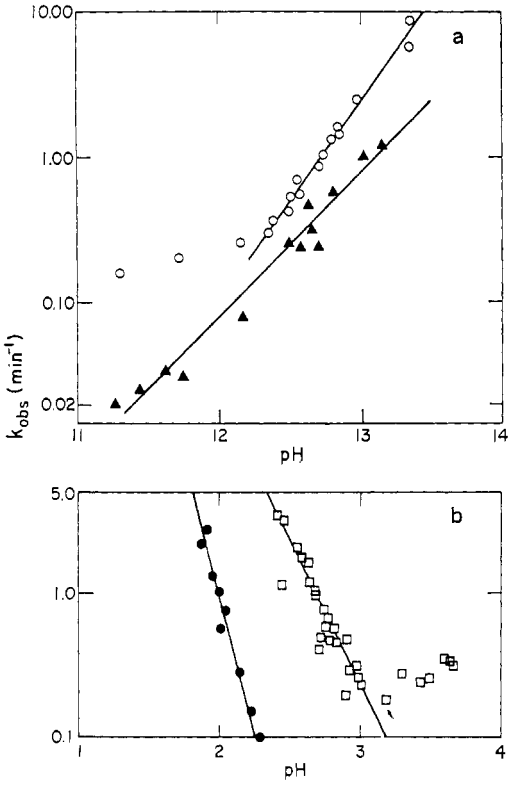



FIGURE 5: The reactions of FA-papain in alkaline and in acidic solutions. (a) (○) The rate of disappearance of FA-papain; (▲) the rate of hydrolysis of sodium dodecyl sulfate denatured FA-papain in buffers containing 1% sodium dodecyl sulfate. (b) The rates of disappearance of FA-papain: (●) in aqueous solution; (□) in 30% dioxane. The conditions are given in the Experimental Section.

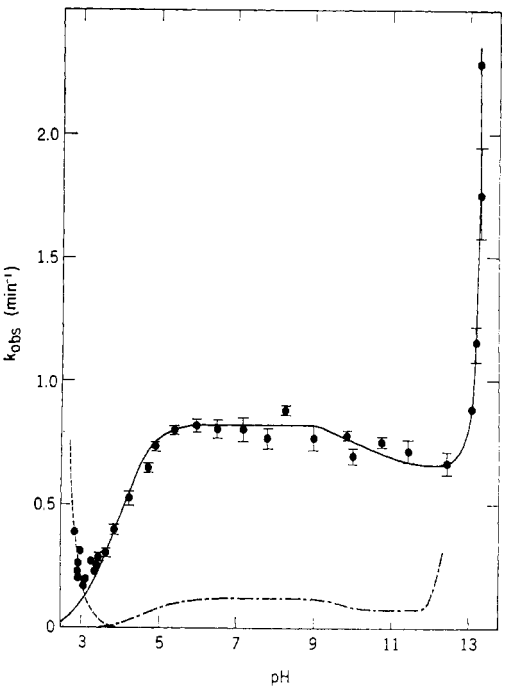



FIGURE 6: The pH dependence of the rates of reaction of FA-papain in 30% dioxane. The solid line is theoretical from pH 3 through pH 9 for the rate of hydrolysis dependent upon a $pK_a$ = 3.91 and $k_{lim}$ = 0.804 min$^{-1}$. (— — —) The rate of denaturation of FA-papain. (-·-) The rate of hydrolysis of FA-papain in aqueous solution (from Figure 4). The conditions are given in the Experimental Section. The error flags are ± the standard deviation of three or more determinations.

However, whether or not deacylation is accompanied by denaturation was not determined. It is possible that both a denaturation reaction and the hydrolysis of denatured and/or native acyl papain contribute to the rate constant obtained from the decrease in absorbance at 360 mμ.

The rates of hydrolysis of sodium dodecyl sulfate denatured FA-papain are also shown in Figures 4 and 5a. The rate of hydrolysis of denatured acyl papain is negligible in acidic and neutral solution and, as shown in Figure 5a, is dependent on the first power of hydroxide ion concentration.

The dotted line in Figure 4 depicts the rate of acid-catalyzed denaturation reaction of FA-papain which does not involve the loss of the acyl group. The denaturation of FA-papain at pH values below 2.5 was characterized by a tight isosbestic point and an infinity spectrum identical with that of denatured FA-papain. After chromatography of the reaction products only denatured FA-papain, and no furylacrylic acid, was found. The rate of denaturation in acid is dependent on approximately the fourth power of hydrogen ion concentration, as shown in Figure 5b.

*Reactions of FA-papain in 30% Dioxane.* The pH–rate profile for the hydrolysis of FA-papain in 30% dioxane solution is shown by the solid line in Figure 6, with the values in aqueous solution included for comparison. These determinations were carried out at 25° and an ionic strength of 0.05. The large increases in the rate of hydrolysis of FA-papain in the presence of organic solvent are observed at all pH values below 12. As shown in Figure 6, the dip in the rate of hydrolysis of FA-papain at pH values above 9 is still observed in 30% dioxane but it is relatively smaller than in water.

Denaturation of FA-papain at acidic pH in 30% dioxane is shown in Figure 6. The acid-catalyzed denaturation occurs at higher pH values in organic solvent and depends on a second-order term in hydrogen ion concentration as shown in Figure 5b. The nature of the reaction was confirmed by product isolation. At pH 2.85 only denatured FA-papain was found, and at pH values above 3.25 only free papain and 3-(2-furyl)-acrylic acid were produced.

An apparent $pK_a$ value for the hydrolysis of FA-papain in 30% dioxane was found by subtracting the fraction of the rate constant due to denaturation, determined from the extrapolation shown in Figure 5b, from the observed rate constants at pH values above 3.25. The corrections involve subtraction of a denaturation rate constant which is less than 10% of the observed rate. In addition the $pK_a$ was determined from only those points at pH values above 4.0, where the contribution of denaturation to the total reaction is negligible and the same value was obtained. The $pK_a$ determined in this manner was 3.91 ± 0.03 and the limiting value of $k$ = 0.804 ± 0.057 min$^{-1}$.

*Temperature Dependence of the Hydrolysis of FA-Papain.* The rates of hydrolysis of FA-papain were determined at various temperatures at neutral pH in aqueous solution and in 30% dioxane. Arrhenius plots are given in Figure 7 and activation parameters, calculated with the aid of a computer program, in Table IV. The rates in dioxane were determined over

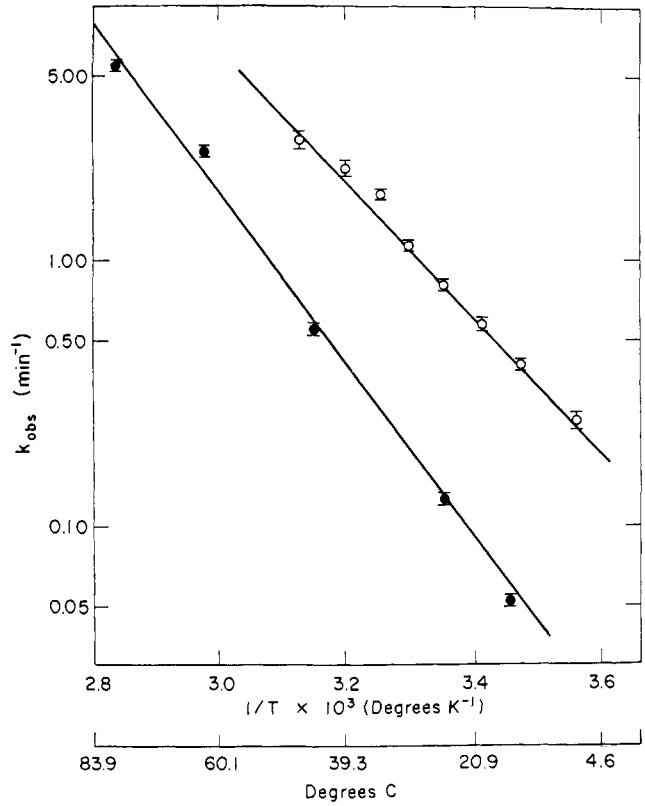



FIGURE 7: Temperature dependence of the rates of hydrolysis of FA-papain at neutral pH: (●) in water; (○) in 30% dioxane. The conditions are given in Table IV. The error flags are ± the standard deviation. The lines are drawn for the values of $E_a$ given in Table IV.

a more limited temperature range since hydrolysis was too rapid to monitor at temperatures above 45°.

## Discussion

*Spectral Characteristics and Rates of Hydrolysis of Acyl Papains.* The metastable acyl-enzymes used in the present studies are considered to be thioesters of the active sulfhydryl group of papain for the following reasons. First, both the free sulfhydryl group and enzymatic activity toward ZGlyNP are required for the formation of the acyl-enzymes and both are lost upon acylation. Second, the deacylation rates of the acyl-enzymes exhibit a pH dependence typical of that observed for $k_{cat}$ in the steady state with substrates where deacylation is known to be the rate-limiting step (Brubacher and Bender, 1966; Bender and Brubacher, 1966; Williams and Whitaker, 1967; Smith and Parker, 1958; Whitaker and Bender, 1965). Finally, the spectra of the denatured acyl-enzymes are essentially the same as the corresponding *S*-acylcysteines (Table I) and the pH dependence of the hydrolysis rates of the denatured acyl-enzymes is typical of thio esters or esters but not of amides or acylimidazoles.

FA-, IA-, and TC-papain are all characterized by absorption maxima occurring at considerably higher wavelengths (19–35 mμ) than those of the corresponding *S*-acylcysteine derivatives or of the denatured acyl-enzymes (Table I). This red shift corresponds to a transition energy of from 4 to 6 kcal relative to the denatured acyl enzymes and is similar in magnitude and direction to those observed for various acyl chymotrypsins and subtilisins, although the serine proteases are characterized by abnormally low extinction coefficients (Table I). Only one stable acyl-enzyme, FA-glyceraldehyde 3-phosphate dehydrogenase (Malhotra and Bernhard, 1968), absorbs light at a wavelength predicted by analogy with a model compound. The reasons for the red shift observed with acyl proteases are not fully understood. In highly polar environments $\lambda_{max}$ of model acyl compounds is shifted to *slightly* higher wavelengths (Bender *et al.*, 1962a). Charney and Bernhard (1967), however, have suggested that when bound to enzymes the acyl groups are in an "s-cis" configuration and that denaturation involves an "s-cis" to "s-trans" rotational isomerization, which accounts for the *magnitude* of the red shift but not the observed change in extinction coefficients (Johansen *et al.*, 1969). The extinction coefficients of native and denatured FA- and IA-papain are the same in the native and denatured states (Table I), but $\epsilon_{max}$ should increase if the acyl groups undergo an "s-cis" to "s-trans" isomerization upon denaturation (Johansen *et al.*, 1969).

TABLE IV: Activation Parameters for the Hydrolysis of FA-Papain in Water and in 30% Dioxane at Neutral pH.[a]

| | FA-Papain in Water[b] | FA-Papain in 30% Dioxane[c] |
|---|---|---|
| $E_a$ (kcal/mole) | 14900 ± 100 | 11500 ± 200 |
| $\Delta H^{\pm}$ (kcal/mole)[d] | 14300 ± 100 | 10900 ± 200 |
| $\Delta F^{\pm}$ (kcal/mole)[d] | 21100 ± 400 | 19800 ± 300 |
| $\Delta S^{\pm}$ (eu)[d] | −22.7 ± 1.5 | −29.8 ± 1.3 |

[a] Activation parameters were determined for the observed rates of hydrolysis. The rate constants measured in dioxane solution were multiplied by 55.5/38.9 to correct for the decreased concentration of water. The values are ± the standard deviation. [b] pH 7.0 in 0.05 M K phosphate buffers containing 0.5 mM EDTA and an ionic strength of 0.5. [c] pH 7.5 in K phosphate buffers containing 0.5 mM EDTA, 30% dioxane, and an ionic strength of 0.05. [d] At 25°.

A plot of the logarithms of the observed rates of deacylation in the pH-independent region of the three acyl papains (FA-, IA-, and TC-) against the p$K_a$ values of the free acids exhibits a slope of 2.5 (not shown). The significance of a free energy relationship based on three points taken over a narrow range is limited, but the data are sufficient to suggest a strong dependence of the rates of deacylation on electron withdrawal in the acyl group. The observed dependence is similar to that reported by Caplow and Jencks (1962) for the deacylation of acyl chymotrypsins and likewise provides presumptive evidence for general base catalysis of the *deacylation* reaction.

*Rate Accelerations Induced by Organic Solvents.* The solvent-mediated increases in the rates of acyl-papain hydrolysis constitute the largest *positive* solvent effect for an enzyme-catalyzed reaction of which we are aware. The variety of solvents which accelerate FA-papain hydrolysis indicates that the effect is general and is not due to some artifact such as solvent contamination, a possibility which was carefully examined in

the case of dioxane (see Results). The products of FA-papain deacylation between pH 3 and 12 were shown quantitatively to be the products of a simple hydrolysis, and the pH dependence and *relative* rate constants of FA-, IA-, and TC-papain in dioxane–water mixtures and in aqueous solution are similar. These results argue against any fundamental change in the hydrolytic mechanism effected by nonreacting organic solvents.

A number of experiments suggest that papain does not undergo any large changes in physical structure in organic solvent–water mixtures. The fact that the absorption spectra of acyl papains are unchanged in concentrations of up to 45% dioxane indicates that there is no gross change in the electronic environment around the acyl group approaching the change seen on denaturation of the acyl-enzymes. Several solvent mixtures, including 20% v/v dioxane, do not change the fluoresence emission spectrum of active papain and cause only a small solvent-dependent change in the ultraviolet absorption spectrum of the enzyme (Barel and Glazer, 1969). Neither the optical rotatory dispersion nor the viscosity of papain is altered by methanol concentrations of up to 70% (Sluyterman, 1967) and papain crystals, which are obtained from 50% methanol, are active in the crystalline state (Sluyterman and De Graaf, 1969). None of these experiments, of course, rules out rather small changes in the environment of the active site in solvent–water mixtures.

The observation of a solvent-mediated increase in the rate of deacylation of proteolytic enzymes is not unprecedented. Inward and Jencks (1965) noted a 30% increase in the rate of hydrolysis of furoyl chymotrypsin in the presence of several alcohols; the rate of hydrolysis of acetyl chymotrypsin is increased slightly in the presence of indole (Foster, 1961) and 3.5-fold in 20% v/v dioxane (Faller and Sturtevant, 1966). Acetonitrile causes a more than 10-fold activation of tyrosinase enzymes toward *p*-nitrophenyl *N*-carbobenzoxy-L-tyrosinate (Marshall *et al.*, 1969). Small solvent-dependent increases in the rates of papain-catalyzed hydrolyses have been observed in acetonitrile (Henry and Kirsch, 1967) and dimethyl sulfoxide (Sluyterman, 1967), in the presence of added alcohol and amine nucleophiles (Fink and Bender, 1969), and in low concentrations of benzamidine, guanidine, and *N*-acetyl- and *N*-benzoylglycine (Whitaker, 1969). None of these previously reported rate increases for acyl papain is greater than 50%, however, and in several other cases the rates of hydrolysis have been unaffected or decreased by organic solvents (Fink and Bender, 1969; Lucas and Williams, 1969). In all cases where papain catalysis has been measured under steady-state conditions any changes in $k_{cat}$, when deacylation is rate determining, have been much smaller than the very large solvent-induced increases in $K_m$ which reflect either a decrease in the rate of acylation or more likely an increase in the dissociation constant of the enzyme–substrate complex. The net effect of these two competing factors (increase in $K_m$ and, sometimes, in $k_{cat}$) is that organic solvents tend to behave as competitive or mixed inhibitors for most substrates.

Large solvent-mediated accelerations of papain-catalyzed hydrolyses occur only with relatively stable acyl-enzymes (FA-, IA-, and TC-), while the deacylation rate constants of less stable acyl-enzymes (Z-glycyl- and Z-L-lysylpapain) are only slightly increased (Table II). These results suggest that organic solvents may act to improve the orientation of the $\beta$-aroyl group with respect to catalytic groups on the enzyme, thereby increasing the rates of acyl transfer to water. In the case of the more reactive acyl-enzymes (Z-glycyl-, Z-L-lysyl-) it is reasonable to assume that orientation is more nearly optimal and therefore less subject to improvement by added solvents. The active-site region of papain is large (Drenth *et al.*, 1968) and capable of binding both large peptide substrates (Schechter and Berger 1967; Berger and Schechter, 1970) and added nucleophiles (Brubacher and Bender 1966, 1967; Fink and Bender, 1969) with considerable specificity, so the catalytic center can probably accommodate solvent molecules in addition to small substrates. The activation parameters shown in Table IV are also consistent with a mechanism involving solvent binding. The entropy of activation for the deacylation of FA-papain decreases from $-22.7$ eu in water to $-29.8$ eu in 30% dioxane, suggesting a shift to a higher kinetic order in organic solvent (Frost and Pearson, 1961; Bruice and Benkovic, 1966). The observed rate increases are due to a more than compensating decrease in the enthalpy of activation of the reactions in organic solvent mixtures. This latter observation may be indicative of some strain in the ground state of the acyl-enzyme or alternatively a relief of strain in the transition state. Alternatively, solvent may interact at some distant site on the papain molecule to bring about an improved alignment of the acyl and catalytic groups; the present data cannot distinguish between these models.

Results interpretable by the mechanism described above are not uncommon. The best studied example for a proteolytic enzyme is the severalfold increase in the rates of trypsin-catalyzed hydrolysis of *N*-acetylglycine ethyl ester, a poor substrate, by alkylguanidines and -amines (Inagami and York, 1968; Inagami and Murachi, 1964). These activators, like the organic solvents used in the present study, become inhibitors for normal substrates such as $\alpha$-*N*-benzoylarginine ethyl ester. The accelerating effects of nitrogen bases with trypsin, unlike the solvent acceleration of acyl-papain hydrolysis, are temperature independent, indicating some difference in mechanism from the present case. Pig liver esterases are subject to pronounced substrate activation. Various organic solvents diminish this activation and concomitantly increase the overall enzymatic activity (Barker and Jencks, 1969; Stoops *et al.*, 1969). A maximum increase in $V_{max}$ of $\sim$ twofold was obtained in 1.3 M acetonitrile in the hydrolysis of phenyl butyrate (Stoops *et al.*, 1969). The rate of carbamylation of acetylcholinesterase is increased up to 14-fold by tetraalkylammonium ions (Metzger and Wilson, 1963), and the citrate-condensing enzyme-catalyzed hydrogen-isotope exchange into acetyl coenzyme A is stimulated by the oxaloacetate analog, *s*-malate (Eggerer, 1965). Boyer has recently suggested the comprehensive term "substrate synergism" for the acceleration of a reaction involving one substrate of a multisubstrate system by others (Bridger *et al.*, 1968). The phenomenon considered above is related to but not identical with "substrate synergism" since the accelerating effect of the solvents under consideration is due to the replacement of a missing part of the more natural substrate rather than the addition of a second substrate.

From the data in Table III it can be seen that substantial rate accelerations were observed with all solvents which were examined. There are no obvious structural requirements for the solvent but the solvents of higher molecular weights effect the greatest rate accelerations. In terms of the model

presented above, the larger, less polar solvent molecules are more effective in reorienting the acyl moiety *vis-à-vis* the catalytic groups, thus "triggering" the deacylation reaction. No saturation of the rate constants was observed with increasing solvent concentration (Figure 3) and in fact the shapes of the curves in Figure 3b are not indicative of a simple saturation process by one molecule of solvent per molecule of acyl-enzyme.

An important consequence of these studies is that any investigations of the reactions of papain with alcohols and amines must include careful product analysis since the magnitude of the solvent effects are comparable with the increases in the rates of deacylation which are attributable to direct nucleophilic displacement.

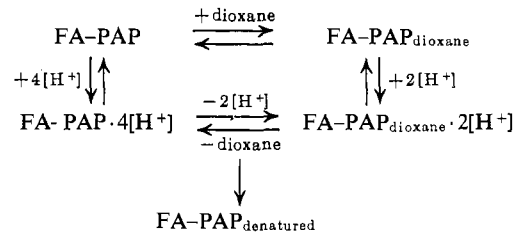
*pH Dependence of the Rates of Hydrolysis.* The pH dependence of the hydrolysis rates of FA-papain in water is quite similar to that of TC-papain (Brubacher and Bender, 1966) and to that observed in steady-state hydrolysis of substrates for which deacylation is known to be the rate-determining step. The dependence of the rates on a group with p$K_a$ between 3 and 5 has been observed with all substrates examined over a sufficiently large range and has been interpreted as representing the ionization of either a histidine or a carboxyl group. The dip in pH–rate profile at high pH values (Figure 6) was observed for the hydrolysis of ZLysNP (Bender and Brubacher, 1966) and TC-papain (Brubacher and Bender, 1966) and is also present with IA-papain (P. M. Hinkle and J. F. Kirsch, unpublished observation), while no decrease in rate at pH values up to 9.5 was found for $k_{cat}$ with ZGlyNP as a substrate (Williams and Whitaker, 1967). Most steady-state studies, however, have been limited to pH values below approximately 9 because of both the high nonenzymatic blank rates at high pH and because $K_m$ depends, in all cases, on a p$K_a$ of about 8.5 so that it is rarely possible to obtain sufficient concentrations of substrates to determine $k_{cat}$ at high pH values.

There are several mechanisms which might explain the "dip" in pH–rate profile. Sluyterman and Wolthers (1969) have suggested that a protonated histidine (p$K_a$ $\sim$ 9.5) may act as an obligatory general acid catalyst in the deacylation reaction. If FA-papain hydrolysis were *completely* dependent on the acidic form of an enzyme group with a p$K_a$ above 9, then the rate constants between pH 9 and 12 would be expected to decrease to values considerably lower than those actually observed. Further evidence bearing on this point will be presented later (P. M. Hinkle and J. F. Kirsch, in preparation). It is of interest in this respect that the strong dependence of the deacylation rate constants on electron withdrawal also suggests general base rather than general acid catalysis. The data at high pH might be explained by a *partial* dependence on the ionization of some enzyme group, presumably a lysine or tyrosine, such that the rates of FA-papain hydrolysis are approximately 30% lower in water and 15% lower in 30% dioxane when the unknown residue is deprotonated. The shape of the observed pH–rate profile is too broad to be explained by any theoretical single ionization; this could, however, be in part the result of the low ionic strengths used in these experiments which are known to broaden protein titration curves (Edsall and Wyman, 1958). A second possibility is that as papain (isoelectric point 8.75) becomes negatively charged its structure changes so that it is a somewhat less efficient catalyst. This would be consistent with the broad pH–rate curves. Finally, it is possible that the acyl papains used in our studies and in those of Brubacher and Bender (1966) were not homogeneous and that the pH dependencies of the various acylated species differed. The acidic pH dependence for the hydrolysis in 30% dioxane is similar to that in water but the p$K_a$ shifts from 4.7 in water to 3.9 in dioxane. Lowe (1970) found a similar effect in 20% dioxane where the p$K_a$ for TC-papain hydrolysis shifted from 4.65 to 4.15.

*Denaturation of Acyl Papains.* The acyl papains used in these studies, like acyl and phosphoryl chymotrypsins (Bender *et al.*, 1962b; Bernhard *et al.*, 1965; Martin and Bhatnagar 1966), are extraordinarily stable compared to the native enzymes. In aqueous solution FA-papain is not denatured at 25° over the pH range 2.5–12, or at neutral pH to temperatures of 80°. In addition FA-papain is not denatured by concentrations of up to 50% dioxane over a wide range of temperature and pH. All of these conditions effect the rapid inactivation of free papain. Bernhard and Rossi (1968) have proposed that *free* enzymes are characterized by a large, open cavity which is readily accessible to denaturants but which, in the presence of bound substrate, becomes closed and inaccessible to denaturing agents accounting for the increased stability of acyl-enzymes in general. Free papain is characterized by a large cleft (Drenth *et al.*, 1968) and this explanation may account for the observed stability of FA-papain.

The rates of denaturation of FA-papain in acidic solution depend on $[H^+]^4$ in aqueous solution and $[H^+]^2$ in 30% dioxane (Figure 5b). Similar dependence on higher powers of hydrogen ion concentration has also been noted in the denaturation of ferrihemoglobin and a number of enzymes (Steinhardt and Kaiser, 1953; Pohl, 1968, 1969). We have no evidence bearing on the reversibility of FA-papain denaturation in acid, but the observed *rates* of denaturation are accommodated by Scheme I.

SCHEME I




Since catalysis of the denaturation reaction requires 4 moles of protons in water and only 2 in 30% dioxane–water, the dioxane causes an instability in the acyl-enzyme which makes it more susceptible to acid-catalyzed denaturation. The increased acid lability of FA-papain in dioxane solution is similar to that observed for trypsin denaturation in the presence of ethanol (Pohl, 1968).

In basic solution FA-papain undergoes denaturation and the denatured and possibly the native forms of the acyl-enzyme are subject to hydroxide ion catalyzed hydrolysis. The rate of hydrolysis of sodium dodecyl sulfate denatured FA-papain is first order in $[OH^-]$, as is the hydrolysis of

urea-denatured TC-chymotrypsin (Bender *et al.*, 1962a). The observed pH dependence of the total rate of disappearance of FA-papain, which includes both hydrolysis and denaturation, appears to depend on $[OH^-]^{1.4}$ over the limited range examined (Figure 5a). It is likely that the reaction of FA-papain in alkali is not a single $[OH^-]$-catalyzed step and that a plot of log $k_{obsd}$ *vs.* pH would not be linear over a larger range of hydroxide ion concentration. In 30% dioxane solution the rates of disappearance of FA-papain in base were too rapid to permit measurement by conventional methods.

The data on the denaturation of FA-papain at pH extremes can be adequately explained by assuming that the acyl-enzyme can react either to give denatured acyl-enzyme or free acid and papain. The acid-catalyzed reactions of IA-chymotrypsin can likewise be explained as independent processes, but at neutral and higher pH values there is evidence to suggest that hydrolysis and denaturation (in the presence of added denaturants) pass through a common intermediate, possibly a tetrahedral addition complex (Bernhard and Rossi, 1968). We have not investigated the possibility that a similar situation obtains for acyl papains.

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# Effect of Monovalent Cations on the Ouabain Inhibition of the Sodium and Potassium Ion Activated Adenosine Triphosphatase*

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ABSTRACT: Inhibition of the ($Na^+$ + $K^+$)ATPase obtained from lamb brain by ouabain is first order in both enzyme and ouabain concentrations. This implies that the interaction of one ouabain per active site inhibits the enzyme. The second-order rate constant for inhibition, $k_i$, depends on both sodium and potassium ion concentrations. The rates of inhibition and binding are the same. The dependence of $k_i$ on sodium and potassium ion concentrations is consistent with a mechanism in which ouabain interacts predominately with the phosphorylated enzyme with a rate constant $k_i'$ which is independent of the monovalent cations bound. The rate constant for the binding of ouabain to the phosphorylated enzyme is $4.35 \times 10^4$ $M^{-1}$ $sec^{-1}$. The dissociation constant, $K_i$, is less than $5 \times 10^{-9}$ M.

The ($Na^+$ + $K^+$)ATPase (ATP phosphohydrolase, EC 3.6.1.3), which is believed to be an integral part of the alkali cation transport system of most mammalian tissues, is specifically inhibited by cardiac glycosides such as ouabain (Albers, 1967). There has been some controversy concerning the stability of the enzyme–ouabain complex. Several reports suggest binding is reversible (Glynn, 1964; Ahmed *et al.*, 1966; Hokin *et al.*, 1966; Tobin and Sen, 1970), difficult to reverse (Baker and Manil, 1968), or virtually irreversible (Albers *et al.*, 1968; Ellory and Keynes, 1969). It has been reported that potassium ion is at least partially competitive of ouabain binding and inhibition (Glynn, 1964; Ahmed *et al.*, 1966; Ellory and Keynes, 1969).

This communication hopes to help clarify some of the controversy over the stability of the enzyme–ouabain complex and the influence of sodium and potassium ions on the ATPase–ouabain interaction. A mechanism for the ($Na^+$ + $K^+$)ATPase is proposed based on the kinetics of ATP hydrolysis and the kinetics of ouabain inhibition and binding.

## Experimental Section

*Materials.* Lamb brain ($Na^+$ + $K^+$)ATPase was prepared by the method of Nakao *et al.* (1965). The specific activity of several preparations was 2.0–2.7 μmoles of ATP hydrolyzed/mg of protein per min at 37°, and 95–98% of the ATPase activity was inhibited by ouabain. Protein was determined by the method of Lowry *et al.* (1951).

Tricyclohexylammonium phosphoenolpyruvate, disodium ATP, NADH, and rabbit muscle lactic dehydrogenase were obtained from Sigma Chemical Co. Ouabain was obtained from Aldrich Chemical Co. and [$^3H$]ouabain, 12 Ci/mmole, was purchased from New England Nuclear Corp. Rabbit muscle pyruvate kinase was prepared by the method of Tietz and Ochoa (1962). Tris-ATP was prepared by the method of Schwartz *et al.* (1962). Lactic dehydrogenase and pyruvate kinase were dialyzed against 0.01 M Tris, pH 7.0, to remove ammonium ion.

*Assay of the ($Na^+$ + $K^+$)ATPase.* In the usual assay procedure, the hydrolysis of ATP was coupled to the oxidation of NADH using pyruvate kinase and lactic dehydrogenase. The advantages of this assay procedure are that ATP hydrolysis can be monitored continuously and inhibitory buildup of ADP is prevented. Pyruvate kinase and lactic hydrogenase activities were always maintained in at least a 100-fold excess over ATPase activity. It was verified for all potassium ion concentrations used that a severalfold variation in the concentration of pyruvate kinase and lactic dehydrogenase had no effect on the ATPase kinetics. Oxidation of NADH was linear for over 2 hr and was directly proportional to the amount of ATPase added. Assay of the ATPase by orthophosphate production (Chen *et al.*, 1956) gave the same specific activity. Neither lactic dehydrogenase nor pyruvate kinase is affected by $10^{-4}$ M ouabain. In a typical experiment the assay conditions would be 0.1 M NaCl–0.01 M KCl–0.003 M $MgCl_2$–0.003 M Tris-ATP–0.03 M Tris (pH 7.0)–0.01 unit/ml of ATPase–20 units/ml of pyruvate kinase–2 units/ml of lactic dehydrogenase–$1.5 \times 10^{-4}$ M NADH–$1.5 \times 10^{-3}$ M phosphoenolpyruvate at 37°. The ionic strength was maintained at 0.3 M with choline chloride. The reaction was followed at 340 mμ.

*Kinetics of Ouabain Inhibition.* Using the assay method above, various concentrations of ouabain were added and the absorbance at 340 mμ was followed until the enzyme was completely inhibited. A typical set of experiments is illustrated in Figure 1. To obtain the kinetics of ouabain inhibition, the ouabain-insensitive base line was extrapolated back to zero time (the dashed line in Figure 1). If $A$ is the absorbance

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