terium even when N–H is *not* hydrogen bonded to O=C.

In addition the present investigations show that, besides H+ and OH−, acids and bases generally catalyze deuterium–hydrogen exchange of amides. The catalytic constants in general acid–base catalysis are much smaller than those for H+ and OH−. On the other hand, H+ and OH− are present in solutions between pH 4 and 10 at very low concentrations, and their net catalytic effectiveness is a product of concentration and catalytic constant. Within the domain of a protein macromolecule, acidic and basic side chains are present in large quantities, corresponding to local concentrations near 1 *M*. Side chains of course are limited in mobility compared to corresponding small molecules such as imidazole or acetic acid. On the other hand, within the region where they reside they provide a very high local concentration of general acid or base. One need only recall how many orders of magnitude more effective an imidazole side chain is within a proteolytic enzyme macromolecule compared to a solution of imidazole. In regard to catalysis of exchange, all types of groups, carboxylic, imidazole, and amino, have been shown to be effective with the model amide (whether it is largely hydrogen bonded or not), and it seems reasonable to expect that they would be catalysts when present as side chains.

In conclusion, then, it seems unlikely that deuterium–hydrogen exchange is a reliable measure of percentage of amide hydrogens in a helical configuration. On the other hand, there seems to be no alternative to the presumption that very slowly exchanging amide hydrogens must be in regions of difficult accessibility. Regions in which solvent can penetrate to various extents should show rates of exchange which reflect the effective local concentration of solvent and of all potentially catalytic acids and bases as well as interamide hydrogen bonding. All of these factors of course reflect the conformation of a protein macromolecule, but it is not a simple matter to assess their relative contributions to the resultant experimental observation.

Kinetics of Papain-Catalyzed Hydrolysis of α-N-Benzoyl-L-arginine Ethyl Ester and α-N-Benzoyl-L-argininamide[1]

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Contribution from the Department of Chemistry, Northwestern University, Evanston, Illinois. Received February 4, 1965

Kinetic data for papain-catalyzed hydrolyses are presented which support the mechanism indicated by eq. 1, in which ES is the enzyme-substrate complex, ES' is the acyl-enzyme, and P_1 and P_2 are the alcohol (or amine) and acid portions of the substrate, respectively. Identical $pH-k_{cat}/K_m(app)$ profiles but different $pH-k_{cat}$ profiles are found in the papain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester and α-N-benzoyl-L-argininamide. These results are not consistent with a one-step catalytic process but have been successfully analyzed in terms of the above two-step catalytic process. Although k_{cat} (the turnover rate constant) for both ester and amide hydrolysis is determined by both k_2 (acylation) and k_3 (deacylation), the predominately rate-determining step for the ester is k_3 while it is k_2 for the amide. K_S for the ester and amide are nearly identical; $k_2(lim)$ for the ester and amide differ by only six- to sevenfold, significantly different from the result found with α-chymotrypsin. The pH–rate profiles of k_2/K_S and k_2 are bell-shaped curves determined by two prototropic equilibria for both ester and amide hydrolysis, while the pH–rate profile of k_3 is a sigmoid curve determined by a single prototropic equilibrium for both ester and amide. A carboxylic acid group and a sulfhydryl group appear to be involved as an acid–base pair in the acylation process while a carboxylate ion appears to be involved as a base in the deacylation process. Deuterium oxide decreases k_3 2.75-fold but decreases k_2 only 1.35-fold.

Recent work on the serine proteolytic enzymes, particularly α-chymotrypsin, leaves little doubt that the hydrolysis of a substrate proceeds *via* the formation of an acyl-enzyme intermediate (eq. 1),[3,4] where ES is the

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} ES' \overset{k_3}{\longrightarrow} E + P_2 \qquad (1)$$
$$+ P_1$$
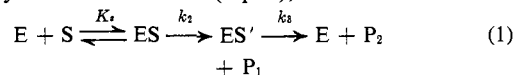
enzyme–substrate complex, K_s its equilibrium dissociation constant, ES' the acyl-enzyme, and P_1 and P_2 are the alcohol and acid portion of an ester substrate, respectively. It has been shown[3–6] that comparison of eq. 1, which involves an acyl-enzyme intermediate, with

(1) This research was supported by grants from the National Institutes of Health and the U. S. Atomic Energy Commission.

(2) National Institutes of Health Special Postdoctoral Fellow on leave from the Department of Food Science and Technology, University of California, Davis.

(3) B. S. Hartley and B. A. Kilby, *Biochem J.*, **50**, 672 (1952); **56**, 288 (1954); H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci. U. S.*, **42**, 719 (1956).

(4) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3669 (1964), and references therein; M. L. Bender and F. J. Kézdy, *ibid.*, **86**, 3704 (1964), and references therein.
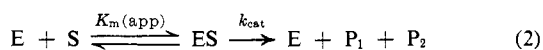
(5) I. B. Wilson and E. Cabib, *ibid.*, **78**, 202 (1956).

(6) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *ibid.*, **86**, 3680 (1964).

the usual Michaelis–Menten equation for an enzymatic process (eq. 2), leads to eq. 3 and 4. The relationship

$$E + S \xrightleftharpoons{K_m(\text{app})} ES \xrightarrow{k_{\text{cat}}} E + P_1 + P_2 \qquad (2)$$

between K_s of eq. 1 and $K_m(\text{app})$ of eq. 2 is given by eq. 3. The relationship between the rate constants,

$$K_m(\text{app}) = [k_3/(k_2 + k_3)]K_s \qquad (3)$$

k_2 and k_3, of eq. 1 and the catalytic (turnover) rate constant, k_{cat}, of eq. 2 is given by eq. 4.

$$k_{\text{cat}} = k_2 k_3/(k_2 + k_3) \qquad (4)$$

In our attempt to understand the mechanism of action of proteolytic enzymes it is of interest to ascertain whether the sulfhydryl proteolytic enzymes exemplified by ficin,[7,8] bromelain,[9] and papain[10-12] behave kinetically like the serine proteolytic enzymes, as typified by α-chymotrypsin. The available data on ficin, bromelain, and papain would support either the three-step mechanism of eq. 1 or the two-step mechanism of eq. 2. Two direct observations of acyl-enzyme intermediates favor eq. 1 as the pathway for sulfhydryl proteolytic enzymes.[13,14] Thus we have decided to obtain more extensive kinetic data on specific substrates.

The sulfhydryl enzyme of choice is papain since it can be obtained readily in the crystalline form[15] and appears to be reasonably homogeneous.[16-19] Smith and collaborators have determined many of the physicochemical characteristics[15-20] of the enzyme, have essentially worked out the primary structure,[21] and have done extensive kinetic work on the enzyme.[10,11,15,17,22] However, further kinetic data are needed to prove the pathway of papain catalysis, since: (1) the previous kinetics were studied only from pH 3.75 to pH 8.5; this incomplete pH–rate profile does not allow a complete kinetic analysis; (2) k_{cat} was found to be essentially pH independent over most of the pH range studied while $K_m(\text{app})$ was pH dependent, particularly at low pH, a result which is unusual in comparison to chymotrypsin; (3) data do not exist to separate the catalytic rate constant, k_{cat}, into its individual rate constants, k_2 and k_3, if indeed the reaction proceeds via an acyl-enzyme; and (4) the reactions were carried out in the presence of an activator, 1,2-dimercapto-3-propanol, which may complicate the kinetics.[23]

It is the purpose of this paper to explore whether the turnover kinetics of hydrolysis of two specific sub-strates of papain, α-N-benzoyl-L-arginine ethyl ester and α-N-benzoyl-L-argininamide, over the pH range 3.5 to 9.6, give evidence for an acyl-enzyme intermediate.

Experimental

Papain. Two-times-crystallized papain (Worthington Biochemical Corp., batch No. PAP 5573) was recrystallized as the mercuripapain derivative from ethanol–water as described by Easley.[24] The procedure is a modification of that reported by Smith, Kimmel, and Brown.[16] The mercuripapain was dissolved in 0.05 M, pH 5.2, acetate buffer and stored in a refrigerator at 4°. Just before use the mercuripapain was activated by the procedure of Soejima and Shimura[25] using 4.5 × 10^{-2} M 4-methylbenzenethiol (Eastman) in toluene. Maximum activation was obtained in the presence of 8 × 10^{-5} M Versene. The activated papain, free of activator, maintained constant activity over a period of 4–6 hr. and lost approximately 5% activity per day when stored in a refrigerator. Papain activated in this manner had the same specific activity as papain activated by 5 × 10^{-3} M cysteine added to the reaction mixture.

Substrates. α-N-Benzoyl-L-argininamide hydrochloride monohydrate (Mann Research Laboratories, Lot No. H2274, chromatographically pure) had m.p. 127–129° and was used without further purification. Two lots of α-N-benzoyl-L-arginine ethyl ester hydrochloride (Mann Research Laboratories, chromatographically pure) were used without further purification. Lot No. H2065 had m.p. 128–130° and lot No. K1590 m.p. 129–131°, lit.[26] m.p. 129.5–131°. α-N-Benzoyl-L-arginine ethyl ester hydrochloride, recrystallized by the method of Kimmel and Smith,[26] had m.p. 131.5–132.5°. All three samples gave identical kinetics with papain. Because of the hygroscopic nature of α-N-benzoyl-L-arginine ethyl ester hydrochloride, its concentration was determined by the change in absorbance at 253.0 mμ on hydrolysis[27] by papain at pH 5.2. A molar absorptivity difference (BA–BAEE) was found to be 1071 M^{-1} cm.$^{-1}$ for the recrystallized sample, pH 5.2, μ = 0.30, acetate buffer, 25.0°, similar to a reported value of 1150 M^{-1} cm.$^{-1}$, pH 8.0, 25.0° phosphate buffer.[28] α-N-Benzoyl-L-arginine (Mann Research Laboratories, Lot No. G2336) was used without further purification.

Buffers. Acetate (pH 3.5 to 5.8), phosphate (pH 6.0 to 7.8, KH_2PO_4 and Na_2HPO_4), and borate (pH 7.5 to 9.6, H_3BO_3 + NaOH) buffers were used. The total ionic strength was maintained at 0.30 by the use of potassium chloride. The kinetic constants appear to be independent of the nature and concentrations of these buffers. Tris·HCl buffers could not be used since the rate of hydrolysis of α-N-benzoyl-L-arginine ethyl ester was found to be dependent upon the buffer concentration. For example, at pH 8.05 and 25.0° the rate of hydrolysis of 4.76 × 10^{-4} M α-N-benzoyl-L-arginine ethyl ester in 0.4 M Tris buffer was 43% that

(7) S. A. Bernhard and H. Gutfreund, *Biochem. J.*, 63, 61 (1956).
(8) B. R. Hammond and H. Gutfreund, *ibid.*, 72, 349 (1959).
(9) T. Inagami and T. Murachi, *Biochemistry*, 2, 1439 (1963).
(10) A. Stockell and E. L. Smith, *J. Biol. Chem.*, 227, 1 (1957).
(11) E. L. Smith and M. J. Parker, *ibid.*, 233, 1387 (1958).
(12) E. L. Smith, *ibid.*, 233, 1392 (1958).
(13) G. Lowe and A. Williams, *Proc. Chem. Soc.*, 140 (1964).
(14) M. L. Bender and L. J. Brubacher, *J. Am. Chem. Soc.*, 86, 5333 (1964).
(15) J. R. Kimmel and E. L. Smith, *J. Biol. Chem.*, 207, 515 (1954).
(16) E. L. Smith, J. R. Kimmel, and D. M. Brown, *ibid.*, 207, 533 (1954).
(17) E. L. Smith, B. J. Finkle, and A. Stockell, *Discussions Faraday Soc.*, 20, 96 (1955).
(18) B. J. Finkle and E. L. Smith, *J. Biol. Chem.*, 230, 669 (1958).
(19) E. O. P. Thompson, *ibid.*, 207, 563 (1954).
(20) E. L. Smith, A. Stockell, and J. R. Kimmel, *ibid.*, 207, 551 (1954).
(21) A. Light, R. Frater, J. R. Kimmel, and E. L. Smith, *Proc. Natl. Acad. Sci. U. S.*, 52, 1276 (1964).
(22) E. L. Smith, V. J. Chavré, and M. J. Parker, *J. Biol. Chem.*, 230, 283 (1958).
(23) T. Sanner and A. Pihl, *ibid.*, 238, 165 (1963).

(24) M. C. Easley, personal communication.
(25) M. Soejima and K. Shimura, *J. Biochem.* (Tokyo), 49, 260 (1961).
(26) J. R. Kimmel and E. L. Smith, *Biochem. Prepn.*, 6, 61 (1958).
(27) G. W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta*, 16, 570 (1955).
(28) I. Trautschold and E. Werle, *Z. Physiol. Chem.*, 325, 48 (1961); W. Rick in "Methods of Enzymatic Analysis, H. U. Bergmeyer, Ed., Academic Press Inc., New York, N. Y., 1963, p. 807.
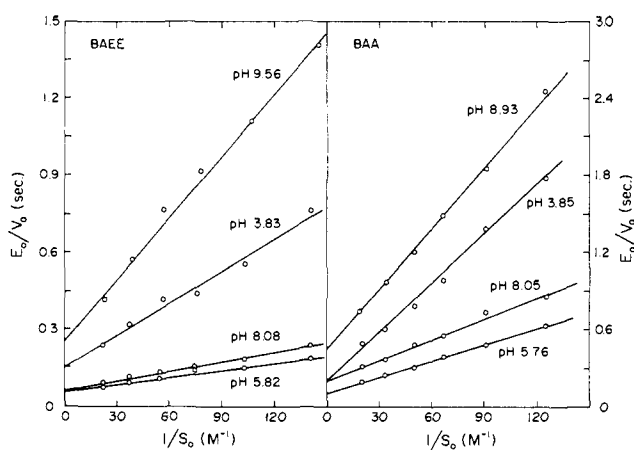
Figure 1. Lineweaver–Burk plots showing the effect of substrate concentration and pH on the velocity of hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) and α-N-benzoyl-L-argininamide (BAA) by papain at 25.0°. Reaction conditions are those described in Tables II and III.

in 0.05 M Tris buffer. Addition of Versene did not alleviate this buffer effect. All reactions were carried out with 1.25×10^{-5} M Versene in the reaction solution. This was found to be necessary because of minute amounts of heavy metal ions in the substrate, particularly Lot No. H2065 of α-N-benzoyl-L-arginine ethyl ester. With this substrate at 0.02 M the enzyme had only 31% the activity in the absence of Versene as it did with 1.25×10^{-5} M Versene. Use of higher concentrations of Versene, up to 4×10^{-3} M Versene, and/or 5×10^{-3} M cysteine did not change the activity from that observed with 1.25×10^{-5} M Versene. With 0.02 M α-N-benzoyl-L-argininamide the enzyme had 93% as much activity in the absence of Versene as in the presence of 1.25×10^{-5} M or 4×10^{-3} M Versene. Glass-distilled water was used in this investigation and all glassware was rinsed with this water.

Enzyme Concentration. The (active) enzyme concentration was determined by titration of the enzyme active sites with N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester at pH 3.2. The initial burst of *p*-nitrophenol liberated was found to be proportional to the active enzyme concentration.[29] For determination of the enzyme concentration each day that kinetic analyses were performed, it was more convenient to use a rate assay employing α-N-benzoyl-L-arginine ethyl ester as the substrate. Since the relationship between the active site titration and the rate assay is known from analyses of the same enzyme solution, the absolute active enzyme concentration could be obtained directly by the latter method. The rate assay was determined using 4.8×10^{-4} M α-N-benzoyl-L-arginine ethyl ester at pH 5.20, $\mu = 0.30$, and 25.0°, and a Cary Model 14 spectrophotometer at 253.0 mμ. The protein content was determined at 280.0 mμ using ϵ 5.10×10^4 M^{-1} cm.$^{-1}$ for papain.[30] On this basis freshly activated papain preparations were found to be 0.719 ± 0.024 enzymatically pure (average of 33 determinations), assuming a molecular weight of papain of 20,700.[17]

Kinetic Measurements. Initial rates of hydrolysis of α-N-benzoyl-L-arginine ethyl ester and α-N-benzoyl-L-

argininamide were determined at $25.0 \pm 0.1°$ using a Cary 14 PM recording spectrophotometer equipped with a thermostated cell compartment. Schwert and Takenaka[27] reported an increase in absorbance with a maximum change at 253.0 mμ when α-N-benzoyl-L-arginine ethyl ester is hydrolysed to α-N-benzoyl-L-arginine and ethyl alcohol. However, there is also strong absorbance of the substrate in this region. At wave lengths above 280 mμ where the substrate does not absorb so strongly, there is a decrease in absorbance when α-N-benzoyl-L-arginine ethyl ester and α-N-benzoyl-L-argininamide are hydrolyzed. At 285.0 mμ, $\Delta\epsilon$ (BAEE–BA) is 17.5 and $\Delta\epsilon$ (BAA–BA) is 30.6 M^{-1} cm.$^{-1}$ (0.02 M, large slide wire). Although these $\Delta\epsilon$ values are small, they are large enough to follow the hydrolysis of 0.007 to 0.05 M substrates on the 0 to 0.1 slide wire of the Cary spectrophotometer.

A typical kinetic experiment is described. Both blank and reaction cuvettes contained 3 ml. of a solution of substrate in buffer. After equilibration in the compartment of the spectrophotometer at 25.0° for at least 25 min. and adjustment of the base-line absorbance to zero, 50 μl. of buffer was added to the blank cuvette and 50 μl. of enzyme solution, in buffer, to the reaction cuvette. After mixing, recording was started within 10 to 20 sec. and continued until at least 20% of the substrate had been hydrolyzed. This was usually 200 to 600 sec., depending on the pH and enzyme concentration. After complete hydrolysis the absorbance difference between the two cuvettes was obtained. The absorbance data were converted to rate data by use of the molar absorptivity difference determined on the reaction solution since it varied with substrate concentration. The variation in molar absorptivities was due to the limitations of the optical system of the spectrophotometer. The validity of the procedure used in the spectrophotometric determinations was confirmed by the fact that a variation in the slit width, and thus the molar absorptivity, produced the same kinetic results. The pH, initially and after complete hydrolysis, was measured on a Radiometer 4C pH meter standardized against pH 6.5 Radiometer standard buffer. When necessary, the absorbance at a final pH was converted to the absorbance at the initial pH using a pK_a for α-N-benzoyl-L-arginine of 3.24. The protonated acid has the same absorbance as the ester. The pK_a of 3.24, determined spectrophotometrically at 25.0°, $\mu = 0.30$, and at 253.0 mμ, is in agreement with reported pK_a values of 3.40[27] and 3.38 (40°).[31]

Data at pH 9.0 and above were corrected for non-enzymatic hydrolysis of α-N-benzoyl-L-arginine ethyl ester. The initial rate data were plotted as E_0/V_0 vs. $1/S_0$ according to eq. 5 which is derived from eq. 2

$$E_0/V_0 = 1/k_{cat} + (K_m(app)/k_{cat})(1/S_0) \qquad (5)$$

(see Figure 1). All rate data were analyzed with an IBM 709 computer program[32] according to the method of least squares, and standard deviations are reported.

Stability of Papain. As described above, papain at pH 5.2 and 0–2° is quite stable, in the absence of activator, after activation with 4-methylbenzenethiol. The stability of the enzyme from pH 3.5 to 9.6 was determined at 25.0° under conditions similar to those

(29) Unpublished work of L. J. Brubacher in this laboratory.
(30) A. N. Glazer and E. L. Smith, *J. Biol. Chem.* **236**, 2948 (1961).

(31) L. A. Æ. Sluyterman, *Biochim. Biophys. Acta*, **85**, 305 (1964).
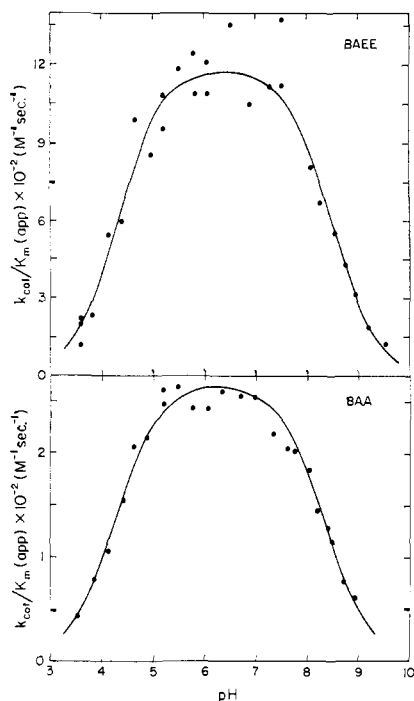(32) We are indebted to J. V. Killheffer for the computer program.

Figure 2. k_{cat}/K_m(app)–pH rate profiles for the papain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) and α-N-benzoyl-L-argininamide (BAA) at 25.0°. Reaction conditions are those described in Tables II and III. The lines are calculated by the use of eq. 9 and the data of Table V.

used for the kinetic analyses. The rate of hydrolysis of 4.8×10^{-4} M α-N-benzoyl-L-arginine ethyl ester was determined at 25.0° using the concentration of enzyme and pH used for the kinetic analyses. Since the time for complete hydrolysis of substrate under these conditions was severalfold longer than the time used in the initial rate studies and since the rate of hydrolysis, in the absence of other perturbing factors, fit extremely good

Table I. Kinetic Constants of Papain-Catalyzed Hydrolysis of α-N-Benzoyl-L-arginine Ethyl Ester and α-N-Benzoyl-L-argininamide[a]

Substrate	Activator	k_{cat}, sec.$^{-1}$	K_m(app), $M \times 10^2$
BAEE	None[d]	15.7 ± 1.5	1.45 ± 0.27
	Cysteine[e]	12.7 ± 1.3	1.02 ± 0.22
BAA	None[d]	8.48 ± 0.44	3.23 ± 0.24
	Cysteine[e]	8.44 ± 0.62	3.33 ± 0.34

[a] In the presence or absence of cysteine at pH 5.20 and 25.0°.[b,c] [b] 0.2 M and 0.05 M acetate buffers, μ = 0.30, were used for the ester and amide, respectively. [c] Active (E_0) was 1.63 to 1.88 × 10^{-6} M. (S_0) was 0.72 to 5.0 × 10^{-2} M. [d] Activated with 4-methylbenzenethiol as described above. [e] 4.9 × 10^{-3} M cysteine.

first-order kinetics, the denaturation of the enzyme could be determined quite readily. Over the pH range 3.5 to 9.6 the enzyme was found to be least stable in the region of pH 8 where the rate constant of denaturation was calculated to be of the order of 2×10^{-7} sec.$^{-1}$. However, over the period of 200–400 sec. needed for the initial rate determinations the enzyme may be considered to be stable. This is in agreement with the results of Stockell and Smith.[10]

Effect of Cysteine on K_m(app) and k_{cat}. An activator, either cysteine or 1,2-dimercapto-3-propanol, is usually
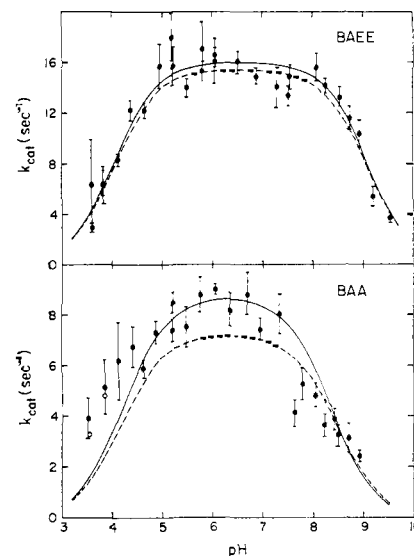

Figure 3. k_{cat}–pH rate profiles for the papain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) and α-N-benzoyl-L-argininamide (BAA) at 25.0°. Reaction conditions are those described in Tables II and III. Data designated by open circles have been corrected for product inhibition. The dashed lines are calculated by use of eq. 4, 6, and 7 and the data of Table V. The solid lines are semiempirical curves calculated by the use of k_{cat} (lim) and the apparent pK values of Table IV.

added to the reaction solution when a sulfhydryl proteolytic enzyme is used.[7,9–11,16–18,22,23] Recently, Sanner and Pihl[23] have reported that k_{cat} and K_m(app) for the hydrolysis of α-N-benzoyl-L-argininamide by papain is increased approximately fourfold in the presence of 5×10^{-3} M cysteine. Our investigations of the effect of cysteine on K_m(app) and k_{cat} for the hydrolysis of both α-N-benzoyl-L-arginine ethyl ester and α-N-benzoyl-L-argininamide, shown in Table 1, indicate little or no effect of cysteine on K_m(app) and k_{cat}. This is in agreement with the results of Bernhard and Gutfreund[7] and Hammond and Gutfreund[8] with ficin. No ready explanation will reconcile these results with those of Sanner and Pihl.

Results

Kinetics of Hydrolysis of α-N-Benzoyl-L-arginine Ethyl Ester and α-N-Benzoyl-L-argininamide. The kinetic constants, k_{cat}, K_m(app), and k_{cat}/K_m(app), for the papain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) and α-N-benzoyl-L-argininamide (BAA) are given in Tables II and III and in Figures 2–4. The hydrolysis of the ester at pH 6.6 conformed to simple Michaelis–Menten kinetics over a 3000-fold range of substrate from 0.05 to 0.000018 M.

A comparison of the pH dependencies of the hydrolysis of the ethyl ester and amide is instructive. In Table IV, the pH dependencies of these reactions are given. The first result to be noted from Table IV is that the two pH dependencies of k_{cat}/K_m(app) are identical for both the ester and amide hydrolyses. This identity must, of course, hold no matter what the pathway of the reaction, if the amide and ester are being hydrolyzed at the same active site *via* the same mechanism.[33] Since the ester and amide have the same specific backbone, since their reactions are in-

(33) L. Peller and R. A. Alberty, *J. Am. Chem. Soc.*, 81, 5907 (1959).

Table II. Kinetic Constants of the Papain-Catalyzed Hydrolysis of α-N-Benzoyl-L-arginine Ethyl Ester at 25.0°[a,b]

pH	Buffer	k_{cat}, sec.$^{-1}$	$K_m(app) \times 10^2$, M	$k_{cat}/K_m(app) \times 10^{-2}$, M^{-1} sec.$^{-1}$
3.60	0.3 M acetate	6.44 ± 3.59	5.36 ± 3.66	1.20
3.61	0.3 M acetate $\mu = 0.25$	2.94 ± 0.12	1.30 ± 0.10	2.25
3.60	0.3 M acetate $\mu = 0.15$	6.44 ± 1.52	3.22 ± 1.04	2.00
3.83	0.3 M acetate	6.62 ± 1.03	2.78 ± 0.62	2.38
4.14	0.2 M acetate	8.35 ± 0.47	1.53 ± 0.15	5.47
4.39	0.3 M acetate	12.2 ± 0.8	2.05 ± 0.21	5.93
4.66	0.2 M acetate	12.2 ± 0.6	1.23 ± 0.11	9.90
4.97	0.05 M acetate	15.7 ± 1.8	1.84 ± 0.35	8.55
5.19	0.2 M acetate	18.0 ± 1.9	1.88 ± 0.33	9.54
5.20	0.2 M acetate	15.7 ± 1.5	1.45 ± 0.27	10.8
5.50	0.2 M acetate	14.0 ± 0.7	1.18 ± 0.12	11.9
5.79	0.2 M acetate	15.3 ± 0.8	1.22 ± 0.12	12.5
5.82	0.3 M acetate	17.1 ± 2.0	1.56 ± 0.33	10.9
6.04	0.19 M phosphate	16.1 ± 1.8	1.33 ± 0.28	12.1
6.04	0.19 M phosphate	16.5 ± 0.7	1.52 ± 0.12	10.9
6.50	0.15 M phosphate	16.0 ± 0.9	1.18 ± 0.14	13.6
6.90	0.10 M phosphate	14.9 ± 0.6	1.42 ± 0.10	10.5
7.28	0.10 M phosphate	14.1 ± 1.6	1.26 ± 0.28	11.2
7.53	0.10 M phosphate	13.4 ± 0.8	0.97 ± 0.12	13.8
7.56	0.10 M phosphate– 0.2 M borate	14.9 ± 1.0	1.45 ± 0.18	10.3
8.08	0.2 M borate	15.6 ± 1.1	1.92 ± 0.21	8.12
8.27	0.2 M borate	14.2 ± 0.6	2.10 ± 0.15	6.75
8.56	0.3 M borate	13.3 ± 0.8	2.38 ± 0.23	5.56
8.76	0.2 M borate	11.7 ± 0.9	2.71 ± 0.29	4.34
8.95	0.2 M borate	10.4 ± 1.1	3.26 ± 0.49	3.19
9.22	0.2 M borate	5.52 ± 0.28	2.94 ± 0.21	1.87
9.56	0.2 M borate	3.71 ± 0.40	2.95 ± 0.44	1.26

[a] Unless otherwise indicated, μ was 0.30. [b] (S$_0$) was 0.65 to 4.5 × 10^{-2} M. Active (E$_0$) was 1.05 to 3.73 × 10^{-6} M. The higher enzyme concentrations were used at the extreme pH values. The rate of reaction was proportional to enzyme concentration over this range.

hibited by the same substances, and since esters and amides are related carboxylic acid derivatives whose nonenzymatic and enzymatic reactions parallel one
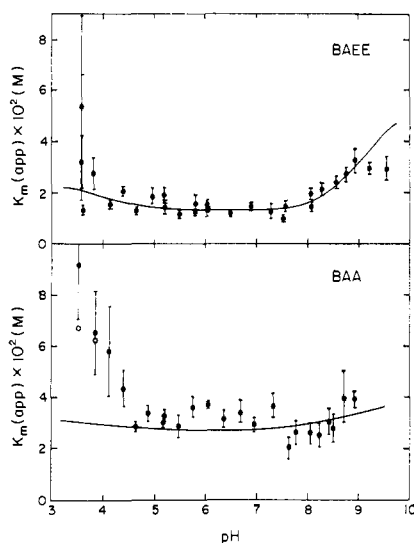

Figure 4. $K_m(app)$–pH profiles for the papain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) and α-N-benzoyl-L-argininamide (BAA) at 25.0°. Reaction conditions are those described in Tables II and III. Data designated by the open circles have been corrected for product inhibition. The lines are calculated by the use of eq. 3, 6, and 7 and the data of Table V.

Table III. Kinetic Constants of the Papain-Catalyzed Hydrolysis of α-N-Benzoyl-L-argininamide at 25.0°[a,b]

pH	Buffer	k_{cat}, sec.$^{-1}$	$K_m(app) \times 10^2$ M	$k_{cat}/K_m(app) \times 10^{-2}$, M^{-1} sec.$^{-1}$
3.53	Acetate	3.95 ± 0.78	9.15 ± 2.07	0.431
3.85	Acetate	5.15 ± 1.07	6.52 ± 1.63	0.790
4.12	Acetate	6.22 ± 1.54	5.80 ± 1.77	1.07
4.42	Acetate	6.77 ± 0.84	4.36 ± 0.71	1.55
4.64	Acetate	5.91 ± 0.29	2.86 ± 0.21	2.07
4.88	Acetate	7.32 ± 0.44	3.39 ± 0.29	2.16
5.19	Acetate	7.40 ± 0.45	3.00 ± 0.26	2.47
5.19	Acetate	8.48 ± 0.44	3.23 ± 0.24	2.62
5.48	Acetate	7.54 ± 0.83	2.86 ± 0.46	2.64
5.76	Acetate	8.81 ± 0.69	3.62 ± 0.39	2.44
6.06	Phosphate	9.00 ± 0.16	3.70 ± 0.09	2.43
6.35	Phosphate	8.23 ± 0.65	3.15 ± 0.36	2.61
6.68	Phosphate	8.84 ± 0.85	3.45 ± 0.46	2.56
6.97	Phosphate	7.47 ± 0.45	2.92 ± 0.26	2.55
7.34	Phosphate	8.08 ± 0.79	3.68 ± 0.49	2.19
7.64	Phosphate	4.13 ± 0.55	2.02 ± 0.45	2.05
7.78	Phosphate	5.30 ± 0.64	2.61 ± 0.48	2.03
8.05	Borate	4.83 ± 0.48	2.60 ± 0.39	1.85
8.23	Borate	3.65 ± 0.44	2.50 ± 0.47	1.46
8.42	Borate	3.88 ± 0.43	3.03 ± 0.49	1.28
8.50	Borate	3.24 ± 0.43	2.80 ± 0.55	1.16
8.72	Borate	3.13 ± 0.59	3.99 ± 1.01	0.784
8.93	Borate	2.41 ± 0.13	3.91 ± 0.30	0.617

[a] 0.05 M buffers; $\mu = 0.30$. [b] (S$_0$) was 0.80 to 5.0 × 10^{-2} M. Active (E$_0$) was 1.40 to 4.23 × 10^{-6} M. The higher enzyme concentrations were used at the extreme pH values. The rate of reaction was proportional to enzyme concentration over this range.

another mechanistically,[34] it can be safely concluded that the ester and amide are being hydrolyzed at the same site via the same mechanism. The identity in

(34) M. L. Bender, Chem. Rev., 60, 53 (1960).

the pH dependencies of the $k_{cat}/K_m(app)$ of the ester and amide hydrolyses by papain of course parallels completely the identity of such hydrolyses of α-chymotrypsin.[6]
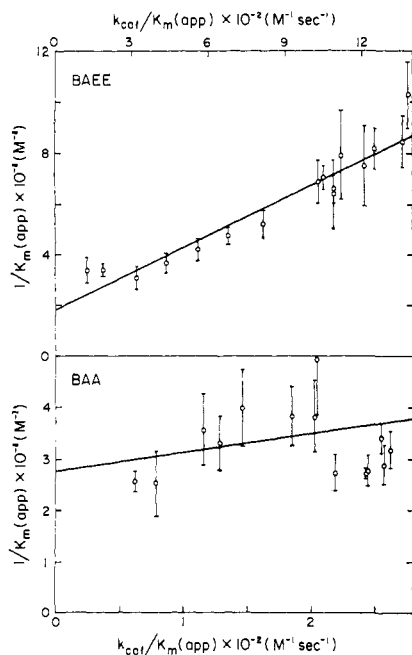
Figure 5. Determination of K_s and k_3(lim) for α-N-benzoyl-L-arginine ethyl ester (BAEE) and α-N-benzoyl-L-argininamide (BAA) according to eq. 8. Experimental conditions are described in Tables II and III.
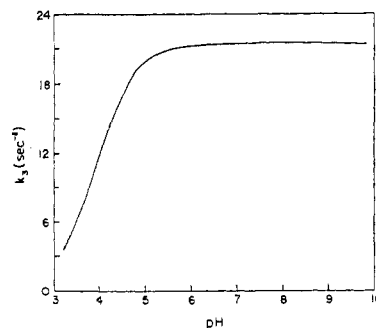

Figure 6. k_3-pH rate profile for the deacylation of α-N-benzoyl-L-argininyl-papain at 25.0°. Experimental conditions are described in Table II. The line is a theoretical curve calculated using eq. 4 and 6, a direct analog of eq. 6 involving k_{cat}(lim), and data of Tables IV and V.

Whereas the pH dependencies of k_{cat}/K_m(app) of the ester and amide hydrolyses are identical, the apparent pH dependencies of k_{cat} of the ester and amide hydrolyses are quite different. The only way to reconcile these differences, assuming a common site and mech-

Table IV. pH Dependencies and Limiting Values of k_{cat} and k_{cat}/K_m(app) in the Papain-Catalyzed Hydrolyses of α-N-Benzoyl-L-arginine Ethyl Ester and α-N-Benzoyl-L-argininamide at 25.0°[a]

Sub-strate	Apparent pK_a[b]				—Limiting values— k_{cat}/K_m (app), M^{-1} sec.$^{-1}$	k_{cat} sec.$^{-1}$
	—k_{cat}/K_m(app)—		—k_{cat}—			
BAEE	4.29	8.49	4.04	9.10	1190	16.1
BAA	4.24	8.35	3.65	8.31	268	8.70

[a] Determined from the data of Tables II and III by the method of Brubacher and Kézdy (L. J. Brubacher and F. J. Kézdy, unpublished work). [b] These values are true pK_a values for k_{cat}/K_m(app) but not for k_{cat} since the latter is a complex constant (see later).

anism for these reactions, is to postulate that two rate steps are involved in these papain-catalyzed hydrolyses, and that the amide and ester hydrolyses have different rate-determining steps. The simplest description of two rate steps, in terms of our understanding of the α-chymotrypsin mechanism and in terms of the observation of acyl-enzyme intermediates in papain-catalyzed reactions, is eq. 1. On this basis, let us now attempt to separate the catalytic rate constants, k_{cat}, into their individual rate constants, k_2 and k_3, and to determine K_s. These constants will then be used to ascertain if the whole set of experimental kinetic data, shown in Tables II and III, is consistent with eq. 1.

From eq. 3 and 4, it is readily seen that k_{cat}/K_m(app) = k_2/K_s. As will be shown later it may be assumed that K_s is pH independent, at least above pH 4.5. If

K_s is pH independent, then the observed pH dependencies of k_2/K_s must indicate the influence of pH on the ionizable groups of the enzyme–substrate complex affecting the k_2 (acylation) step. From Table IV, these groups are two in number, with pK values in the region of 4.24–4.29 and 8.35–8.49, respectively. This dependence of the acylation step in papain-catalyzed hydrolyses on two ionizable groups is reminiscent of the dependence of the acylation step of α-chymotrypsin-catalyzed hydrolyses on two ionizable groups. The deacylation of trans-cinnamoyl-papain is dependent on a single ionizable group,[14] as is the deacylation of trans-cinnamoyl-α-chymotrypsin. Thus, we can reasonably assume that k_3 (deacylation) of the ethyl ester and amide hydrolyses investigated here are dependent on a single ionizable group. We may therefore describe the pH dependencies of k_2 and k_3 of papain-catalyzed hydrolyses by the same set of equations as those previously used for α-chymotrypsin-catalyzed hydrolyses.

$$k_2 = k_2(\text{lim})/\{1 + [(H^+)/K_1] + [K_2/(H^+)]\} \quad (6)$$

$$k_3 = k_3(\text{lim})/\{1 + [(H^+)/K_1']\} \quad (7)$$

Equations 3 and 4 can be combined and rearranged to eq. 8.[6]

$$1/K_m(\text{app}) = 1/K_s + (k_{cat}/K_m(\text{app}))(1/k_3) \quad (8)$$

At high pH, where $k_3 = k_3$(lim), a plot of $1/K_m$(app) vs. k_{cat}/K_m(app) should be linear with a slope of $1/k_3$(lim) and an intercept of $1/K_s$. Such plots for both α-N-benzoyl-L-arginine ethyl ester and amide are shown in Figure 5. As mentioned above, k_{cat}/K_m(app) = k_2/K_s. Since k_{cat}/K_m(app)(lim) is known from Table IV and, since K_s has been calculated above, it is possible to determine k_2(lim). At this point, the values of k_2(lim), pK_1, and pK_2 are known for these hydrolyses; thus, using eq. 6, values of k_2 at any pH are known. Furthermore, values of k_{cat}(lim) and apparent pK values of this reaction are also known (Table IV); thus, using a direct analog of eq. 6, values of k_{cat} at any pH are known. Using these values of k_2 and k_{cat} at any pH, values of k_3 at any pH may be calculated, using eq. 4, as shown in Figure 6 which utilizes the data of α-N-benzoyl-L-arginine ethyl ester. As assumed previously, the deacylation rate constant, k_3, of α-N-benzoyl-L-argininyl-papain is dependent upon a single ionizable group whose pK_1' is found to be 3.91. The results of these calculations are summarized in Table V.

Sub-strate	k_2 (lim), sec.$^{-1}$	k_3(lim),^{c} sec.$^{-1}$	$K_s \times 10^2$,^{c} M	—Acylation—— p$K_1{}^a$	p$K_2{}^a$	Deacylation p$K_1{}'^a$
BAEE	64.9 ± 13.9^b	20.2 ± 1.7	5.45 ± 1.17	4.29	8.49	3.91^d
BAA	9.70 ± 2.09^b	28.7 ± 25.1	3.62 ± 0.78	4.24	8.35	3.91^e

^{a} Determined by the method of Brubacher and Kézdy, Table IV, footnote a. ^{b} Calculated from the limiting value of k_{cat}/K_m(app) (Table IV) and K_s, using the equation k_{cat}/K_m(app) $= k_2/K_s$. ^{c} From data plotted according to equation 8 (see Figure 5). The reason for the large error in k_3(lim) for α-N-benzoyl-L-argininamide is that K_m(app) is almost equal to K_s and thus k_{cat} is almost equal to k_2. ^{d} From pH dependence of k_3 which was calculated from k_2(lim) and k_{cat}(lim) (Table IV) and their pH dependencies (see Figure 6). ^{e} Assuming that pK_1' of deacylation is the same for both substrates. pK_1' cannot be calculated from the experimental data for α-N-benzoyl-L-argininamide hydrolysis because of perturbation of the kinetic parameters in this region by product inhibition and/or change in K_s (see text).

The following general conclusions can be drawn from the data of Table V. (1) While k_{cat} for both ester and amide hydrolysis is determined by both k_2 and k_3, the predominantly rate-determining step for the ester hydrolysis is k_3, while it is k_2 for the amide hydrolysis. (2) K_s for the ester and amide hydrolyses is not greatly different, as was predicted by Zerner and Bender[4] for α-chymotrypsin substrates. (3) k_2(lim) for the ester and amide hydrolyses differs by only six- to sevenfold. (4) Both (k_2/K_s)–pH and k_2–pH profiles are bell-shaped curves determined by two prototropic equilibria for both the ester and amide hydrolyses. (5) k_3 is a sigmoid curve determined by a single prototropic equilibrium for both ester and amide hydrolyses. (6) k_3 of the ester and amide hydrolyses are the same within experimental error, as required by eq. 1.

If the assumptions made above are correct it should be possible to calculate, from the kinetic constants listed in Tables IV and V and eq. 3, 4, 6, and 7, the shape of the experimental curves of pH vs. k_{cat}/K_m(app), k_{cat}, and K_m(app). These calculations are described below.

Theoretical pH–k_{cat}/K_m(app) profiles were calculated according to eq. 9, derived from eq. 6 assuming K_s is pH independent. The data of Table V were used.

$$k_2/K_s = \{k_2(\text{lim})/\{1 + [(H^+)/K_1] + [K_2/(H^+)]\}\}/K_s \quad (9)$$

The theoretical curves for the two substrates, shown as solid lines in Figure 2, show excellent agreement with the experimental data.

The theoretical k_{cat}–pH profiles calculated by the use of the data of Table V and eq. 4, 6, and 7 are shown as dashed lines in Figure 3. There is excellent agreement between the experimental data and theoretically calculated curve for the ester and fair agreement for the amide. The agreement between theoretical curves and experimental data confirms the mechanistic pathway of eq. 1 for papain reactions. A possible explanation for the discrepancy in the amide profile below pH 4.5 will be discussed later.

The theoretical K_m(app)–pH profiles, calculated by the use of eq. 3, 6, and 7, are shown as solid lines in Figure 4. The data of Table V were used. In general, the agreement between theory and experiment is good above pH 4.5. Below pH 4.5 the experimental values increase much more rapidly than is predicted by the oretical considerations. Above pH 4.5, the assumption made earlier that K_s is pH independent appears to be confirmed.

Let us examine the discrepancy below pH 4.5 between theory and experimentally determined K_m(app) and k_{cat} values, especially for α-N-benzoyl-L-arginin-

amide. In addition to the assumption of a pH-independent K_s, we have assumed that the mechanism of hydrolysis of α-N-benzoyl-L-arginine ethyl ester and α-N-benzoyl-L-argininamide by papain can be described by eq. 1 and that the k_3–pH rate profile curve is sigmoid with a dependence upon a single ionizable group. The general agreement between the experimental results and the theoretically calculated curves indicates the essential correctness of our assumptions. However, it is possible that inhibition by product is pH dependent and/or that K_s is not pH independent below 4.5–5.0. It should be emphasized that if K_i(app) $<$ K_m(app) it may be impossible to observe correctly initial rates. Therefore, the equation which correctly describes the results is no longer eq. 5 but rather is eq. 10, where K_i is the dissociation constant of the

$$E_0/V = [1 - (K_m(\text{app})/K_i)]/k_{cat} +$$
$$[K_m(\text{app})/k_{cat}][1 + (S_0/K_i)](1/S) \quad (10)$$

inhibitor–enzyme complex. Therefore, an interpretation of the intercept and slope as $1/k_{cat}$ and K_m(app)/k_{cat} will result in values of k_{cat} and K_m(app) which are too large.

K_i *of* α-*N*-Benzoyl-L-*arginine for Papain.* Inhibition experiments were carried out at pH 3.61 and 5.50 at constant substrate and enzyme concentrations and with variable α-N-benzoyl-L-arginine concentrations. K_i was obtained from the data plotted according to eq. 11,

$$E_0/V_0^{(\text{inhib})} = (1/k_{cat})[1 + (K_m(\text{app})/S_0)] +$$
$$(K_m(\text{app})/k_{cat}S_0K_i)(i) \quad (11)$$

where $V_0^{(\text{inhib})}$ is the observed velocity in the presence of i concentration of inhibitor.[35] The results are shown in Table VI. The observed K_i is a combination of the inhibition constants for the ionized (RCOO$^-$) and protonated α-N-benzoyl-L-arginine (RCOOH). By use of eq. 12 and p$K_a = 3.24$ for α-N-benzoyl-L-arginine the inhibition constants for the two species can be calculated and are given in Table VI.

$$1/K_i^{\text{obsd}} = \alpha/K_i^0 + (1 - \alpha)/K_i^- \quad (12)$$

where K_i^0 and K_i^- are the dissociation constants for the protonated α-N-benzoyl-L-arginine–enzyme complex and the ionized α-N-benzoyl-L-arginine–enzyme complex, respectively, and α is the fraction of protonated α-N-benzoyl-L-arginine at a given pH. It is seen that the protonated acid is at least 20 times more inhibitory than is the ionized acid. This result, which is in agreement with the results of Stockell and Smith,[10] may be

(35) M. Dixon, *Biochem. J.*, **55**, 170 (1953).

pH	K_i^{obsd}, $M \times 10^2$	$K_i^{obsd,a}$ $M \times 10^2$	K_i,[b] $M \times 10^2$	K_i,[c] $M \times 10^2$
5.50	14.8 ± 2.0	~20		14.8
3.61	2.05 ± 0.20	...	0.69 ± 0.07	
4.0	...	~2.8	~0.61	

[a] L. A. A. Sluyterman, *Biochim. Biophys. Acta*, **85**, 316 (1964); at 38°. [b] Calculated K_i for protonated acid using $pK_a = 3.24$ for our data and $pK_a = 3.38$ for Sluyterman's data as reported by him for α-N-benzoyl-L-arginine. [c] K_i of the ionized α-N-benzoyl-L-arginine.

due to the fact that the protonated acid is in equilibrium with an acyl-papain at low pH values.

K_i^{obsd}, the important parameter experimentally, at pH 3.61 is essentially the same as the predicted K_m(app) for α-N-benzoyl-L-arginine ethyl ester and smaller than the predicted K_m(app) for α-N-benzoyl-L-argininamide (Figure 4). Therefore, one would expect product inhibition to be observable with the amide before it is observable with the ester. In Figures 3 and 4 our attempts to correct some of our data for product inhibition do not explain completely the discrepancy between the experimental and theoretical values.

Effect of D_2O on the Kinetics of Hydrolysis of α-N-Benzoyl-L-arginine Ethyl Ester and α-N-Benzoyl-L-argininamide. K_m(app) and k_{cat} were determined for both the ester and amide in D_2O. All reagents except the enzyme were dissolved in 99.64% D_2O (Volk Radiochemical Company). Mercuripapain, in aqueous buffer, was diluted with 1×10^{-4} M Versene in D_2O, before activation with 4-methylbenzenethiol. The maximum amount of H_2O added by this procedure to the reaction was 0.41%. The activated papain appeared to be stable in the D_2O–H_2O solution as indicated by enzymatic assays carried out in aqueous solution. The pD was determined using a glass electrode and a correction of +0.40 unit.[36,37] The kinetic results in D_2O are shown in Table VII. The effect of D_2O on k_{cat} for α-N-benzoyl-L-arginine ester and for α-N-benzoyl-L-argininamide at pD 6.5–6.6 is quite different. This result is consistent with the hydrolysis of these two substrates occurring *via* two rate steps with the rate-determining step of the two hydrolyses being different.

The effect of D_2O on k_{cat} of α-N-benzoyl-L-argininamide was found to be quite small, in agreement with the results of Stockell and Smith[10]; this small D_2O effect may be attributed to the effect on k_2, the rate-limiting step for the hydrolysis of the amide (see Table V). On the other hand, k_{cat} of α-N-benzoyl-L-arginine ethyl ester was found to be 2.45-fold higher in H_2O at pH 6.04 to 6.50 (where k_{cat} is essentially independent of pH) than in D_2O at pD 6.54. This large D_2O effect may be attributed to the effect on k_3, the rate-limiting step of the ester hydrolysis (see Table V). Thus it appears that the k_2 step (acylation) is only slightly affected by D_2O while the k_3 step (deacylation) is affected significantly. From the data for the ester hydrolysis at pD 6.54, k_3 is calculated to be 7.35 sec.$^{-1}$

(36) R. Lumry, E. L. Smith, and R. R. Glantz, *J. Am. Chem. Soc.*, 73, 4330 (1951).
(37) B. Zerner and M. L. Bender, *ibid.*, 83, 2267 (1961), and also ref. 12 and 13 of this paper.

while it is 20.2 sec.$^{-1}$ in H_2O at pH 5.94; thus k_3 is 2.75-fold larger in H_2O than in D_2O. This is in agreement with the effect of D_2O on the rate of deacylation of *trans*-cinnamoyl-papain,[14] and on the rate of deacylation of *trans*-cinnamoyl-α-chymotrypsin.[38]

Our previous analysis indicates that k_{cat} of the amide hydrolysis approximates k_2 very closely. Thus to a fair approximation $k_2^{H_2O}/k_2^{D_2O} = 1.35$. The reason for the smaller deuterium oxide effect in acylation than in deacylation is not clear at present. At pD 6.6 there is a 2.4- and 2.9-fold decrease in K_m(app) for α-N-benzoyl-L-arginine ethyl ester and α-N-benzoyl-L-argininamide, respectively, in D_2O as compared with H_2O. These results are in agreement with those on α-chymotrypsin.[38]

Discussion

In the absence of any perturbing effects produced by the ionization of a prototropic group of the substrate, the effect of pH on the experimentally determined ratio k_{cat}/K_m(app) (= k_2/K_s) reveals the prototropic groups of the *free* enzyme essential to the activity of the enzyme.[33] This is true regardless of the number of intermediates formed in the reaction. Table VIII data gives the pH dependence of k_2/K_s for three plant sulfhydryl proteolytic enzymes, papain, ficin, and bromelain, for a number of substrates. For these three enzymes, pK_1 and pK_2 are identical for all substrates, with the exception of N-benzyloxycarbonyl-glycylglycine. This result is true even though the magnitude of k_2/K_s varies some 4600-fold among the substrates, even though some of the substrates are esters whereas others are amides, and even though some of the substrates are positively charged whereas others are neutral. k_2/K_s for N-benzyloxycarbonylglycyl-glycine increases at low pH[22] presumably due to protonation of the substrate molecule. The imidazole nitrogen of N-benzyloxycarbonyl-L-histidinamide loses a proton within the pH region 6 to 7; although this appears to affect K_m(app) and k_{cat} individually, the ratio of the two constants does not appear to be perturbed.[22] Thus all the reactions of Table VIII appear to depend on a pair of acid-base groups of pK_a' values 3.9–4.5 and 8.0–9.0.

Smith and collaborators have emphasized the apparent interrelationship between k_1 and k_0, defined by eq. 13 and 14, for papain, and have used the ratio

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_0}{\longrightarrow} E + products \tag{13}$$
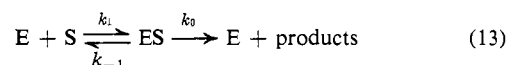
$$K_m = \frac{k_{-1} + k_0}{k_1} \tag{14}$$

k_0/K_m for the calculation of k_1.[10–12,17,22] Comparison of these equations with eq. 1 and 2 indicates that $k_1 = k_{cat}/K_m$(app) $= k_2/K_s$. The interrelationship between $k_1(k_{cat}/K_m$(app)) and $k_0(k_{cat})$ is now obvious. Using these relationships and the data of Table VIII, it is apparent that the present results are in substantial agreement with the results of Smith and co-workers[10,11] on the kinetics of the papain-catalyzed hydrolyses of α-N-benzoyl-L-arginine ethyl ester and amide. The only real differences are: (1) differences in k_{cat}(lim) which reflect different methods of determining enzyme concentration; and (2) more complete pH–rate pro-

(38) M. L. Bender and G. A. Hamilton, *ibid.*, 84, 2570 (1962).

Table VII. Papain-Catalyzed Hydrolysis of α-N-Benzoyl-L-arginine Ethyl Ester and α-N-Benzoyl-L-argininamide in Water and Deuterium Oxide at $25.0°$[a,b]

Sub-strate	pH (pD)	k_{cat}, sec.$^{-1}$	K_m(app) $\times 10^2$ M	k_{cat}, sec.$^{-1}$	K_m(app) $\times 10^2$ M
		H$_2$O		D$_2$O	
BAEE	6.04	16.5 ± 0.7	1.52 ± 0.12		
	6.50	16.0 ± 0.9	1.18 ± 0.14		
	(6.54)			6.63 ± 0.31	0.572 ± 0.088
BAA	6.06	9.00 ± 0.16	3.70 ± 0.09		
	6.68	8.84 ± 0.85	3.45 ± 0.46		
	(6.58)			6.64 ± 0.29	1.21 ± 0.11

[a] (S$_0$) was 0.67 to 5.0×10^{-2} M. Active (E$_0$) was 1.07 to 2.79×10^{-8} M. [b] The D$_2$O reaction was carried out in $99+\%$ D$_2$O.

Table VIII. Kinetic Constants for Papain, Ficin, and Bromelain

Enzyme	Sub-strate[a]	$[k_2/K_s]$lim, M^{-1} sec.$^{-1}$	pK_1	pK_2	K_m(app), M	k_{cat}(lim), sec.$^{-1}$	Temp., °C.	Ref.
		k_2/K_s						
Papain	BAEE	390	4.30	8.20	0.023	9	25	11
		660	4.30	8.02	0.018	12	37	11
		1190	4.29	8.49	0.014	16.1	25	Present report
	BAA	280	3.9	8.2	0.040	11.0	38	10
		268	4.24	8.35	0.032	8.70	25	Present report
	BGEE	147	4.1	8.0	0.021	3.1	40	31
	CHA	31	4.3	8.0	0.02[b]	4.0[c]	38	22
	BGA	3.8	3.9	8.2	0.16	0.6[d]	38	22
	CGG	0.26[e]	...	8.0	0.32[e]	0.083[e]	38	22
Ficin	BAEE	100	4.40	8.46	0.025	2.5	25	8
	BAA	46	4.40	...	0.048	2.2	25	8
	BGME	73	4.40	8.46	0.048	3.5	25	8
	BGA	0.77	4.40	...	0.13	0.10	25	8
Bromelain	BAEE	2.9[f]	4.2	9.0	0.17[f]	0.50[f]	25	9
	BAA	2.9[f]	4.5	...	0.0012[f]	0.0035[f]	25	9

[a] The following abbreviations are used: BAEE, α-N-benzoyl-L-arginine ethyl ester; BAA, α-N-benzoyl-L-argininamide; BGEE, N-benzoylglycine ethyl ester; BGME, N-benzoylglycine methyl ester; BGA, N-benzoylglycinamide; CHA, N-benzyloxycarbonyl-L-histidinamide; CGG, N-benzyloxycarbonylglycylglycine. [b] Minimum value reported at pH 5.2. [c] At pH 6.7 and probably is not maximal. [d] At pH 5.2 and is not maximal. [e] At pH 7.22. [f] At pH 6.0.

files determined in this research which have resulted in the uncovering of undisclosed, different pH dependencies of k_{cat} for the amide and ester reactions at high pH.

We have shown that $k_{cat} = k_2 k_3/(k_2 + k_3)$ is dependent upon two prototropic groups in papain; further our kinetic data are consistent with an analysis that k_2 (acylation) is dependent upon groups with pK_1 of 4.24–4.29 and pK_2 of 8.35–8.49 while k_3 (deacylation) is dependent upon a group with a pK_1' of 3.90. In the bromelain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester,[9] the effect of pH on k_{cat} has been extended far enough into the alkaline region to observe a definite pH effect on k_{cat}. In this reaction there is an indication of an apparent pK of 8.6. Data for the bromelain-catalyzed hydrolysis of α-N-benzoyl-L-argininamide[9] shows the same general trend but studies were not extended beyond pH 8.2. There is disagreement concerning the effect of pH on k_{cat} in the acid region. The data on ficin[7] and bromelain[9] are in agreement with ours on papain: k_{cat} for the ficin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester was found to be dependent upon a pK(app) of 4.35,[7] and k_{cat} for the bromelain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester and α-N-benzoyl-L-argininamide was found to be dependent upon pK(app) values of 3.9 and 3.4, respectively.[9] On the other hand, k_{cat} for the papain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester was reported to be independent of

pH in the acid region.[11,31,39,40] In addition, the papain-catalyzed hydrolysis of N-benzoylglycine ethyl ester was reported to increase 3.5-fold at pH 3.8 compared to that above pH 4.2.[31] Finally, the deacylation (k_3) of $trans$-cinnamoylpapain was found to be dependent solely upon a prototropic group of pK = 4.7.[14]

We have assumed that K_s is independent of pH at least above pH 4.5, and agreement between the experimental data and theoretically calculated curves appear to confirm this hypothesis. K_m(app) (suggested to be equivalent to K_s), for the ficin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester, was found to be independent of pH from 4.0 to 8.4 although there may be an indication of a small increase in K_m(app) at 4.0.[8] K_m(app) for the bromelain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester was found to be essentially pH independent in the acid region while it decreased markedly in the alkaline region (pK(app) of 9.0).[9] On the other hand we, together with Stockell and Smith,[10] have found for papain, and Inagami and Murachi[9] have found for bromelain, that K_m(app) appears to increase below pH 4.5–5 for the enzyme-catalyzed hydrolysis of α-N-

(39) A. Williams, personal communication.

(40) According to a communication to Sluyterman,[31] the previous data[11] were not corrected for incomplete ionization of α-N-benzoyl-L-arginine at low pH.

benzoyl-L-argininamide. Increases in K_m(app) at low pH for the papain-catalyzed hydrolyses of α-N-benzoyl-L-arginine ethyl ester,[11,31] N-benzoylglycine ethyl ester,[31] and N-benzyloxycarbonyl-L-histidinamide[22] have also been observed. k_{cat}/K_m(app) curves for bromelain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester and α-N-benzoyl-L-argininamide appear to be perturbed below pH 4.5.[9]

That there is a perturbing influence on the kinetic parameters below pH 4.5–5 appears certain. We have shown that part, but not all, of this effect may be due to inhibition by protonated α-N-benzoyl-L-arginine. The remainder of the effect may be due to the influence of pH on an anionic binding site, presumably a carboxylic acid group, of the enzyme. It appears from the data on N-benzyloxycarbonyl-L-histidinamide[22] that the positive charge is quite important in binding the substrate to the enzyme. On the other hand, α-N-benzoyl-L-arginine ethyl ester and N-benzoylglycine ethyl ester, two substrates whose charge types are different, are reported to have identical K_m(app) values and identical effects of pH on these K_m(app) values (Table VIII). Thus, at the moment there is insufficient data to assess the relative importance of the hydrophobic and ionic parts of the binding site.

The mechanism of eq. 1 involving an acyl-enzyme intermediate has been suggested several times for the action of the sulfhydryl-containing proteolytic enzymes, papain, ficin, and bromelain.[8–10,12,41] The acyl-enzyme intermediate has been postulated on the basis of: (1) the equivalence of k_{cat} for the papain- and ficin-catalyzed hydrolyses of α-N-benzoyl-L-arginine ethyl ester and amide[8,12]; (2) the essentiality of a single sulfhydryl group of the enzymes on the basis of chemical inhibition studies[8–10]; and (3) the spectrophotometric observation of the acyl-enzymes, thionohippuryl-papain[13] and trans-cinnamoyl-papain.[14] The different pH dependencies found here for the k_{cat} of the papain-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester and amide are not consistent with the one-step catalytic process of eq. 2 but have been successfully analyzed in terms of the two-step catalytic process of eq. 1.

The k_{cat} values of the papain-catalyzed hydrolyses of α-N-benzoyl-L-arginine ethyl ester and amide found here differ from one another only by a factor of two (Table IV). Similarities in the k_{cat} values of hydrolyses of this ethyl ester–amide pair have been noted before for reactions catalyzed by papain[10] and by ficin,[8] but not by bromelain.[9] Since the reactivities of ethyl esters and amides toward nucleophiles such as hydroxide ion usually differ by over a thousandfold, the similarity in the k_{cat} values of hydrolyses of ethyl esters and amides has previously been attributed to a common rate-determining deacylation, k_3, step. Our analysis of the papain-catalyzed hydrolyses of α-N-benzoyl-L-arginine ethyl ester and amide does not indicate this conclusion (Table V). Rather, it must be postulated either that there is a substantial electrophilic component in papain-catalyzed reactions or that the reactivity of a nucleophilic sulfhydryl group toward esters and amides is essentially identical. These possibilities are being investigated.

The prototropic groups of pK_a 4.24–4.29 and 8.49–

8.35 found here in the acylation step may be designated as a carboxylic acid group and a sulfhydryl group, respectively; the group of pK_a 3.91 found in the deacylation step may be designated as a carboxylic acid group. Previously, Smith and collaborators[10,22] had suggested the carboxylic acid and sulfhydryl groups as essential to the action of papain on the basis of pH dependencies, heats of ionization, and chemical inhibition studies. The spectrophotometric properties of the acyl-enzymes, thionohippuryl-papain[13] and trans-cinnamoyl-papain,[14] are consistent with the assignment of the structure of the acyl-enzyme as a thiol ester and thus are in agreement with designating the group of $pK_a \sim 8.4$ as a sulfhydryl group.

Of the two groups of $pK_a \sim 4.3$ and ~ 8.4, it is known that one acts in its acidic form while the other acts in its basic form. However, it is not possible to designate which is the acid and which is the base in the absence of independent evidence. This statement of uncertainty follows from the equilibrium $HA + B \rightleftharpoons A^- + BH^+$. Thus, the set $HA + B$ differs from the set $A^- + BH^+$ only by an equilibrium constant, and a simple kinetic analysis does not distinguish between the two sets. Since the (carboxylate) group of $pK_a = 3.9$ acts as a base in deacylation, it may be presumed that the (carboxylate) group of $pK_a = 4.3$ in acylation acts as a base, and thus, the sulfhydryl group of $pK_a = 8.4$ acts as an acid.

In ficin, a kinetically important group of $pK_a = 8.46$ has been designated as an ammonium ion.[8] This identification was made on the basis that the profile being observed was a deacylation profile, and therefore that the pK_a of 8.46 could not be that of a sulfhydryl group. However, an analysis of these data in terms of the present paper indicates that the profile is probably that of k_{cat}/K_m(app) and thus the pH–rate profile represents not deacylation but the free enzyme. Thus, this group of $pK_a = 8.46$ could easily be the sulfhydryl group.

In conclusion, it appears that eq. 1 adequately describes the pathway of papain catalysis and thus papain behaves similarly to α-chymotrypsin. In α-chymotrypsin the acyl-enzyme appears to be an oxygen ester, whereas in papain it appears to be a thiol ester. As with α-chymotrypsin, papain-catalyzed reactions show a pK_a of ~ 8.5 in acylation, but not in deacylation. However, in contrast to α-chymotrypsin, this group may be directly identified as a sulfhydryl group which is acylated in the acylation step and whose pK_a thus disappears. By process of elimination, the catalytic entity (seen in both acylation and deacylation) which is an imidazole group in α-chymotrypsin may be a carboxylic acid group in papain. Papain reactions show a deuterium oxide isotope effect like chymotrypsin reactions, as found here and previously,[14] implying a rate-determining proton transfer. The imidazole group has been proposed as the optimum group to take part in enzymatic proton transfers.[42] Thus, it appears that a suboptimal group participates in papain proton transfers. The low pK_a in chymotrypsin therefore is easily accounted for and the higher pK_a is not, while the opposite is true in papain. These possible inconsistencies must be answered before a rational mechanism of papain catalysis may be presented.

(41) H. Gutfreund, Discussions Faraday Soc., 20, 167 (1955).

(42) M. Eigen and G. G. Hammes, Advan. Enzymol., 25, 28 (1963).