Kinetics of Papain-Catalyzed Hydrolysis of α-*N*-Benzoyl-L-arginine-*p*-nitroanilide*

John E. Mole‡ and H. Robert Horton*

ABSTRACT: Papain, freed from inactive protein contaminants by affinity chromatography, was employed in a kinetic study of its action on *N*-benzoyl-L-arginine-*p*-nitroanilide. The Michaelis constant of papain toward this substrate was found to be approximately 3 mM, and a k_{cat} of 0.7–0.8 sec^{-1} was observed in the region of optimal pH. Analysis of the pH dependence of the steady-state kinetic parameters provided a K_s of 7.86 mM and a "deacylation" rate constant, k_3(lim), of 1.2 sec^{-1}. This latter value is markedly different from that obtained in similar kinetic analyses using *N*-benzoyl-L-arginine ethyl ester, for which the k_3(lim) of papain is 30 sec^{-1}. Previous kinetic measurements of anilide hydrolyses catalyzed by papain and by trypsin have been interpreted assuming a k_3(lim) equal to that for the corresponding ester. Such an attempt in the present instance, however, would provide K_s and k_{cat} values which are significantly out of the range of the scatter of kinetic data for this *p*-nitroanilide. Thus the present findings reveal that the action of papain in catalyzing the hydrolysis of *N*-benzoyl-L-arginine-*p*-nitroanilide may not involve simply the familiar three-step mechanism established for esters, which employs a common *N*-benzoyl-L-arginylpapain intermediate.

Papain (EC 3.4.4.10) has been the most extensively studied of the sulfhydryl proteases. Data obtained in recent years have dealt primarily with the correlation of molecular structure and biochemical function. Considerable evidence has accumulated in support of a three-step mechanism for the papain-catalyzed hydrolysis of esters since its postulation by Smith and his colleagues (Smith *et al.*, 1955; Smith, 1958). A variety of studies from several laboratories (Lowe and Williams, 1965a; Lowe and Yuthavong, 1971a,b; Whitaker and Bender, 1965; Williams and Whitaker, 1967) have provided evidence for the participation of an acylated enzyme intermediate, in which cysteine residue 25 forms a thiol ester linkage, according to the general equation

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{ES'} \xrightarrow[H_2O]{k_3} E + P_2 \qquad (1)$$

where P_1 and P_2 are the alcohol and acid formed in the hydrolysis of the ester substrate, S; ES is the Michaelis complex; ES′ is the acylated enzyme intermediate; and k_2 and k_3 are the rate constants governing the enzyme's acylation and deacylation steps, respectively. For this three-step kinetic model, the apparent Michaelis constant, K_m(app), and the catalytic coefficient, k_{cat}, are given by eq 2 and 3.

$$K_m(\text{app}) = \frac{k_3(k_{-1} + k_2)}{(k_2 + k_3)k_1} \approx \left(\frac{k_3}{k_2 + k_3}\right)K_s \qquad (2)$$

$$k_{cat} = \frac{k_2k_3}{k_2 + k_3} \qquad (3)$$

A serious limitation in kinetic studies of papain reported heretofore has been the presence of 30–70% catalytically inactive protein in the enzyme preparations. Although active site titrations have usually been performed in an effort to correct for the presence of inactive enzyme (Bender and Brubacher, 1966; Whitaker and Bender, 1965; Williams and Whitaker, 1967), these corrections have been found to be approximations, at best. The recent introduction and successful application of affinity chromatography (Cuatrecasas and Anfinsen, 1971) have made possible the preparation of extremely pure papain (Blumberg *et al.*, 1970; Sluyterman and Wijdenes, 1970) which possesses two–three times the observed k_{cat} of earlier preparations.

Two general approaches have been applied to detailed kinetic studies of papain: evaluation of the pH dependence of K_m(app) and k_{cat}, as used by Bender and his colleagues (Bender and Brubacher, 1966; Whitaker and Bender, 1965), and measurement of the kinetics of a variety of substrates containing a common acyl moiety, as exemplified by work of Kirsch and Igelström (1966), Lowe and Yuthavong (1971a,b), and Lucas and Williams (1969). Both approaches are based on certain major assumptions, which will be summarized here to re-emphasize the problems encountered in kinetic analyses.

In the former case, it is assumed that K_s remains constant over the pH range in which kinetic measurements are made, and that k_3 remains constant at pH values from 5 to 9.6. Lucas and Williams (1969) have pointed out that the assumption of k_3 and K_s values which are independent of pH was not directly tested with the substrates under investigation by Bender and Brubacher (1966) and Whitaker and Bender (1965). However, the finding of a constant rate of deacylation of *trans*-cinnamoylpapain (Brubacher and Bender, 1966) and a constant K_s over the pH range of 3.7–9.3 (Lowe and Yuthavong, 1971a,b) provided the basis for this approach in the evaluation of K_s, k_2, and k_3.

In the latter approach, rates of hydrolysis of several esters

† From the Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27607. *Received June 29, 1972.* This investigation has been supported in part by National Institutes of Health Research Grant GM 13878, and in part by National Science Foundation Grant GB-33472, and is a contribution from the School of Agriculture and Life Sciences and the School of Physical and Mathematical Sciences. Paper 3765 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, North Carolina.

‡ Present address: Department of Microbiology, The University of Alabama in Birmingham, Birmingham, Ala. 35233.

of a common acid are measured, and in those cases in which the k_{cat} values are identical, it is asumed that the deacylation step is rate limiting; *i.e.*, $k_{cat} = k_3$. However, difficulties are encountered when different k_{cat} values are obtained in such a series of esters. Lucas and Williams (1969) obtained k_{cat} values of 13.3 sec^{-1} for the benzyl and *p*-methoxyphenyl esters of *N*-methanesulfonylglycinates, but a value of 1.0 sec^{-1} for the corresponding isopropyl ester. Their interpretation of these data was to assume that k_3 for the isopropyl ester was 13.3 sec^{-1}, and that k_2 controlled the measured k_{cat} of this substrate.

Such kinetic differences in k_{cat} could, in fact, reflect differences in rates attributed to the k_3-controlled step, a possibility which becomes even more significant when amides or peptides are compared with esters as substrates for proteases. Thus, as had been suggested by Caplow and Jencks (1963) for serine proteases such as chymotrypsin, amide and ester hydrolysis may proceed by different enzymatic mechanisms, rather than through the formation of a common acyl-enzyme intermediate; or, as suggested more recently by Caplow (1969), chymotrypsin may form additional (tetrahedral) intermediates with its substrates prior to enzyme acylation and subsequent deacylation. Studies of the action of papain on amide substrates have been limited to a few kinetic analyses (Lowe and Williams, 1965b; Lowe and Yuthavong, 1971a,b; Whitaker and Bender, 1965), and the kinetic data obtained have been interpreted according to the three-step mechanism given in eq 1 for ester hydrolysis (but with P_1 representing the amine). However, careful inspection of the data reveals that evaluation of rate constants k_2 and k_3 for the papain-catalyzed hydrolysis of *N*-benzoyl-L-argininamide was subject to large errors so that the reported identity (Glazer and Smith, 1971) of k_3 (28.7 ± 25.1 sec^{-1}) to that of *N*-benzoyl-L-arginine ethyl ester (20.2 ± 1.7 sec^{-1}) could not be clearly demonstrated.

The recent synthesis of *N*-benzoyl-L-arginine-*p*-nitroanilide (L-BAPNA)[1] (Nishi *et al.*, 1970; Tokura *et al.*, 1971) provided a sensitive means for measuring the initial, steady-state rates of hydrolysis of this amide in direct comparison to those of the corresponding ethyl ester in an effort to test the identity of their "deacylation" rates.

Experimental Procedure

Materials. Papain (2× crystallized) was purchased from Worthington Biochemicals and P-L Laboratories. L-BAEE·HCl (lots 60C-1340 and 81C-0060) and CGNP (lot 58B-1000) were purchased from Sigma Chemical Co.; L-BAPNA·HCl (lot 72046) was obtained from the Protein Research Foundation, Peptide Institute, Osaka, Japan, and had a melting point of 223°. β-Mercaptoethanol was obtained from Matheson Coleman and Bell, DTNB (lot 010022) from Calbiochem, and PCMB (control no. 6209) from Nutritional Biochemicals.

Enzyme Purification. Commerical preparations of 2× crystallized papain were subjected to mercurial column purification by a modification of the procedure of Sluyterman and Wijdenes (1970). The papain was activated in 0.3 M β-mercaptoethanol prior to its transfer to sodium sulfite buffer for affinity chromatography, since sulfite alone did not provide full activation of the papain solutions. Following elution of the mercuripapain in a volume of *ca.* 250 ml, dimethyl sulfoxide was removed by dialysis against four changes (4 l. each) of 0.05 M sodium acetate, pH 5.0, over a period of 24 hr, and the enzyme was then concentrated to *ca.* 30 ml by ultrafiltration (PM-10 Diaflo Membrane, Amicon Corporation). The purified mercuripapain was stored at 5° in stock solutions of 25 mg/ml in the presence of a five-fold molar excess of $HgCl_2$. In this form, the purified papain was stable over periods of several months as evidenced by full recoveries of enzymatic activity toward L-BAEE and sulfhydryl groups (1.0 mol/mol) upon activation.

Kinetic Measurements. A Cary Model 15 spectrophotometer with thermostated cell compartment (25°) was used to monitor continuously the release of *p*-nitroaniline from L-BAPNA at 410 nm, employing a $\Delta\epsilon$ of 8800 M^{-1} cm^{-1}. The $\Delta\epsilon$ remained unchanged over the pH range of this investigation. Protein concentration was determined by absorption at 280 nm using an extinction coefficient of 25 for a 1.0% solution and molecular weight of 23,000 (the value used in this determination was verified through amino acid analyses). All assays contained 0.1 M buffer, and ionic strength was maintained at 0.3 M with KCl. Buffers used were: acetate, pH 4.0–5.6; phosphate, pH 5.9–7.5; and borate, pH 7.6–9.5. Measurements of pH were made and verified by using both a Radiometer pH meter 25-SE and a Corning pH meter Model 10, each of which was standardized at pH 4.0, 7.0, and 10.1.

A typical assay is described. Papain was activated for at least 15 min in 0.015 M EDTA containing 0.075 M β-mercaptoethanol, pH 6.0, and was used within 3 hr. Stock solutions of L-BAPNA at 4.0, 5.0, 6.67, 10.0, and 20.0 mM were made in water (L-BAPNA, unlike DL-BAPNA, is highly soluble), and their exact concentrations were ascertained by absorption at 315 nm (ϵ = 13,000 M^{-1} cm^{-1}). Stock buffer solutions, 0.12 M, of ionic strength 0.36 M, were prepared. Each 3.0-ml assay solution consisted of 2.5 ml of buffer, 0.3 ml of stock substrate, and 0.2 ml of freshly activated papain (or EDTA–mercaptoethanol no-enzyme blank), giving final concentrations of 1.0 mM EDTA and 5.0 mM β-mercaptoethanol, 0.3 M in ionic strength. The pH of each assay was checked in the no-enzyme blank after enzymatic hydrolysis. No hydrolysis was detected at any pH studied when enzyme was omitted from the assay solutions.

Assays using L-BAEE as substrate (4.0–20.0 mM) were performed at an ionic strength of 0.3 M, in the presence of 5.5 mM β-mercaptoethanol and 3.3 mM EDTA by titration using a Radiometer pH-Stat (pHM 25/TTT-11/ABU-1b/SBR 2c) to deliver 0.01 N NaOH. All assays were made in duplicate, and verified by a second series of duplicate assays. Appropriate corrections were applied to observed rates for the ionization of *N*-benzoyl-L-arginine (pK_a = 3.40), and for electrode poisoning by the assay mixture.

Analysis of Kinetic Data. Kinetic data for both substrates were analyzed for k_{cat} and K_m(app) values at various pH values by computer fitting to the hyperbolic Michaelis–Menten equation according to a modification of the program of Cleland (1967).

Values for the three constants, K_1, K_2, and k_{cat}/K_m(lim), were determined by fittng the individual k_{cat}/K_m(app) ratios measured at various pH values and the H^+ concentrations, respectively, to eq 4 using a nonlinear least-squares computer program (Marquardt, 1963), where K_1 and K_2 represent the dissociation constants of two ionizable groups on the enzyme,

[1] Abbreviations used are: L-BAPNA, *N*-benzoyl-L-arginine-*p*-nitroanilide; D-BAPNA and DL-BAPNA, the D isomer and racemic derivative, respectively; L-BAEE, *N*-benzoyl-L-arginine ethyl ester; CGNP, *N*-carbobenzoxyglycine *p*-nitrophenyl ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate; EDTA, ethylenediaminetetraacetate.

TABLE I: Kinetic Comparisons of Purified Papain with "Corrected" Twice-Crystallized Papain.[a]

Substrate	Purified Papain, Obsd Values			2× Cryst Papain, Lit. Values		
	pH	K_m(app) (mM)	k_{cat} (sec^{-1})	pH	K_m(app) (mM)	k_{cat} (sec^{-1})
L-BAEE	5.85	12.8 ± 0.7	23.2 ± 0.7	5.82[b]	15.6 ± 0.3	17.1 ± 2.0
L-BAEE	7.22	13.7 ± 0.8	28.9 ± 0.9	7.28[b]	12.6 ± 0.3	14.1 ± 1.6
CGNP	5.11	0.0062 ± 0.0015	6.5 ± 0.3	5.00[c]	0.0048 ± 0.0003	4.8 ± 0.3

[a] L-BAEE assays contained: 5.5 mM β-mercaptoethanol and 3.3 mM EDTA, μ = 0.3 M with KCl; $[S]_0$ = 3.52–17.8 mM; $[E]_0$ = 0.743 μM. CGNP assays contained: 5.8 mM β-mercaptoethanol, 2.2 mM EDTA and 0.23 M acetate buffer, 12% (v/v) CH_3CN; $[S]_0$ = 0.02–0.10 mM; $[E]_0$ = 0.020–0.108 μM; $\Delta\epsilon$ = 8700 M^{-1} cm^{-1}. Each of the observed values represents the average of three separate determinations, reported with their standard deviations. [b] Whitaker and Bender (1965). [c] Williams and Whitaker (1967).

$$k_{cat}/K_m(app) = \frac{k_{cat}/K_m(lim)}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} \quad (4)$$

and k_{cat}/K_m(lim) represents the limiting (maximum) value of this kinetic ratio. Analysis of k_2 and k_3 as a function of pH (by the method presented in eq 6 under Results) provided values which were similarly fitted to eq 4a and 5, respectively.

$$k_2 = \frac{k_2(lim)}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} \quad (4a)$$

$$k_3 = \frac{k_3(lim)}{1 + \frac{[H^+]}{K'}} \quad (5)$$

Results

A major assumption in previous kinetic investigations of papain has been that active site titrations could be employed to correct for inactive enzyme present and thus to obtain accurate values of k_{cat}. Although, as shown in Table I, the K_m values for papain-catalyzed hydrolysis of L-BAE and CGNP were found to agree with those peviously reported, the k_{cat} values with fully active papain were found to be as much as twofold greater than those based on active site corrections.

Use of the *p*-nitroanilide of *N*-benzoyl-L-arginine as substrate provides a simple, sensitive spectrophotometric assay of papain activity. Figure 1 shows traces of progress curves recorded for the papain-catalyzed hydrolysis of L-BAPNA at pH 5.97 at several substrate concentrations. Moreover, in contrast to observations with *p*-nitrophenyl esters, there is no measurable nonenzymatic hydrolysis of the anilide even at pH 9.0 and in the presence of 5 mM β-mercaptoethanol. Initial velocities are linear with no curvature due to product inhibition for periods of 5 min or longer.

Figure 2 gives typical Lineweaver–Burk plots of kinetic data obtained at several pH values, demonstrating that the hydrolysis of L-BAPNA by papain follows Michaelis–Menten kinetics throughout the concentration range employed. However, analysis of kinetic data was made by directly fitting them to the hyperbolic Michaelis–Menten equation (see Experimental Procedures), rather than to the double

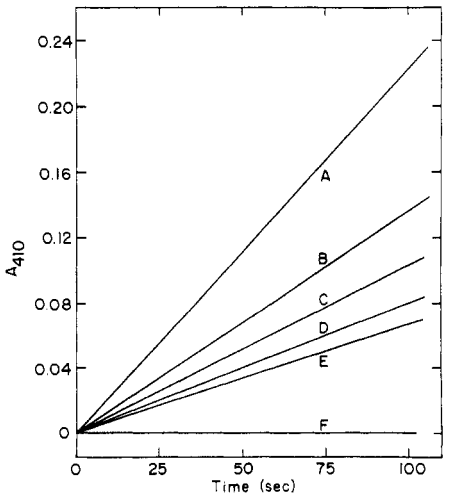

FIGURE 1: Progress curves for hydrolysis of L-BAPNA. Curves A–E, papain-catalyzed hydrolysis at pH 5.97, 0.1 M phosphate buffer, 1.0 mM EDTA, 5.0 mM β-mercaptoethanol, μ = 0.3 M with KCl, $[E]_0$ = 0.826 M; $[S]_0$: curve A, 212 mM; B, 1.05 mM; C, 0.713 mM; D, 0.519 mM; E, 0.392 mM. Curve F, no enzyme blank, $[S]_0$ = 2.2 mM, 5.0 mM β-mercaptoethanol, pH 5.97 and 9.0 (results at both pH values superimposable).

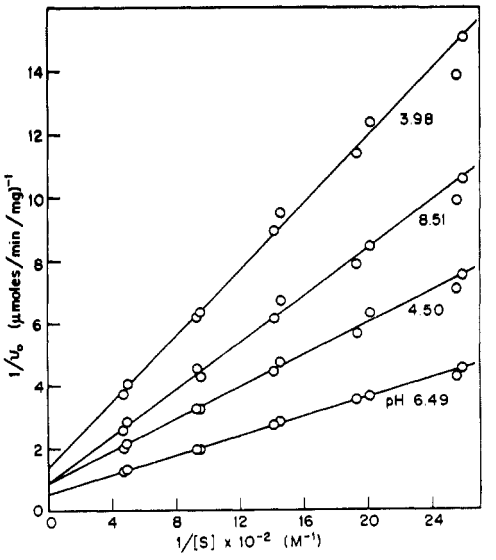

FIGURE 2: Typical Lineweaver–Burk plots for papain-catalyzed hydrolysis of L-BAPNA at varied pH and substrate concentrations. Curves are identified by numerical pH values. Experimental conditions as given in Table II.

TABLE II: Kinetics of Papain-Catalyzed Hydrolysis of *N*-Benzoyl-L-arginine-*p*-nitroanilide.[a]

pH	K_m(app) (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m(app) (M^{-1} sec^{-1})	k_2 (sec^{-1})	k_3 (sec^{-1})
3.98	4.07 ± 0.44	0.29 ± 0.02	71.7 ± 13.4	0.53	0.65
4.50	3.23 ± 0.25	0.47 ± 0.03	146.4 ± 19.3	1.08	0.84
4.99	3.29 ± 0.14	0.72 ± 0.02	218.5 ± 16.2	1.61	1.30
5.49	3.17 ± 0.16	0.76 ± 0.03	239.7 ± 20.6	1.77	1.33
5.96	2.86 ± 0.13	0.74 ± 0.03	257.3 ± 19.6	1.90	1.20
6.49	2.95 ± 0.16	0.73 ± 0.03	247.8 ± 22.3	1.83	1.22
7.01	3.00 ± 0.35	0.69 ± 0.06	231.0 ± 45.7	1.70	1.17
7.48	3.18 ± 0.41	0.63 ± 0.06	199.1 ± 43.6	1.47	1.11
7.65	3.59 ± 0.57	0.63 ± 0.08	176.0 ± 48.4	1.30	1.23
8.28	5.06 ± 0.40	0.53 ± 0.03	104.7 ± 14.3	0.75	1.80
8.51	4.39 ± 0.57	0.45 ± 0.04	101.6 ± 23.0	0.75	0.93
8.82	5.47 ± 0.55	0.40 ± 0.03	73.1 ± 12.8	0.54	1.54
8.91	5.76 ± 1.28	0.34 ± 0.06	59.4 ± 23.8	0.44	1.53
9.25	6.06 ± 0.58	0.21 ± 0.02	35.3 ± 5.0	0.26	1.09
9.51	6.20 ± 0.70	0.14 ± 0.12	22.5 ± 4.5	0.17	0.80

[a] Conditions: 0.1 M buffers, acetate, pH 3.98–5.49; phosphate, pH 5.96–7.48; borate, pH 7.65–9.51; μ = 0.3 M; $[S]_0$ = 0.40–2.00 mM; $[E]_0$ = 0.413–0.826 μM.

reciprocal transformations. The resulting kinetic parameters, k_{cat}, K_m(app), and k_{cat}/K_m(app), are tabulated in Table II, together with their standard deviations.

Plotting k_{cat}/K_m(app) as a function of pH produces the anticipated bell-shaped pH–activity relationship shown in Figure 3. The curve shown is the computer-generated fit of the data to eq 4. The estimated pK_1 and pK_2 values of 4.3 and 8.2, respectively, are in excellent agreement with values obtained by Whitaker and Bender (1965) for L-BAEE and *N*-benzoyl-L-argininamide.

Solving eq 2 and 3 for k_2 and combining give the linear equation

$$k_{cat} = k_3 - \frac{k_3 K_m(app)}{K_s} \qquad (6)$$

The values of k_{cat} and K_m(app) obtained at pH 4.99 and above, where k_3 is constant, were fitted by computer to this equation by the method of least squares, and a plot of the data is presented in Figure 4a. From this plot, a K_s of 7.86 mM and a k_3(lim) of 1.16 sec^{-1} for L-BAPNA were obtained. The reliability of fit is evidenced by a correlation coefficient of 0.95 and by the fact that the value for k_3 is in excellent agreement with a value of 1.22 obtained by averaging the k_3 values of Table II at pH $\geq$ 5.

Equation 6 can be rearranged to the reciprocal equation (6a)

$$\frac{1}{K_m(app)} = \frac{1}{K_s} + \left(\frac{k_{cat}}{K_m(app)}\right)\frac{1}{k_3} \qquad (6a)$$

To permit direct comparison with the results published for L-BAEE and *N*-benzoyl-L-argininamide (Whitaker and Bender, 1965), the data shown in Figure 4a were fitted to eq 6a as shown in Figure 4b. The values for K_s (7.37 mM) and k_3(lim) (1.23 sec^{-1}) are essentially the same as those derived from fitting the data to eq 6. Despite the fact that the data appear to fit eq 6a (correlation coefficient 0.98) better than eq 6, the use of the latter equation is preferred because it avoids the reciprocal transformation and the presence of the K_m(app) term in both variables.

Values thus obtained for the individual rate constants governing acylation (k_2) and deacylation (k_3) are presented in Table II, and plotted as a function of pH in Figure 5. The similarity in magnitude of k_2 and k_3 indicates that both enter into the k_{cat} value for L-BAPNA. Limiting values for each of the kinetic parameters determined in eq 4, 4a, and 5, together with the respective pK values, are presented in Table III.

Because the k_3(lim) of 1.2 sec^{-1} obtained for L-BAPNA differed widely from that reported by Whitaker and Bender (1965) for L-BAEE and *N*-benzoyl-L-argininamide, which share the common acyl group (*N*-benzoyl-L-arginine), and because of the difficulties encountered in the spectrophoto-

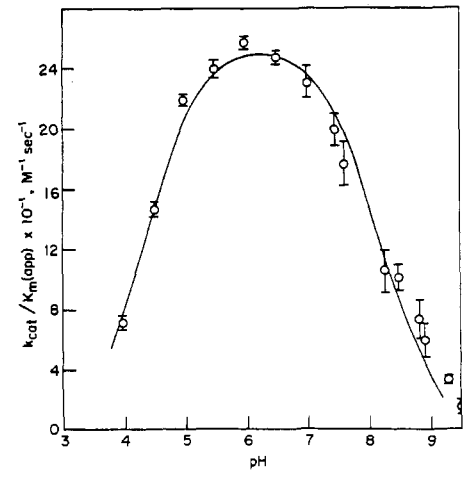

FIGURE 3: Variation of k_{cat}/K_m(app) of papain for L-BAPNA as a function of pH. Reaction conditions as described in Table II. The solid line represents the computer-generated theoretical curve of the data fitted to eq 4 as described in Experimental Procedure.

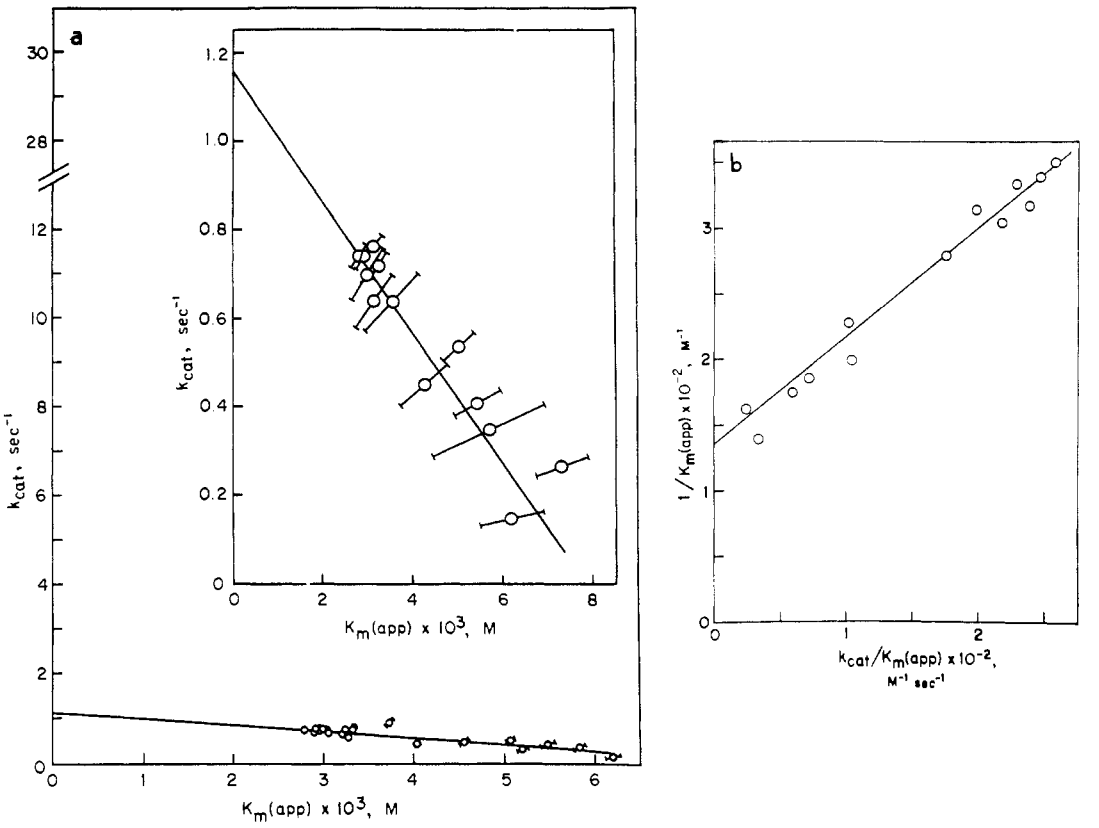

FIGURE 4: (a) k_{cat} values (at various pH values above 4.99) plotted as a function of K_m(app) according to eq 6, in order to evaluate K_s and k_3(lim). The lines represent a computer fit of the data presented in Table II. The main graph is a plot of *individual* kinetic analyses, together with standard error estimates provided by Cleland's computer program, on a scale which emphasizes the significant departure of the data from an assumed k_{cat} intercept of 30 sec^{-1}. The inset provides a plot of *averaged* values (three values obtained by repetitive kinetic analyses at each pH) together with standard errors of the average values obtained. (b) The data of Figure 4a (inset) plotted according to eq 6a.

TABLE III: Limiting Kinetic Values and pK Values for Papain-Catalyzed Hydrolysis of L-BAPNA.

k_{cat}/K_m(app)(lim) (M^{-1} sec^{-1})	k_2(lim) (sec^{-1})	k_3(lim) (sec^{-1})	K_s (mM)	Acylation Step pK_1	Acylation Step pK_2	Deacylation Step pK'
251	1.85	1.2	7.86	4.32	8.24	3.95

metric assay of L-BAEE,[2] we sought to verify the k_3(lim) for L-BAEE by kinetic measurements with the pH-Stat. Analysis of our kinetic data for L-BAEE by the procedure given above for L-BAPNA resulted in a k_3(lim) value of 30 sec^{-1}, which verifies the value of 20.2 sec^{-1} reported by Whitaker and Bender (1965) when corrections are made for the differences in the reported k_{cat} approximations (see Table I). Our results indicate that papain-catalyzed hydrolysis of the ethyl ester and the *p*-nitroanilide of *N*-benzoyl-L-arginine do not proceed by the simple three-step mechanism (eq 1) involving a common deacylation step with a single value for k_3.

Since earlier measurements of papain activity toward the *p*-nitroanilide had been made with the *N*-benzoyl-DL-arginine derivative, in which dimethyl sulfoxide was used to effect solubilization of the racemic substrate (Erlanger *et al.*, 1961; Kirschenbaum, 1971), the effect of dimethyl sulfoxide on the kinetics of L-BAPNA hydrolysis was of interest. Values for k_{cat} and K_m(app) at neutral pH are given in Table IV. It can be seen that dimethyl sulfoxide at concentrations up to 5% (v/v) does not affect the k_{cat} of papain for L-BAPNA, and increases the K_m only slightly. Through comparison of the kinetic constants obtained for DL-BAPNA with those for L-BAPNA under otherwise identical conditions (lines 1 and 2, Table IV), a value for K_I of 5.8 mM was obtained for the D isomer. A value of 2.3 mM had been previously reported by Tokura *et al.* (1971).

Discussion

The newly available substrate, L-BAPNA, provides a means for rapid and accurate spectrophotometric assays of papain activity. Such assays afford several advantages over those frequently employed—increased sensitivity, ease, and accuracy, even at high substrate concentrations, and a lower

[2] Due to limitations of instrument optics, the very small $\Delta\epsilon$ must be calculated separately for each substrate concentration employed in the spectrophotometric assay of L-BAEE hydrolysis. Determination of such values, which range from 12 to 26 M^{-1} cm^{-1}, involves sizable percentage errors.

TABLE IV: Effect of Dimethyl Sulfoxide on Papain Catalysis.

Conditions	Substrate	pH	k_{cat} (sec^{-1})	K_m(app) (mM)
0.06 M phosphate, μ = 0.10 M, 5% (v/v) Me_2SO	DL-BAPNA	7.06	0.314 ± 0.009	3.92 ± 0.18
0.06 M phosphate, μ = 0.10 M, 5% (v/v) Me_2SO	L-BAPNA	7.15	0.702 ± 0.032	3.72 ± 0.26
0.1 M phosphate, μ = 0.3 M, no Me_2SO	L-BAPNA	7.01	0.693 ± 0.056	3.00 ± 0.35

K_m(app) (3 mM) than those of L-BAEE (14 mM) and *N*-benzoyl-L-argininamide (33 mM). Its greater water solubility[3] and stability toward nonenzymatic hydrolysis result in significant improvements over the more commonly employed assays using *p*-nitrophenyl esters of N-substituted glycine and lysine derivatives.

In virtually all papain preparations which have been studied kinetically, the thiol content has been less than 1 mol/mol following activation (see Glazer and Smith, 1971). Accordingly, attempts have been made to correct observed k_{cat} values to account for the presence of inactive protein in papain assays (Bender and Brubacher, 1966; Whitaker and Bender, 1965; Williams and Whitaker, 1967). The efficacy of affinity chromatography in obtaining fully active papain has been demonstrated both by Sluyterman and Wijdenes (1970) and by Blumberg *et al.* (1970). From specific activities toward L-BAEE measured at pH 6.0, Sluyterman and Wijdenes estimated k_{cat} to be 26 sec^{-1} based on an assumed K_m of 18 mM. Blumberg *et al.* evaluated k_{cat} as 28.5 sec^{-1} with K_m(app) = 18 mM. At pH 7.2, the papain preparation used in the present study exhibited a k_{cat} toward L-BAEE of 28.9 ± 0.9 sec^{-1} (see Table I), as compared to 14.1 ± 1.6 sec^{-1} which had been previously reported on the basis of active site corrections (Whitaker and Bender, 1965). On the other hand, the K_m values of this substrate were not significantly altered by removal of inactive papain. In the case of the *p*-nitrophenyl ester of *N*-carbobenzoxyglycine, the kinetic values obtained for purified papain more nearly agreed with those reported as "corrected" values (Williams and Whitaker, 1967). The active site titrations employed in "correcting" the earlier kinetic observations were based on measurement of the initial burst of *p*-nitrophenol liberated upon reaction of papain with *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (Whitaker and Bender, 1965; Williams and Whitaker, 1967), and thus subject to inherent errors in evaluation. The present studies serve to emphasize the value of utilizing fully active enzyme preparations in kinetic determinations, rather than attempting to "correct" kinetic constants on the basis of active site titrations.

Examination of the kinetics of papain's action on the *p*-nitroanilide of *N*-benzoyl-L-arginine provides interesting contrasts to the enzyme's behavior with the corresponding ethyl ester. Some investigators have assumed K_s to be independent of the leaving group in the substrate, and have attributed differences in K_m(app) values of ethyl and *o*-, *m*-, and *p*-nitrophenyl esters of *N*-carbobenzoxyglycine to the relative ease of nucleophilic displacement at the carbonyl carbon atom (Kirsch and Igelström, 1966). The present study provides a K_s value of 7.86 mM for L-BAPNA, which is somewhat lower than 54.5 mM for L-BAEE and 36.3 mM for *N*-benzoyl-L-argininamide (Whitaker and Bender, 1965). Similar dependence of the K_s of papain on the bulk of the substrate's leaving group had also been noted by other investigators; thus, K_s values of 10.7, 0.52, and 0.03 mM were reported for the methyl, phenyl, and *p*-nitrophenyl esters, respectively, of *N*-carbobenzoxy-L-lysine (Bender and Brubacher, 1966), and Lowe and Williams (1965b) observed a K_s of 10.2 mM for *n*-butyl hippurate as compared to 202 mM for hippuramide.

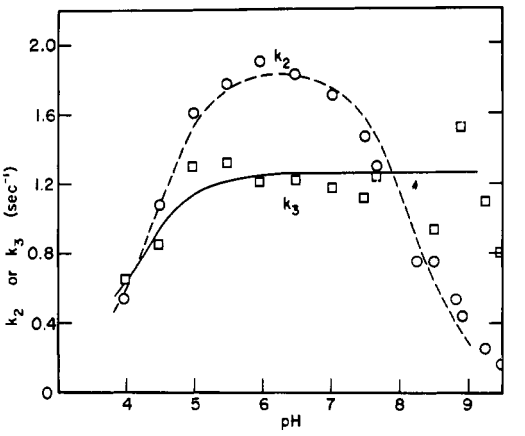

FIGURE 5: Variation of acylation and deacylation rate constants of papain for L-BAPNA as a function of pH. Broken line represents computer-generated curve derived from fitting the k_2 data (Table II) to eq 4a; solid line represents that from fitting k_3 data to eq 5.

Evaluation of the apparent deacylation rate constant, k_3(lim), for the papain-catalyzed hydrolysis of L-BAPNA, led to the surprising discovery that it is more than an order of magnitude smaller (1.2 sec^{-1}) than the k_3(lim) obtained for L-BAEE (30 sec^{-1}), although the pH dependence appears identical.

The data presented in Figure 4a reflect the reproducibility of the kinetic measurements made using the *p*-nitroanilide substrate at each pH, and emphasize the departure of these data from fitting a similar linear relationship (eq 6) with a k_{cat} intercept, *i.e.*, k_3(lim), of 30 sec^{-1}, or even 20.2 sec^{-1} as previously recorded (Whitaker and Bender, 1965).[4]

If the action of papain on both amide and ester substrates of *N*-benzoyl-L-arginine occurred simply by the generally accepted three-step mechanism (ordered Uni-Bi, as shown in eq 1), the value of k_3(lim), which governs the rate of deacylation of the common *N*-benzoyl-L-arginylpapain intermediate, should be identical for each substrate. Such identity has previously been assumed on the basis of the values reported

[3] At concentrations >2 mM, L-BAPNA solutions become cloudy at the higher pH values; however, at assay concentrations <2 mM, no solubility problems were encountered at any pH studied.

[4] In reciprocal form, Figure 4b, the nitroanilide data would have to correspond to a line intersecting the 1 K_m(app) axis at 3.33 (to give a K_s of 3.0 mM) in order to provide a k_3(lim) value which conforms to that of the ester.

by Whitaker and Bender (1965) for L-BAEE (20.2 ± 1.7 sec^{-1}) and *N*-benzoyl-L-argininamide (28.7 ± 25.1 sec^{-1}). It now appears that the scatter resulting from kinetic measurements of amide hydrolysis (compare Figure 5 of Whitaker and Bender (1965) with Figure 4b of this article) may have precluded accurate evaluation of k_3.

Thus, the steady-state kinetics of papain's action on L-BAPNA suggest that the hydrolysis of amide bonds (and perhaps peptide bonds, by inference) catalyzed by this enzyme may proceed by a mechanism which does not involve the obligatory formation of an acyl-enzyme intermediate such as that found with ester substrates. It is, of course, premature to propose a distinctly different mechanism for each type of substrate, particularly in view of the complications associated with such a mechanistic diversity (see Caplow, 1969). Evidence has been recorded for the existence of nonproductive binding of acyl-amino acid substrates to papain (Hinkle and Kirsch, 1971); however, the kinetic consequences of such ineffectual binding are to give a low estimate for the rate constant governing acylation, k_2, but to provide the correct value for k_3(lim) by the Whitaker and Bender analysis, as shown by Brocklehurst *et al.* (1968). Thus, in order to account for an apparent k_3(lim) of 1.2 sec^{-1}, assuming an actual deacylation rate constant of 30 sec^{-1} were to exist for the anilide as well as the ester, "wrong-way" binding would have to actually occur in such a manner as to permit a very slow deacylation of "wrong-way" acylated papain. In any event, additional kinetic analyses are needed to determine the extent to which "k_3" values for amides and peptides may differ from those of analogous ester substrates.

As pointed out by Lucas and Williams (1969), errors in determinations of k_{cat} and K_m may be proportional to each other, so that when k_{cat} is close to k_2, serious error may be introduced in evaluation of k_3 by the Whitaker and Bender procedure. In the case of papain's action on L-BAPNA, however, we have observed that hydroxynitrobenzylation of the enzyme leads to a sevenfold increase in acylation rate (k_2), so that it is considerably higher than k_{cat}, with no increase in the k_3(lim) value (Mole and Horton, manuscript in preparation). Thus, the value of k_3(lim) for the papain-catalyzed hydrolysis of benzoyl-L-arginine-*p*-nitroanilide does appear to be truly smaller in magnitude than does that for benzoyl-L-arginine ethyl ester.

Examination of pre-steady-state kinetics of amides should provide useful information with respect to mechanism(s) of papain-catalyzed hydrolyses of amides, anilides, and peptides. Unfortunately, pre-steady-state kinetics requiring [E] $\gg$ [S] (see Hubbard and Kirsch, 1968) to provide data concerning the order of product release from the enzyme would require papain concentrations impossible to achieve (*ca.* 1.6 g/ml) in the case of L-BAPNA, because of its relatively large K_s (7.86 mM).

Acknowledgments

The authors would like to thank Dr. Samuel B. Tove for his assistance with the computer programs used in this study and helpful comments concerning evaluation of the data. We are also indebted to Mrs. Jackie Mole for introduction to the computer programs and for her assistance in computations.

References

Bender, M. L., and Brubacher, L. J. (1966), *J. Amer. Chem. Soc. 88*, 5880.

Blumberg, S., Schechter, I., and Berger, A. (1970), *Eur. J. Biochem. 15*, 97.

Brocklehurst, K., Crook, E. M., and Wharton, C. W. (1968) *FEBS (Fed. Eur. Biochem. Soc.) Lett. 2*, 69.

Brubacher, L. J., and Bender, M. L. (1966), *J. Amer. Chem. Soc. 88*, 5871.

Caplow, M. (1969), *J. Amer. Chem. Soc. 91*, 3639.

Caplow, M., and Jencks, W. P. (1963), *J. Biol. Chem. 238*, PC1907.

Cleland, W. W. (1967), *Advan. Enzymol. 29*, 1.

Cautrecasas, P., and Anfinsen, C. B. (1971), *Methods Enzymol. 22*, 345.

Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961), *Arch. Biochem. Biophys. 95*, 271.

Glazer, A. N., and Smith, E. L. (1971), *in* The Enzymes, 3rd ed, Vol. 3, Boyer, P. D., Ed., New York, N. Y., Academic Press, p 501.

Hinkle, P. M., and Kirsch, J. F. (1971), *Biochemistry 10*, 2717.

Hubbard, C. D., and Kirsch, J. F. (1968), *Biochemistry 7*, 2569.

Kirsch, J. F., and Igelström, M. (1966), *Biochemistry 5*, 783.

Kirschenbaum, D. M. (1971), *Biochim. Biophys. Acta 235*, 159.

Lowe, G., and Williams, A. (1965a), *Biochem. J. 96*, 189.

Lowe, G., and Williams, A. (1965b), *Biochem. J. 96*, 199.

Lowe, G., and Yuthavong, Y. (1971a), *Biochem. J. 124*, 107.

Lowe, G., and Yuthavong, Y. (1971b), *Biochem. J. 124*, 117.

Lucas, E. C., and Williams, A. (1969), *Biochemistry 8*, 5125.

Marquardt, D. W. (1963), *J. Soc. Ind. Appl. Math. 11*, 431.

Nishi, N., Tokura, S., and Noguchi, J. (1970), *Bull. Chem. Soc. Jap. 43*, 2900.

Sluyterman, L. A. AE., and Wijdenes, J. (1970), *Biochim. Biophys. Acta 200*, 593.

Smith, E. L. (1958), *J. Biol. Chem. 233*, 1392.

Smith, E. L., Finkle, B. J., and Stockell, A. (1955), *Discuss. Faraday Soc. 20*, 96.

Tokura, S., Nishi, N., and Noguchi, J. (1971), *J. Biochem. (Tokyo) 69*, 599.

Whitaker, J. R., and Bender, M. L. (1965), *J. Amer. Chem. Soc. 87*, 2728.

Williams, D. C., and Whitaker, J. R. (1967), *Biochemistry 6*, 3711.