Effects of Proflavine on Papain's Catalytic Activity. A Proflavine-Mediated Decrease in K_M†

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ABSTRACT: Proflavine is a noncompetitive activator of papain which induces a 2.7–6.0-fold decrease in K_M for the hydrolysis of *N*,α-benzoyl-L-arginine ethyl ester and *N*-benzoylglycine ethyl ester. Proflavine alters V_{max} by less than 50%. Dissociation constants (K_D) for papain–proflavine complexes were determined fluorometrically by observing the quenching of proflavine fluorescence which accompanies formation of a papain–proflavine complex. At pH 6, mercuripapain (Hg-papain) has a greater affinity for proflavine (K_D = 107 μM) than papain (K_D = 210 μM), whereas at pH 4 both proteins have essentially the same affinity for proflavine (K_D = 300–310 μM). This result indicates that in Hg-papain the p*K* of an ionizable group involved in proflavine binding is perturbed. At pH 6, nonactivatable papain has essentially the same affinity as papain for proflavine. This result coupled with the inability of the peptide inhibitor Gly-Gly-Tyr(Bzl)-Arg to displace proflavine from papain indicates that proflavine is not binding at the substrate binding site. The dissociation constant of the proflavine–papain complex was determined kinetically from its effects on K_M. The kinetically determined dissociation constant (K_D = 220 μM) is essentially identical with the one determined fluorometrically, suggesting that the complex characterized fluorometrically is responsible for inducing the observed changes in K_M.

The dye proflavine (3,6-diaminoacridine) has been an extremely effective probe for elucidating the mechanism of action of certain proteolytic enzymes. Spectral changes accompanying displacement of proflavine by substrate from chymotrypsin's active site have facilitated characterization of several steps in the catalytic pathway (Bernhard and Gutfreund, 1965; Bernhard *et al.*, 1966; Himoe *et al.*, 1967, 1969; Brandt *et al.*, 1967; Glazer, 1965). Whereas proflavine competes with substrate for chymotrypsin's active site, it binds noncompetitively to the thiolproteinase ficin (Hollaway, 1968). Hollaway (1968) showed that proflavine increases the rate of the acylation step in ficin-catalyzed reactions.

Papain's substrate binding site is capable of interacting with seven aminoacyl residues of a polypeptide substrate (Schechter and Berger, 1967). Berger and Schechter (1970) showed that papain, like chymotrypsin, contains a subsite for aromatic groups in its substrate binding site. However, the amide bond cleaved during papain catalysis is not part of the aminoacyl residue which interacts with this aromatic binding site. In papain, this aromatic binding site is one aminoacyl residue removed from the labilized amide bond. In order to further characterize this aromatic binding site and determine how interactions at this site or another hydrophobic site might alter catalytic efficiency, studies of the interactions between papain and proflavine were undertaken. In this work, a noncompetitive interaction between proflavine and papain is characterized, in which proflavine decreases K_M for papain-catalyzed hydrolyses of BzArgOET[1] and BzGlyOEt.[2] Effects of other ligands on the papain–proflavine interaction presented in this work provide new information about the active-site region of papain.

Materials and Methods

Papain (EC 3.4.4.10), twice crystallized in suspension in 0.05 M acetate buffer (pH 4.5), was obtained from Worthington Biochemical Corp., Freehold, N. J. The papain was further purified by the method of affinity chromatography (Blumberg *et al.*, 1970), which separated the active from the nonactivatable papain (NAP). L-Cysteine (free base) (CP) and *N*-benzoylglycine ethyl ester (CP) (BzGlyOEt) were from Mann Research Laboratories, New York, N. Y. *N*,α-Benzoyl-L-arginine ethyl ester hydrochloride (BzArgOEt) (Aldrich Analyzed) was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Proflavine bisulfate monohydrate was obtained from Calbiochem, La Jolla, Calif. The spectrum of proflavine in aqueous solution exhibited the expected maxima at 260 and 444 nm (Millich and Oster, 1959; Glazer, 1965). The molar absorptivity at 260 nm relative to the molar absorptivity at 444 nm was 1.36 in good agreement with the previously reported value of 1.42 (Glazer, 1965). Assuming the sample of proflavine used was all bisulfate monohydrate (as labeled), it had a molar absorptivity of 39,700 as opposed to previously reported values of 33,400–36,000 (Millich and Oster, 1959; Glazer, 1965; Fersht and Requena, 1971). The discrepancy between these values is attributed to the proflavine used in this work being a mixture of bisulfate and sulfate salts. Titration of the bisulfate with standardized base supports this conclusion. The titration showed that on a weight basis the sample contained less than 58% of the expected bisulfate. Assuming the remainder of the sample is proflavine sulfate one obtains an expected value of 35,000–36,000 for the molar absorptivity of the proflavine.

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[1] Abbreviations used are: BzArgOEt, *N*,α-benzoyl-L-arginine ethyl ester; BzGlyOEt, *N*-benzoylglycine ethyl ester; NAP, nonactivatable papain; Hg-papain, mercuripapain.

[2] A report of a proflavine mediated decrease in K_M for the hydrolysis of BzArgOEt and BzGlyOEt appeared after his work was submitted for publication (Hall *et al.*, 1972). However, that report did not deal with the interaction of papain with proflavine in the absence of substrate nor did it quantitatively relate the proflavine mediated decrease in K_M to the formation of a proflavine–papain complex.

Gly-Gly-Tyr(Bzl)-Arg (I) was prepared using the method of Blumberg *et al.* (1970). High voltage electrophoresis (75 V/cm, 90 min) at pH 3.5 (2.5% acetic acid, neutralized to pH 3.5 with pyridine) gave a single spot (ninhydrin). On acid hydrolysis in 6 N HCl at 108° for 22 hr the peptide gave 1.1 Arg, 2.0 Gly, and 0.85 Tyr. Presumably this treatment liberates most of the tyrosine from the *O*-benzyltyrosyl residue. Amino acids were determined by the method of Spackman *et al.* (1958) except that after development of the color the absorbance at 570 nm was measured continuously on a Gilford Model 2000 multiple sample absorbance recorder with 0.5- and 1-cm flow-through cells. Areas under the peaks were measured with a compensating polar planimeter sold by Gelman Instrument Co., Ann Arbor, Mich. The amino acid analyzer was calibrated with an 18 amino acid standard solution from Calbiochem.

EDTA, disodium salt, reagent grade, was purchased from Matheson Coleman and Bell, Norwood, Ohio. Potassium chloride, reagent grade, was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. The distilled water supplied to the laboratory was passed through a demineralizer and redistilled in an all-glass still. Carbon dioxide and oxygen were removed from the water with a stream of nitrogen prior to all kinetic and fluorescence measurements.

Measurements of pH were made using a Radiometer Model PHM-4C pH meter or a Radiometer TTT-1c pH-Stat, which was standardized with a 1:1 phosphate NBS primary standard solution (Bates, 1964). The response of the glass electrode was checked with another NBS primary standard solution (phthalate). Any nonideality in the glass electrode response was corrected with the temperature compensator.

Fluorescence measurements were made using an Aminco Bowman spectrofluorometer, Model 4-8106.

The concentrations of papain were determined from the absorbancies at 280 nm measured on a Model 240 Gilford spectrophotometer, using a value of 5.77×10^4 cm^{-1} M^{-1} for the molar absorptivity of papain, based on a value of $E_{1\%}^{1\,cm} = 24.7$ (Bender *et al.*, 1966; Glazer and Smith, 1961) and a mol wt of 23,350 (Wolthers *et al.*, 1970). Proflavine concentrations in sample solutions were determined from absorbancies of diluted solutions at 444 nm using a molar absorptivity of 3.5×10^4 cm^{-1} M^{-1} (Millich and Oster, 1959; Glazer, 1965). Absorbancies in 1-cm path-length cells were in the region of 0.1–0.8 absorbance unit, well within the range where proflavine absorbance follows Beer's law (Glazer, 1965). Activation of papain was achieved with 50 mM cysteine and 5 mM EDTA at least 10 min prior to use in all assays.

Assays of the catalytic activity of papain were performed by following the papain-catalyzed hydrolysis of BzArgOEt and BzGlyOEt using a Radiometer TTT-1c pH-Stat using 0.10 M KOH–0.05 M KCl as the titrant. The reaction mixture was maintained at 25 ± 0.2° with water from a constant-temperature circulator. In different assays, concentrations varied from 9.1×10^{-4} to 4.1×10^{-2} M BzArgOEt, 3.6×10^{-4} to 1.5×10^{-2} M BzGlyOEt, 4.9×10^{-5} to 6.8×10^{-4} M proflavine, and 4.8×10^{-7} to 1.9×10^{-6} M papain. For each proflavine concentration a series of assays was run in which the substrate concentration was varied. After each assay series, a reassay of the papain with no proflavine was done. This reassay enabled the V_{max} of a particular series to be corrected for papain's slight loss of catalytic activity during the course of the day.

K_I for the peptide inhibitor Gly-Gly-Tyr(Bzl)-Arg was determined at $\Gamma/2 = 0.15$ and $\Gamma/2 = 0.33$ by observing the decrease in initial velocity of hydrolysis of 9.9×10^{-3} M BzArgOEt caused by 9.9×10^{-4} M inhibitor. The ratio V/V_I was related to K_I by the following equation.

$$K_I = \frac{[I]}{\dfrac{(V/V_I)[1 + (K_M/[S])] - 1}{K_M/[S]} - 1}$$

Proflavine fluorescence in the presence of varying concentrations of enzyme and constant proflavine concentration (0.5 μM) was measured relative to the fluorescence of free proflavine at the same concentration. Cuvets were placed in a thermoregulated cell compartment at 25 ± 0.2°. The wavelength of exciting light was 444 nm. The fluorescent light was measured at 500 nm, the wavelength at which fluorescence was maximal. Fluorometric titrations were run at constant concentrations of buffer, proflavine, and inhibitor, when present. A phosphate buffer prepared by adding 0.05 M K_2HPO_4 to 0.05 M KH_2PO_4–0.1 M KCl until pH 6 was obtained was used to maintain pH 6. An acetate buffer prepared by adding 0.05 M NaAc–0.1 M KCl to 0.05 M HAc–0.15 M KCl, until pH 4 was obtained, was used to maintain pH 4. The concentration of papain was varied in the range of 2.0×10^{-5}–3.7×10^{-4} M.

The fluorescence of the enzyme solution alone (before the addition of proflavine) was measured to check for scattering and it never exceeded 1.2% of the fluorescence of free proflavine.

The peptide inhibitor (I), when present, was at 5×10^{-3} M or 10^{-2} M. The fluorescence of free proflavine was measured against proflavine–peptide inhibitor solutions to ensure that the peptide inhibitor did not alter free proflavine fluorescence. All fluorescence values were within 3% of each other. Ultraviolet difference spectra were obtained with a Cary 15 recording spectrophotometer. In the spectrophotometric titrations, the concentration of proflavine was held constant at 7 μM and the papain concentration varied in the range 5.7×10^{-5}–1.8×10^{-4} M.

Results

Table I illustrates the effect of proflavine on the Michaelis–Menten parameters, K_M and V_{max}, for the papain-catalyzed hydrolysis of BzArgOEt. At pH 6, proflavine decreases K_M almost threefold without changing V_{max}. A similar effect of proflavine on K_M is seen at pH 4; however, at pH 4, proflavine appears to cause a small increase in V_{max}. A proflavine-induced sixfold decrease in K_M for the papain-catalyzed hydrolysis of BzGlyOEt is also depicted in Table I. This sixfold decrease in K_M for the papain-catalyzed hydrolysis of BzGlyOEt is accompanied by only a 25% decrease in V_{max}. To further characterize the interactions responsible for proflavine's ability to mediate these changes in K_M, the interactions between papain and proflavine were studied separately under conditions of equilibrium.

Binding of proflavine to papain is accompanied by a shift in the proflavine spectrum to longer wavelengths. Difference spectra of papain and proflavine *vs.* proflavine exhibit a trough at 430 nm and a peak at 464 nm. Similar difference spectra have been reported for chymotrypsin and ficin whose difference spectra exhibit peaks at 465 and 466 nm, respectively (Bernhard *et al.*, 1966; Hollaway, 1968). The perturbation of the absorbance spectrum of proflavine mediated by these enzymes is very similar to the spectral perturbation observed when proflavine is dissolved in an apolar solvent (Bernhard *et al.*, 1966).

TABLE I: Effect of Proflavine on the Kinetics of the Papain-Catalyzed Hydrolysis of BzArgOEt and BzGlyOEt at 25°, $\Gamma/2 = 0.15$.

Substrate, pH	[Proflavine] (μM)	K_M[a] (mM)	V_{max}[a] / V_{max}^0
BzArgOEt, 6	0	14.3	1.0
	48.6	8.9	0.95[b]
	58.2	9.6	1.04
	69.1	8.0	1.01
	83.1	8.0	1.01
	98.2	7.2	0.98
	112	8.0	1.06
	134	6.5	1.01
	172	7.0	1.07
	191	6.6	1.02
	196	5.9	1.04
	260	5.0	0.91
	676	5.3	1.14
BzArgOEt, 4	0	14.0	1.00
	254	5.0	1.15
	672	5.0	1.47
BzGlyOEt, 6	0	12.8	1.00
	267	2.9	0.806
	504	2.6	0.766
	679	2.1	0.726

[a] K_M and V_{max}/V_{max}^0 were obtained from plots of $[S]/V$ *vs.* [S]. The standard errors in K_M and V_{max}/V_{max}^0 were between 1 and 5% of the listed values for BzArgOEt and between 3 and 12% for BzGlyOEt. The limited solubility of BzGlyOEt prevented a more precise determination of these parameters for BzGlyOEt. [b] Papain, 2× crystallized from Worthington, was used directly. All other runs were made with 2× crystallized papain which was purified by affinity chromatography. There was no difference in the values of K_M obtained for both these samples of papain when [proflavine] = 0.

Plots of $1/\Delta A_{465}$ *vs.* the reciprocal of the papain concentration ($1/[P]_t$) were linear (Figure 1). Equation 1 was used to relate the dependence of ΔA_{465} on the papain concentration to the dissociation constant of the papain–proflavine complex (K_D) and the change in molar absorptivity (ΔE) which accompanies binding of proflavine to papain. Since the total concentration of papain, $[P]_t$, was always much greater than the concentrations of bound dye, $[P]_t$ is essentially equal to [P], the concentration of papain not complexed with dye. Therefore, no significant error was introduced by using $[P]_t$ instead of [P] in interpreting eq 1. At pH 6, a value of 11,000 M^{-1} cm^{-1}

$$\frac{1}{\Delta A_{465}} = \frac{K_D}{\Delta E[D]}\frac{1}{[P]} + \frac{1}{\Delta E[D]} \quad (1)$$

was obtained for the change in molar absorptivity of proflavine on binding to papain, along with a value of 300 μM for the dissociation constant of the papain–proflavine complex. Similar spectrophotometric titrations using Hg-papain rather than activated papain yielded the same value for ΔE, but a value of 175 μM for K_D. In addition to shifting the absorbance spectrum, the binding of proflavine to papain causes dramatic quenching of proflavine fluorescence. Because of the greater sensitivity of the fluorescence response, interactions between proflavine and papain were studied in detail by fluorometric titration at constant low proflavine concentrations. Equation 2

$$\frac{1}{1-R} = \frac{F_i}{F_i - F} = \frac{f_D}{f_D - f_{PD}}\frac{K_D}{[P]} + \frac{f_D}{f_D - f_{PD}} \quad (2)$$

was used to relate the observed fluorescence, F, to the dissociation constant of the protein–dye complex, K_D, and the intrinsic fluorescence of free and bound proflavine (f_D and f_{PD}). R equals F/F_i and F_i represents the fluorescence of the proflavine solution in the absence of protein. Linear plots of $1/(1 - R)$ *vs.* $1/[P]$ were obtained. These plots were used to determine the values of K_D (see eq 2). Typical plots shown in Figure 2 indicate that at pH 6 the fluorescence of proflavine bound to papain (f_{PD}) is indistinguishable from zero and certainly not more than 0.1–0.2 that of the fluorescence of free proflavine (f_D). At pH 4, however, the fluorescence of bound proflavine appears to increase somewhat to about 0.3 that of the fluorescence of free proflavine. Interestingly, the fluorescence of proflavine bound to Hg-papain decreases from about 0.2 that of free proflavine to <0.1 that of free proflavine on going from pH 6 to 4 (Figures 2 and 3). The values of K_D obtained in this work are presented in Table II. The value of 210 (μM) for K_D determined by the fluorometric method is

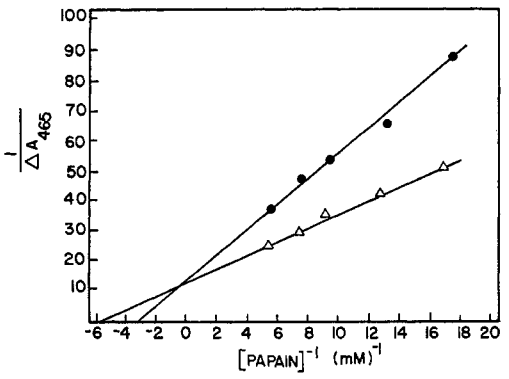


FIGURE 1: Determination of the dissociation constants for complexes of papain and Hg-papain with proflavine at pH 6.0 from uv difference spectra: ●, papain; △, Hg-papain.

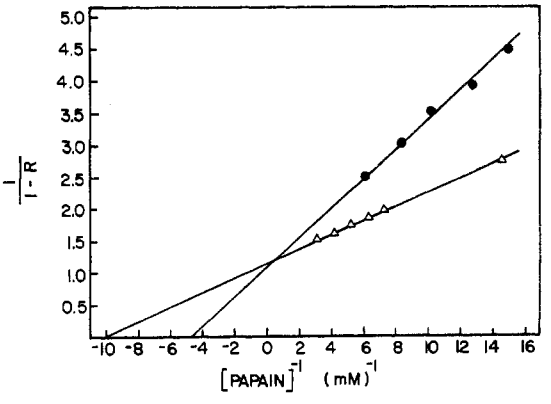


FIGURE 2: Determination of the dissociation constants for complexes of papain and Hg-papain with proflavine at pH 6.0 from fluorometric titrations of proflavine with protein: ●, papain; △, Hg-papain.

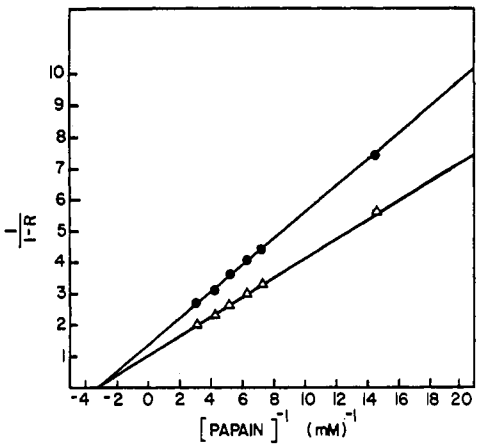


FIGURE 3: Determination of the dissociation constants for complexes of papain and Hg-papain with proflavine at pH 4.0 from fluorometric titrations of proflavine with protein: ●, papain; △, Hg-papain.

somewhat lower than the value of 300 μM determined from difference absorbance spectroscopy. Because of the larger changes involved, the fluorometric method is more precise and probably yields a more accurate determination of K_D. The conclusion from difference absorbance spectroscopy that Hg-papain binds proflavine more tightly than papain at pH 6.0 is substantiated using the fluorometric titration. Interestingly, papain and Hg-papain have similar affinities for proflavine at pH 4. Table II also reveals that nonactivatable papain, which is separated from active papain by affinity chromatography (Blumberg *et al.*, 1970), is indistinguishable from papain in its binding of proflavine at pH 6. Nonactivatable papain, a catalytically inactive form of papain, is thought to have a primary structure identical with papain except with regard to the state of the active site Cys-25 (Glazer and Smith, 1971).

In an effort to determine whether proflavine interacts with part of the substrate binding site, the effect of the peptide inhibitor Gly-Gly-Tyr(Bzl)-Arg (I) on the proflavine–papain interaction was studied. Unlike the small substrate BzArgOEt, the peptide inhibitor occupies a large portion of the aromatic subsite of the substrate binding site on papain (Blumberg *et al.*, 1970; Berger and Schechter, 1970). The dissociation constant (K_I) of the papain–inhibitor complex (in the absence of proflavine) was determined kinetically at pH 6 from its competitive inhibition of the papain-catalyzed hydrolysis of BzArgOEt. K_I was found to decrease from 483 to 111 μM, as Γ/2 was increased from 0.15 to 0.33. The value determined at Γ/2 = 0.33 is in reasonable agreement with the value of 150 μM at Γ/2 = 0.30 reported by Blumberg *et al.* (1970).

Where inhibitor is present (data in Table II), the inhibitor concentration is greater than the papain concentration, which is greater than the proflavine concentration. At pH 6 and inhibitor concentrations of 5–10 mM, 91–95% of the papain should contain bound inhibitor. Thus, an 11–20-fold increase should be seen for the apparent dissociation constant of the papain–proflavine complex, if the inhibitor occupies the proflavine binding site. Instead, only a 1.9–2.2-fold increase is observed in the apparent dissociation constant of the papain–proflavine complex in the presence of inhibitor. Thus, papain appears capable of binding inhibitor and proflavine simultaneously. Similar effects of peptide inhibitor on the apparent dissociation constant for the papain–proflavine complex are seen at pH 4. Since the dissociation constant for the peptide–papain complex markedly decreases as the pH is lowered (Blumberg *et al.*, 1970) the less than twofold increase in K_D mediated by 5 mM inhibitor suggests that proflavine and inhibitor also bind to the enzyme simultaneously at pH 4.

TABLE II: Apparent Dissociation Constants (K_D) for Papain–Proflavine Complexes at 25°, Γ/2 = 0.15.

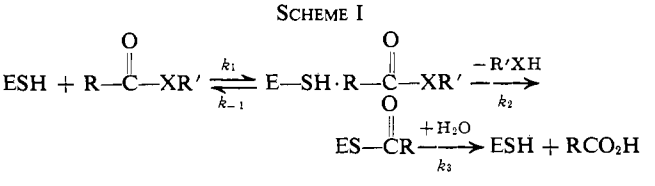
pH	Protein	Additions	K_D[a] (μM)	f_{PD}/f_D[b]
6	Papain	None	210 ± 10[c]	0.05 ± 0.05
6	Hg-papain	None	107 ± 7[d]	0.20 ± 0.03
6	Nonactivatable papain	None	217	0.09
6	Papain	5 mM I	395 ± 23[d]	0
6	Papain	10 mM I	455	0
4	Papain	None	300 ± 1[c]	0.29 ± 0.04
4	Hg-papain	None	310 ± 10[c]	0.02 ± 0.02
4	Papain	5 mM I	510 ± 20[d]	0.09 ± 0.09

[a] Calculated from the negative reciprocal of the x intercept of plots of $1/(1 - R)$ *vs.* 1/[P], where $R = F/F_i$. [b] The difference between unity and the reciprocal of the y intercept of plots of $1/(1 - R)$ *vs.* 1/[P]. The closeness of the intercepts to unity prevented a more precise determination of f_{PD}/f_D. [c] Average of three separate titrations. [d] Average of two separate titrations.

Discussion

A substantial body of evidence has accumulated indicating that the papain-catalyzed hydrolysis of many *N*-acyl-L-amino acid derivatives proceeds through an acyl-enzyme intermediate (Scheme I) in which the thiol group of Cys-25 is

SCHEME I



acylated by the substrate. (See Lowe, 1970, for a review of some of this evidence.)

At pH 6.0, the rate of hydrolysis of the acyl-enzyme is rate controlling in the papain-catalyzed hydrolysis of BzArgOEt. The rate of hydrolysis of the acyl-enzyme is three- to fivefold slower than the rate of attack of the active-site thiol group on the substrate to form the acyl-enzyme (Whitaker, 1969; Whitaker and Bender, 1965).

Equations 3 and 4 relate the Michaelis–Menten parameters K_M and V_{max} to the individual rate constants of Scheme I.

$$\frac{V_{max}}{[P]_t} = \frac{k_2 k_3}{k_2 + k_3} \tag{3}$$

$$K_M = \frac{k_{-1} + k_2}{k_1} \frac{k_3}{k_2 + k_3} \tag{4}$$

assuming $k_{-1} \gg k_2$, $K_M = K_S [k_3/(k_2 + k_3)]$, where $K_S = k_{-1}/k_1$. For the substrate BzArgOEt, $k_2 > k_3$ and the pro-

flavine-mediated decrease in K_M without a significant effect V_{max} might might be attributed to a proflavine-mediated decrease in K_S and/or increase in k_2.

Equations 5 and 6 relate the dependence of the kinetic parameters V_{max} and K_M on the concentration of proflavine assuming binding of proflavine and substrate is rapid and proflavine binding to the enzyme causes only k_2 to change to k_{2D} (eq 5) or proflavine binding to the enzyme causes only

$$Y = \left(\frac{V_{max}}{K_M V_{max}^0} - \frac{1}{K_M^0}\right)^{-1} = \frac{K_M^0}{[(k_{2D}/k_2) - 1]}\frac{K_D}{[D]} + \frac{K_M^0}{[(k_{2D}/k_2) - 1]} \quad (5)$$

K_S to change to K_{DS} (eq 6). The superscript zeros represent

$$Y = \left(\frac{V_{max}}{K_M V_{max}^0} - \frac{1}{K_M^0}\right)^{-1} = \frac{K_M^0}{[(K_S/(K_{DS}) - 1]}\frac{K_D}{[D]} + \frac{K_M^0}{[(K_S/(K_{DS}) - 1]} \quad (6)$$

values at [D] = 0. Regardless of whether proflavine binding causes an increase in papain's affinity for BzArgOEt or an increase in the rate constant for the acylation step (k_2), a plot of Y *vs.* 1/[D] should yield an x intercept which is the negative reciprocal of K_D. Figure 4 illustrates the determination of K_D suggested by eq 5 and 6. This method for determining K_D is less precise than the fluorometric determination, since small errors in $V_{max}/K_M V_{max}^0$ can result in large errors in Y. The error bars in Figure 4 represent the effect on Y of a 5% error in $V_{max}/K_M V_{max}^0$. The average standard error in this quantity was less than 5%. A regression analysis of the data in Figure 4 yields a value for K_D of 220 μM ± a standard error of 40 μM.

Although this value of K_D is less precise than the value of 210 μM obtained from fluorometric titration, the agreement between these two values is certainly significant. The correspondence between these values strongly suggests that proflavine interacts with papain in a specific manner. The kinetic determination of K_D was carried out at high proflavine concentrations and low enzyme concentrations, whereas the fluorometric determinations were carried out at low proflavine concentrations and high papain concentrations. Thus, nonspecific medium effects caused by increasing the proflavine concentration in the kinetic experiments are an unlikely cause of the proflavine-induced perturbation of K_M. The coincidence of the values of K_D determined fluorometrically and kinetically and the correspondence to eq 2 of papain's perturbation of proflavine fluorescence strongly suggest that self-association of papain, if it occurs, does not affect proflavine binding. It may be argued that the peptide inhibitor, I, did not significantly alter papain–proflavine interactions, because self-association of papain weakens the papain–inhibitor interaction. However, the fact that the papain–proflavine interaction is independent of self-association of papain argues against proflavine binding at an inhibitor site which is destroyed by self-association of papain.

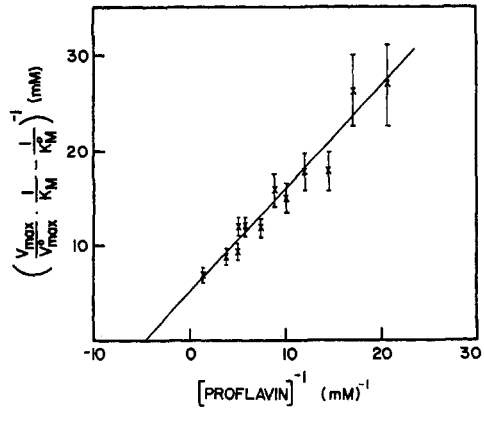


FIGURE 4: Determination of the dissociation constant for the papain–proflavine complex at pH 6.0 from the dependence of V_{max}/K_M on the proflavine concentration.

Papain's and nonactivatable papain's similar affinities for proflavine and their different affinities for the peptide inhibitor (papain, but not nonactivatable papain, binds Sepharose containing covalently linked peptide inhibitor; Blumberg *et al.*, 1970), also lead to the conclusion that the proflavine and peptide binding sites are two distinct sites. These observations strongly suggest that proflavine binding causes a conformational change at the substrate binding site which results in either increased affinity of papain for its substrate or in an increase in a rate constant for a step in papain's catalytic cycle (*e.g.*, acylation). The small increase observed at pH 4, in V_{max} for BzArgOEt hydrolysis at high proflavine concentrations, may be reflecting a proflavine-mediated increase in k_2 coupled with a decrease in the ratio k_2/k_3 on going from pH 6 to 4. As k_2/k_3 decreases, V_{max} becomes sensitive to changes in k_2. From the data of Whitaker and Bender (1965), one can calculate that k_2/k_3 should decrease by about 35% on going from pH 6 to 4. However, a small proflavine-induced increase in k_3 at pH 4 could also be responsible for the observed increase in V_{max}. The proflavine-induced decrease in K_M for BzGlyOEt can also be attributed to an increase in the rate of active thiol attack (of Cys-25) on this ester or an increased affinity of the enzyme for BzGlyOEt on complexing with proflavine. In the case of BzGlyOEt, proflavine appears to cause a slight reduction in the rate constant for the deacylation step, which is reflected in a small proflavine-mediated decrease in V_{max}. It should be noted that the effects of proflavine reported here are different from the effects of small organic molecules like dioxane on the activity of papain reported by Hinkle and Kirsch (1970). These molecules at high concentrations (*e.g.*, 30% dioxane), cause both an increase in the rate constant for deacylation and an increase in K_M (Hinkle and Kirsch, 1970). The fact that at pH 4.0 papain and Hg-papain have similar affinities for proflavine, whereas at pH 6.0 Hg-papain binds proflavine twice as tightly, strongly suggests that in Hg-papain the pK of an ionizable group involved in proflavine binding is perturbed. The small difference in the fluorescence of proflavine bound to Hg-papain and papain may also be indicative of a difference in the environment of the proflavine binding site in these two proteins. Studies of the influence of the state of the active-site thiol group on the conformation of the protein are continuing.

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Effect of Insulin and Growth Hormone on Rat Liver Cyclic Nucleotide Phosphodiesterase†

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ABSTRACT: Rat liver cyclic adenosine monophosphate phosphodiesterase activities, which have apparent K_m values of 6.3×10^{-7} and 7.3×10^{-7} M cyclic adenosine monophosphate, displayed negatively cooperative kinetic behavior, and appeared bound to membrane particles of differing sedimentation rates, were differentiated from cyclic guanosine monophosphate phosphodiesterase activity and lower affinity cyclic adenosine monophosphate phosphodiesterase activity by zonal centrifugation with discontinuous sucrose gradients. Thirty minutes after injection of insulin (3 units/100 g) into streptozotocinized diabetic rats, or of bovine growth hormone (1 mg/100 g) into hypophysectomized rats, the separated membrane-bound high affinity cyclic adenosine monophosphate phosphodiesterases were activated, but there was no effect on the cytosol phosphodiesterases. The activated cyclic adenosine monophosphate phosphodiesterases retained negatively cooperative kinetic behavior and showed no change in the apparent K_m values, but had increased apparent maximum velocities. Neither insulin nor growth hormone had any effect on phosphodiesterase activity *in vitro* under the conditions of these measurements. The results of these investigations support the hypothesis that membrane-bound, high affinity cyclic adenosine monophosphate phosphodiesterase may be involved in the mechanisms of insulin and growth hormone actions.

Recognition of multiple cyclic nucleotide phosphodiesterases and their associated kinetic complexities and substrate specificities (Thompson and Appleman, 1971a,b; Brooker *et al.*, 1968; Kakiuchi *et al.*, 1971; Beavo *et al.*, 1970) necessitates evaluation of hormone interaction with phosphodiesterase activity of enzyme preparations other than that of homogenate or differentially centrifuged enzyme preparations. The high affinity form of cyclic adenosine monophosphate (cyclic AMP[1]) phosphodiesterase activity in several tissues appears to have a subcellular distribution and unique regulatory properties which enable this enzyme to react to changes of substrate level (Thompson and Appleman, 1971b; Russell *et al.*, 1972b). Relatively little is known about hormonal regulation of this form of phosphodiesterase.

Recently, the multiplicity of rat liver cyclic nucleotide phosphodiesterases has been established by enzyme separation techniques and kinetic characterization (Russell *et al.*, 1972a). Previous attempts to characterize rat liver enzyme revealed that liver phosphodiesterase differs in its properties from that of other rat tissues (Beavo *et al.*, 1970; Thompson and Appleman, 1971b; Hemington *et al.*, 1971; Menahan *et al.*, 1969). However, there appears to be a low K_m, negatively cooperative, cyclic AMP phosphodiesterase in liver which may also be a membrane-bound system (Russell *et al.*, 1972a; Hemington *et al.*, 1971).

Insulin stimulation of phosphodiesterase activity has been suggested as a mechanism to account for decreased cyclic

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[1] Abbreviations used are: cyclic AMP, cyclic 3′,5′-adenosine monophosphate; cyclic GMP, cyclic 3′,5;-guanosine monophosphate; iv, intravenous; ip, intraperitoneal.