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Proflavine Interactions with Papain and Ficin. II. Effects of Dye Binding upon Reversible Inhibition†

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ABSTRACT: In the absence of proflavine, competitive inhibition by benzamidoacetonitrile and by the tetrapeptide Gly-Gly-Tyr(Bzl)-Arg was observed for the papain- or ficin-catalyzed hydrolysis of both α-*N*-benzoyl-L-arginine ethyl ester and *N*-carbobenzoxyglycine *p*-nitrophenyl ester. The kinetics of inhibition in the presence of proflavine were examined as a means of probing the possible effects of this dye upon the binding affinities of the papain and ficin active site toward these substrate analog inhibitors, and, presumably, toward substrates as well. With papain, inhibition in the presence of proflavine is again competitive and the kinetically determined inhibition constants remain unchanged. Thus the dye appears not to affect inhibitor or substrate binding with this enzyme. With ficin, the failure of benzamidoacetonitrile to perturb the proflavine–ficin difference spectrum indicates that, as with papain, dye binding and inhibitor binding are mutually independent. However, with ficin in the presence of proflavine noncompetitive or mixed inhibition is observed instead of competitive inhibition. Mechanistic schemes are proposed which are consistent with these findings and provide a reasonable rationale to explain proflavine-induced enhancements of papain and ficin activity.

In the accompanying paper (Hall and Anderson, 1974) it was concluded that the inactivation of papain or ficin in the presence or absence of proflavine by *N*-alkylmaleimides is a two-step process in which the irreversible alkylation of the catalytically essential thiol group is preceded by a reversible binding step in which the maleimide associates with the enzyme active site to form a noncovalent complex. The accelerating effect of proflavine upon these inactivations can be attributed entirely to an enhancement of this reversible binding step. There appears to be no effect of proflavine on the reactivity of the thiol group itself toward alkylation.

As it is currently understood (Glazer and Smith, 1971) the series of events which characterizes the catalytic action of papain and ficin on normal substrates is quite similar to the maleimide inactivation process just described. A reversible enzyme–substrate binding interaction is followed by a nucleophilic attack of the active-site thiol group on the carbonyl group of the scissile ester or amide linkage of the substrate. This results in acylation of the enzyme, presumably in much the same way that thiol attack upon a bound maleimide species results in alkylation. However, acylation by a normal substrate is followed by rapid hydrolytic cleavage of the thiol ester linkage to regenerate active enzyme. No such hydrolytic cleavage of the thiol ether linkage of the alkylated enzyme is possible.

It has already been established that proflavine does not accelerate the deacylation step of papain or ficin catalysis (Hollaway, 1968; Hall *et al.*, 1972; Skalski *et al.*, 1973). But proflavine does indeed accelerate papain- and ficin-catalyzed hydrolyses of certain simple ester substrates, so the question arises: can these effects be attributed to an enhanced enzyme–substrate binding interaction such as that which characterizes maleimide inactivation, or to an enhanced acylation rate? Some kinetic data have been interpreted to imply that with papain the observed activation by proflavine is the result of enhanced substrate binding (Hall *et al.*, 1972). On the other hand, the proflavine effect upon ficin catalysis appears to be due to an enhanced acylation rate (Hollaway, 1968), quite in contrast to the effect of proflavine on the maleimide inactivation of ficin.

Unfortunately, the relative magnitudes of the individual rate constants for the acyl-enzyme mechanism which characterizes papain or ficin catalysis often make it impossible to determine dissociation constants (K_s) for enzyme–substrate complexes from steady-state kinetic data. However, one can obtain an estimation of the net binding affinity of papain or ficin toward substrates by determining dissociation constants (K_i) for complexes of these enzymes with substrate analogs which are competitive inhibitors (see, for example, Williams *et al.* (1972)). These competitive inhibition constants (K_i) are of course readily obtained from steady-state kinetic data (Webb, 1963).

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TABLE I: Observed Michaelis–Menten Parameters for Papain-Catalyzed Hydrolysis in the Presence and Absence of Proflavine and/or Inhibitors.[a]

Substrate (Inhibitor)	[Proflavine] (mM)	[Inhibitor] (mM)	$K_{m(app)}$ (mM)	k_{cat} (sec^{-1})
BzArgOEt[b] (benzamidoacetonitrile)	0	0	17.3 ± 0.3	23.1 ± 0.3
	0.10	0	8.7 ± 0.8	23.4 ± 1.2
	0	0.10	26.8 ± 0.7	20.4 ± 0.4
	0	0.25	39.5 ± 2.2	19.3 ± 0.9
	0.10	0.10	13.3 ± 0.3	20.3 ± 0.3
	0.10	0.25	21.0 ± 0.6	20.6 ± 0.5
BzArgOEt[c] (Gly-Gly-Tyr(Bzl)-Arg)	0	0	17.9 ± 0.7	20.9 ± 0.7
	0.10	0	8.7 ± 0.4	22.5 ± 0.7
	0	0.43	37.2 ± 5.1	22.8 ± 2.7
	0	0.85	44.8 ± 3.6	21.2 ± 1.5
	0.10	0.43	16.2 ± 0.7	23.3 ± 0.7
	0.10	0.85	24.3 ± 1.2	23.5 ± 0.9
CbzGlyONPh[d] (benzamidoacetonitrile)	0	0	0.0044 ± 0.0002	5.54 ± 0.02
	0.10	0	0.0047 ± 0.0002	5.52 ± 0.03
	0	1.04	0.0229 ± 0.0005	5.61 ± 0.07
	0.10	1.04	0.0225 ± 0.0009	5.55 ± 0.07
BzArgPNA[e]	0		6.8 ± 1.7	0.61 ± 0.15
	0.10		4.5 ± 2.5	0.35 ± 0.18

[a] pH 6.60, 0.3 M ionic strength (added KCl), 25°. All initial velocities determined in duplicate at five substrate concentrations in the presence of 1.0 mM EDTA and thiol activator. [b] Initial velocities determined by pH-Stat titration with $[S]_0$ = 4.0–50.0 mM, 0.17% acetonitrile, 5 mM cysteine, and $[E]_0$ = 1.17 μM. [c] Initial velocities determined by pH-Stat titration with $[S]_0$ = 4.0–50.0 mM, 5 mM cysteine, and $[E]_0$ = 1.14 μM. [d] Initial velocities determined spectrophotometrically (400 nm in 0.02 M phosphate buffer, 6.9% acetonitrile) with $[S]_0$ = 0.02 – 0.10 mM, 0.33 mM cysteine, and $[E]_0$ = 0.0922 μM. [e] Initial velocities determined spectrophotometrically (410 nm in 0.10 M phosphate buffer, 5% dimethyl sulfoxide) with $[S]_0$ = 0.18–0.89 mM, 5 mM mercaptoethanol, and $[E]_0$ = 0.761 μM.

Thus, as an indirect means of determining the effects of proflavine upon the binding affinity of papain and ficin toward substrates, we have in this investigation sought to determine the effects of the dye on kinetically determined competitive inhibition constants.

Experimental Section

Materials. Papain (EC 3.4.4.10), recrystallized in the presence of mercury and supplied as a suspension in 70% ethanol, was purchased from Worthington Biochemical Corp. It was further purified by affinity chromatography according to the method of Blumberg *et al.* (1970) and stored as mercuripapain. A detailed description of this purification procedure has recently been published (Furlanetto and Kaiser, 1973). Ficin (EC 3.4.22.3) was obtained from a crude fig latex preparation supplied by Sigma Chemical Co. and purified by the method of Englund *et al.* (1968) as described in the accompanying paper (Hall and Anderson, 1974).

The substrates BzArgOEt,[1] Cbz-GlyONPh, and BzArgPNA were obtained from Sigma Chemical Co. Benzamidoacetonitrile was prepared as described by Klages and Haack (1903), and the tetrapeptide Gly-Gly-Tyr(Bzl)-Arg·2HCl was synthesized by the method of Blumberg *et al.* (1970). Cysteine and proflavine were purchased from Mann Laboratories, Inc., and EDTA (the disodium salt) from Sigma Chemical Co. Buffer salts and organic solvents were of reagent grade and glass-distilled water was used throughout. Titrants used in pH-Stat titrations were Fisher Certified standard sodium hydroxide solutions.

Methods. BzArgOEt hydrolyses were monitored by pH-Stat titration using Radiometer equipment as described previously (Hall *et al.*, 1972). Hydrolyses of Cbz-GlyONPh and BzArgPNA were followed spectrophotometrically using a Cary 14 spectrophotometer as described for these substrates by Kirsch and Igelstrom (1966) and by Horton (1973), respectively. Enzyme concentrations were determined from the 280-nm absorbance of stock solutions employing the values of ϵ_{280} 5.78×10^4 M^{-1} cm^{-1} (Husain and Lowe, 1969) for papain and ϵ_{280} 5.2×10^4 M^{-1} cm^{-1} (Englund *et al.*, 1968) for ficin.

In all kinetic experiments, initial velocities were found to obey Michaelis–Menten kinetics (eq 1) as indicated by the linearity of double reciprocal plots according to the Lineweaver–Burk expression (eq 2). The parameters k_{cat} and

$$v_0 = \frac{k_{cat}[E]_0[S]_0}{K_{m(app)} + [S]_0} \quad (1)$$

$$\frac{[E]_0}{v_0} = \frac{K_{m(app)}}{k_{cat}}\frac{1}{[S]_0} + \left(\frac{1}{k_{cat}}\right) \quad (2)$$

$K_{m(app)}$ with their standard error estimates were calculated from the slopes and intercepts of computer-generated least-squares fits of initial rate data to eq 2.

Results

Hydrolyses of three substrates by each enzyme were studied in the presence and absence of inhibitors, both with and without added proflavine. The results are recorded in Table I and

[1] Abbreviations used are: BzArgOEt, α-*N*-benzoyl-L-arginine ethyl ester; Cbz-GlyONPh, carbobenzoxyglycine *p*-nitrophenyl ester; BzArg-PNA, α-*N*-benzoyl-DL-arginine *p*-nitroanilide; Gly-Gly-Tyr(Bzl)-Arg, glycylglycyl-*O*-benzyl-L-tyrosyl-L-arginine.

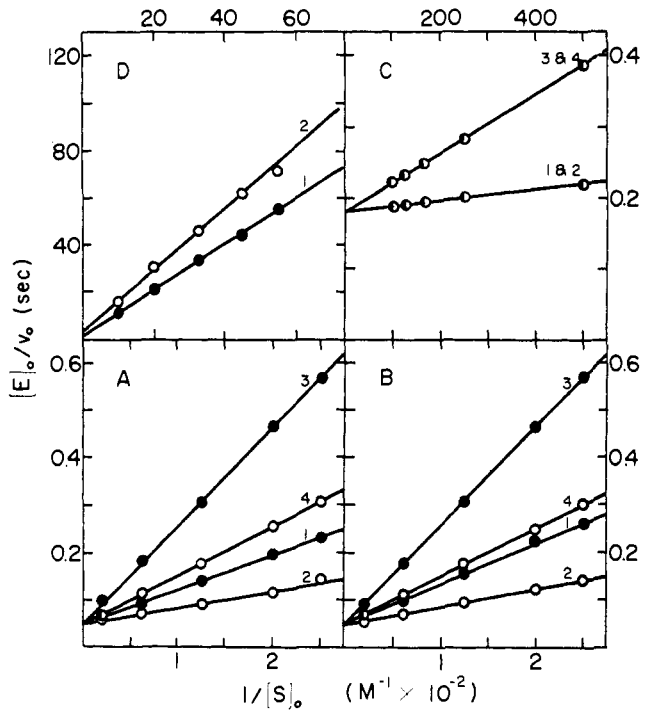


FIGURE 1: The effects of inhibitors upon papain catalysis in the presence (O), or absence (●), or presence and absence (◐) of proflavine: (A) BzArgOEt and benzamidoacetonitrile; (B) BzArgOEt and Gly-Gly-Tyr(Bzl)-Arg; (C) Cbz-GlyONPh and benzamidoacetonitrile; (D) BzArgPNA. In each set of plots, lines 1 and 2 represent initial velocities in the absence of inhibitor, and lines 3 and 4 represent initial velocities in the presence of inhibitor. For Figures 1A and 1B, lines 3 and 4 represent the higher of two inhibitor concentrations cited in Table I. Conditions are otherwise as noted in Table I.

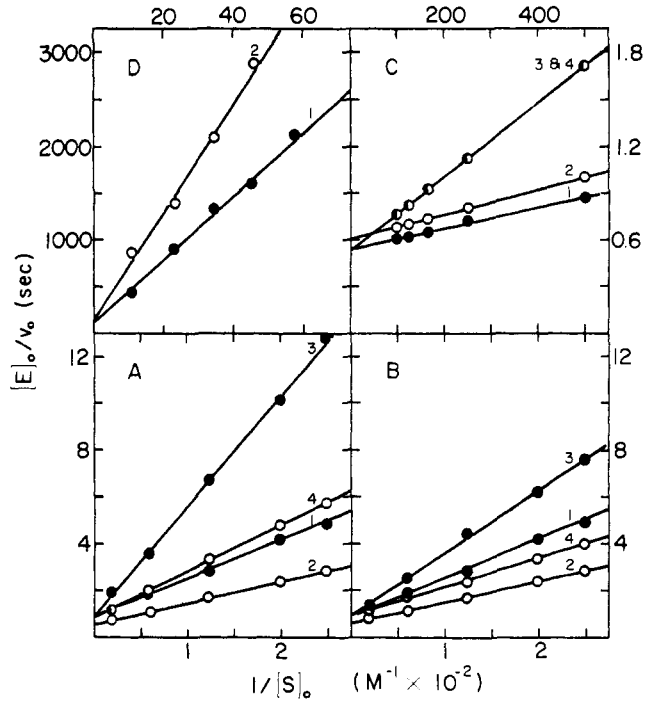


FIGURE 2: The effects of inhibitors upon ficin catalysis in the presence (O), or absence (●), or presence and absence (◐) of proflavine: (A) BzArgOEt and benzamidoacetonitrile; (B) BzArgOEt and Gly-Gly-Tyr(Bzl)-Arg; (C) Cbz-GlyONPh and benzamidoacetonitrile; (D) BzArgPNA. In each set of plots, lines 1 and 2 represent initial velocities in the absence of inhibitor, and lines 3 and 4 represent initial velocities in the presence of inhibitor. Conditions are as noted for Table II.

Figure 1 for papain, and in Table II and Figure 2 for ficin. The substrates employed were α-*N*-benzoyl-L-arginine ethyl ester (BzArgOEt), *N*-carbobenzoxyglycine *p*-nitrophenyl ester (Cbz-GlyONPh), and α-*N*-benzoyl-DL-arginine *p*-nitroanilide (BzArgPNA). Two inhibitors, both used previously in papain studies but neither of which has been previously reported as an inhibitor of ficin catalysis, were used in this investigation. They were benzamidoacetonitrile (Lucas and Wllliams, 1969) and the tetrapeptide derivative Gly-Gly-Tyr(Bzl)-Arg·2HCl (Blumberg *et al.*, 1970).

In the absence of proflavine, both benzamidoacetonitrile and Gly-Gly-Tyr(Bzl)-Arg appear from the Lineweaver–Burk plots of Figures 1 and 2 to behave as competitive inhibitors for the papain- or ficin-catalyzed hydrolyses of the esters BzArgOEt or Cbz-GlyONPh. Also, Dixon plots (Webb, 1963) for inhibition of papain- or ficin-catalyzed hydrolysis of BzArgOEt by benzamidoacetonitrile are linear, indicating that benzamidoacetonitrile inhibition is fully competitive under the conditions of this investigation. However, inhibition by the peptide Gly-Gly-Tyr(Bzl)-Arg may not be fully competitive at pH 6.6, particularly in the case of papain for which the Dixon plot shows a definite departure from linearity.

Inhibitor constants (K_i) for benzamidoacetonitrile and Gly-Gly-Tyr(Bzl)-Arg in the absence of proflavine were calculated directly from the relative slopes of the appropriate Lineweaver–Burk plots using eq 3. The results are tabulated in Table III, and the K_i values for benzamidoacetonitrile and Gly-Gly-Tyr(Bzl)-Arg as competitive inhibitors of papain catalysis are in reasonable agreement with previously published values (Sluyterman and Wijdenes, 1973; Lucas and Williams, 1969; Blumberg *et al.*, 1970) when allowances are made for differences in experimental conditions.

$$K_i = \frac{[\text{inhibitor}]}{\left(\frac{\text{slope with inhibitor}}{\text{slope without inhibitor}}\right) - 1} \qquad (3)$$

The amide BzArgPNA is a relatively poor substrate for papain and ficin, and Michaelis–Menten parameters for its hydrolysis by these enzymes are difficult to determine with precision due to large uncertainties in the magnitude of the ordinate intercept in Lineweaver–Burk plots. However, a qualitative inspection of the data in Figures 1D and 2D reveals that proflavine is an inhibitor rather than an accelerator of the papain- or ficin-catalyzed hydrolysis of BzArgPNA.

In the presence of proflavine, competitive inhibition by benzamidoacetonitrile and by Gly-Gly-Tyr(Bzl)-Arg in the papain-catalyzed hydrolysis of both ester substrates is again observed. Furthermore, the K_i values obtained in the presence of proflavine are very closely similar to those obtained in the absence of dye (compare Table IV with the data for papain in Table III). Thus there is no indication that proflavine binding to papain results in any perturbation of the binding affinity of the papain active site toward these inhibitors.

With ficin, on the other hand, inhibition of ester hydrolysis in the presence of proflavine is no longer competitive, but rather may tentatively be characterized as noncompetitive inhibition or perhaps mixed inhibition. Due to the indeterminate character of this inhibition in the presence of proflavine, no attempt was made to calculate inhibitor binding constants from these data for comparison with corresponding constants in the absence of proflavine. However, nonkinetic evidence was obtained which indicates that with ficin as with papain, proflavine binding and inhibitor binding are in fact mutually indepen-

TABLE II: Observed Michaelis–Menten Parameters for Ficin-Catalyzed Hydrolysis in the Presence and Absence of Proflavine and/or Inhibitors.[a]

Substrate (Inhibitor)	[Proflavine] (mM)	[Inhibitor] (mM)	$K_{m(app)}$ (mM)	k_{cat} (sec^{-1})
BzArgOEt[b] (benzamidoacetonitrile)	0	0	21.3 ± 0.9	1.29 ± 0.05
	0.10	0	19.7 ± 1.1	2.11 ± 0.09
	0	1.00	65.3 ± 1.2	1.38 ± 0.23
	0.10	1.00	26.6 ± 0.9	1.34 ± 0.04
BzArgOEt[c] (Gly-Gly-Tyr(Bzl)-Arg)	0	0	21.3 ± 0.9	1.29 ± 0.05
	0.10	0	19.7 ± 1.1	2.11 ± 0.09
	0	3.40	36.8 ± 2.4	1.35 ± 0.08
	0.10	3.40	16.7 ± 1.1	1.32 ± 0.06
CbzGlyONPh[d] (benzamidoacetonitrile)	0	0	0.0125 ± 0.0006	1.85 ± 0.02
	0.10	0	0.0131 ± 0.0003	1.67 ± 0.01
	0	5.21	0.0432 ± 0.0012	1.87 ± 0.03
	0.10	5.21	0.0412 ± 0.0015	1.80 ± 0.03
BzArgPNA[e]	0		3.3 ± 3.6	0.0096 ± 0.0097
	0.10		4.8 ± 8.5	0.0083 ± 0.0139

[a] pH 6.60, 0.3 M ionic strength (added KCl) (0.1 M ionic strength for BzArgOEt substrate), 25°. All initial velocities determined in duplicate at five substrate concentrations. Ficin preactivated for 30 min with thiol activator. [b] Initial velocities determined by pH-Stat titration with $[S]_0$ = 4.0–50.0 mM, 0.67% acetonitrile, 0.07 mM EDTA, 1.0 mM cysteine, and $[E]_0$ = 13.8 μM. [c] Initial velocities determined by pH-Stat titration with $[S]_0$ = 4.0–50.0 mM, 0.07 mM EDTA, 1.0 mM cysteine, and $[E]_0$ = 13.8 μM. [d] Initial velocities determined spectrophotometrically (400 nm in 0.02 M phosphate buffer, 10.8% acetonitrile) with $[S]_0$ = 0.02–0.10 mM, 0.03 mM cysteine, 1.0 mM EDTA, and $[E]_0$ = 0.283 μM. [e] Initial velocities determined spectrophotometrically (410 nm in 0.10 M phosphate buffer, 5% dimethyl sulfoxide) with $[S]_0$ = 0.18–0.88 mM, 5 mM mercaptoethanol, 0.1 mM EDTA, and $[E]_0$ = 91.3 μM.

TABLE III: Competitive Inhibition of Papain and Ficin in the Absence of Proflavine.[a]

Enzyme	Inhibitor (concn, mM)	Substrate	K_i (mM)
Papain	Benzamidoacetonitrile (0.10)	BzArgOEt	0.132 ± 0.001
	(0.25)	BzArgOEt	0.145 ± 0.002
	(1.04)	Cbz-GlyONPh	0.246 ± 0.014
	Peptide[b] (0.43)	BzArgOEt	0.471 ± 0.014
	(0.85)	BzArgOEt	0.582 ± 0.012
Ficin	Benzamidoacetonitrile (1.00)	BzArgOEt	0.536 ± 0.014
	(5.21)	Cbz-GlyONPh	2.15 ± 0.11
	Peptide[b] (3.4)	BzArgOEt	5.28 ± 0.11

[a] Conditions as described in Tables I and II. [b] The peptide inhibitor is Gly-Gly-Tyr(Bzl)-Arg.

dent. It was found that the difference spectrum in the visible absorption band of proflavine which characterizes the interaction between ficin and the dye (Hollaway, 1968) is not detectably perturbed by the inhibitor benzamidoacetonitrile, even with the highest attainable concentrations of inhibitor and enzyme.[2]

Discussion

In an earlier paper (Hall *et al.*, 1972) arguments were advanced to support the hypothesis that the rate-enhancing effects of proflavine on the papain-catalyzed hydrolysis of certain simple ester substrates such as BzArgOEt could be accounted for in terms of dye-induced substrate-binding enhancements. However, the findings reported in this paper lead

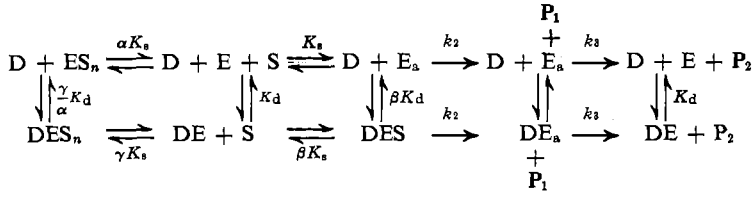
TABLE IV: Competitive Inhibition of Papain in the Presence of 0.10 mM Proflavine.[a]

Inhibitor (concn, mM)	Substrate	K_i (mM)
Benzamidoacetonitrile (0.10)	BzArgOEt	0.131 ± 0.006
(0.25)	BzArgOEt	0.143 ± 0.006
(1.04)	Cbz-GlyONPh	0.273 ± 0.018
Peptide[b] (0.43)	BzArgOEt	0.484 ± 0.015
(0.85)	BzArgOEt	0.511 ± 0.015

[a] Conditions as described in Table I. [b] The peptide inhibitor is Gly-Gly-Tyr(Bzl)-Arg.

[2] Identical split-compartment cuvets, each containing proflavine (100 μM) and a selected concentration of ficin (0–118 μM) in one compartment and an equal volume of 8000 μM benzamidoacetonitrile in the other, were placed in the reference and sample beams of the Cary 14 spectrophotometer. Spectra were recorded before and after mixing the contents of the sample cell. The difference spectra so obtained were identical with those obtained in a control set of experiments in which the benzamidoacetonitrile was omitted.

SCHEME I



to the conclusion that proflavine does *not* perturb the affinity of papain toward compounds which are both structural analogs of papain substrates and competitive inhibitors of the papain-catalyzed hydrolysis of BzArgOEt and other ester substrates. Since this conclusion implies that net substrate binding to papain is itself independent of dye–enzyme interaction, alternatives to the rationale advanced earlier must be considered.

One alternative scheme is that advanced by Hollaway (1968) to account for the effects of proflavine he observed upon ficin-catalyzed BzArgOEt hydrolysis. In that scheme, it was assumed that proflavine binding results in an enhancement of the microscopic rate constant for acylation in the three-step acyl-enzyme pathway generally thought to constitute a minimally adequate representation of ficin (or papain) catalysis in the absence of added effectors (Glazer and Smith, 1971). Arguments can be constructed which demonstrate that this scheme of Hollaway (1968) can accommodate most of our kinetic data on papain- or ficin-catalyzed BzArgOEt hydrolysis. However, the scheme is not consistent with our data on other substrates. Furthermore, we have found (Hall and Anderson, 1974) that the active-site thiol group of papain or ficin, which is assumed to be the attacking nucleophile in the acylation step of catalysis by these enzymes, is no more reactive in the presence of proflavine toward alkylation by *N*-alkylmaleimides than it is in the absence of the dye.

The scheme which we now propose as a viable working hypothesis to account for the effects of proflavine on both ficin and papain catalysis focuses upon the probability that substrates can bind at the active site of papain (or presumably ficin as well) either productively or nonproductively (Brocklehurst *et al.*, 1968; Hinkle and Kirsch, 1971). It is conceivable that without perturbing the *absolute* affinity of the papain or ficin active site for certain substrates (or competitive inhibitors), dye binding might change the *ratio* of productive to nonproductive substrate binding at that site. In terms of Scheme I, this situation may be expressed by the equality $(1 + 1/\alpha) = (1/\beta + 1/\gamma)$.

The steady-state Michaelis–Menten parameters for Scheme I, assuming that all reversible steps prior to the acylation step represent equilibria and that $(1 + 1/\alpha) = (1/\beta + 1/\gamma)$, are defined in eq 4–6. Clearly, the net effect of the presence of dye in Scheme I is to perturb the *apparent* acylation rate constant $k_{2(app)}$. Thus Scheme I is kinetically equivalent to the simpler scheme proposed by Hollaway (1968).

$$k_{2(app)} = k_2(1 + ([D]/\beta K_d))/(1 + (1/\alpha))(1 + ([D]/K_d)) \quad (4)$$

$$k_{cat} = k_{2(app)}k_3/(k_{2(app)} + k_3) \quad (5)$$

$$K_{m(app)} = K_s k_3/(1 + (1/\alpha))(k_{2(app)} + k_3) \quad (6)$$

Two limiting cases must be considered in accounting for the kinetics of BzArgOEt hydrolysis by ficin on the one hand and by papain on the other. Ficin catalyzes BzArgOEt hydrolysis with rate-limiting acylation (Hollaway, 1968). In terms of Scheme I this corresponds to the condition, $k_{2(app)} \ll k_3$, so that eq 5 simplifies to $k_{cat} = k_{2(app)}$ and eq 6 to $K_{m(app)} = K_s/(1 + 1/\alpha)$. It is apparent that this limiting condition implies an observable effect of dye concentration upon k_{cat}, but no such effect upon $K_{m(app)}$, which in fact is the result obtained (Table II). Conversely, for the case in which the deacylation step of Scheme I is rate-limiting ($k_{2(app)} \gg k_3$), eq 5 simplifies to $k_{cat} = k_3$ and eq 6 to $K_{m(app)} = (K_s k_3/k_{2(app)})/(1 + 1/\alpha)$. In this second limiting case, dye concentration will have no observable effect upon k_{cat}, but $K_{m(app)}$ will be affected. This is just the sort of behavior observed for the papain-catalyzed hydrolysis of BzArgOEt in the presence and absence of proflavine. The data of Table I show that a concentration of proflavine (0.10 mM) approximately equal to its dissociation constant K_d (0.108 ± 0.039 mM, see the accompanying paper) is just sufficient to reduce the value of $K_{m(app)}$ for BzArgOEt hydrolysis by a factor of 2. Within the experimental uncertainty of our determinations, this dye concentration effects no change in k_{cat}; however, a dye-induced increase of less than 10% in k_{cat} might not be detected experimentally.

It has been determined under conditions quite similar to those under which our experiments were conducted that deacylation is only marginally rate limiting in the papain-catalyzed hydrolysis of BzArgOEt, with a k_2/k_3 ratio of approximately 3 (Whitaker and Bender, 1965) to 5 (Whitaker, 1969). But using eq 4–6 with $[D]/K_d = 1$ and $k_{2(app)}/k_3 = 5$, it may be calculated that if $1/\beta = 3.4$ (regardless of the relative values of α and γ) one would in fact observe a twofold reduction in $K_{m(app)}$ accompanied by an increase in k_{cat} by a factor of just 1.1. Thus our BzArgOEt data are consistent with Scheme I.

We turn now to our observation that benzamidoacetonitrile and Gly-Gly-Tyr(Bzl)-Arg, which are simple competitive inhibitors of ficin catalysis in the absence of proflavine, give rise to a more complex apparently noncompetitive or mixed inhibition in the presence of the dye. This unanticipated observation cannot be attributed to any interdependence between proflavine binding and net inhibitor binding since benzamidoacetonitrile fails to perturb ficin–proflavine difference spectra. We therefore speculate that the interaction of proflavine with ficin makes it possible for the enzyme to bind substrate and inhibitor simultaneously to give a quaternary DEIS complex which either does not lead to products at all (noncompetitive inhibition) or undergoes acylation much more slowly than the DES complex (mixed inhibition). Scheme I can be expanded to represent this situation with the inclusion of EI, DEI, DEIS, and DE_aI complexes. Somewhat unwieldy steady-state kinetic expressions corresponding to eq 4–6 can be derived for this expanded scheme. Although these expressions are too complex to allow evaluation of individual microscopic kinetic constants, they do show that only when acylation is kinetically rate limiting (*i.e.*, when $k_{2(app)} \ll k_3$ as in the case of ficin-catalyzed BzArgOEt hydrolysis) will noncompetitive or mixed inhibition actually be observed in the presence of dye. In cases where deacylation is rate limiting as in the papain–BzArgOEt case, $k_{cat} = k_3$ whether or not dye and/or inhibitor are present and only $K_{m(app)}$ will reflect the presence of inhibitor, giving rise to an apparent competitive inhibition with an observed K_i value (eq 3) which is independent of dye concentration.

Thus, although our papain data do not in themselves warrant the consideration of a quaternary DEIS complex for this enzyme in the presence of both dye and inhibitor, the overall similarity between papain and ficin suggests that the same kinetic scheme might well apply to both enzymes.

So far in our discussion we have considered only the data for BzArgOEt hydrolysis. Although our data for other substrates are much more limited than those for BzArgOEt and warrant no detailed analysis, it is clear that the nature of the proflavine effects varies considerably from one substrate to another. This is what one would expect if these effects are indeed attributable to productive *vs.* nonproductive substrate binding, or even multiple substrate binding, the qualitative as well as quantitative nature of which should be a sensitive function of variations in substrate structure. In the particular case of the anilide substrate BzArgPNA, whose papain- or ficin-catalyzed hydrolysis is inhibited rather than accelerated by proflavine, it may be significant to note that BzArgPNA hydrolysis by papain may not even follow the same mechanism which characterizes ester hydrolysis (Mole and Horton, 1973). In any case it seems likely that the binding interactions between papain or ficin and simple substrates and inhibitors are complex, and that in slightly altering enzyme conformation, proflavine can perturb these binding interactions in different ways and to different extents depending upon substrate structure.

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