# Proflavine Interactions with Papain and Ficin. I. Dye Binding and Its Effects upon Enzyme Inactivation by *N*-Alkylmaleimides†

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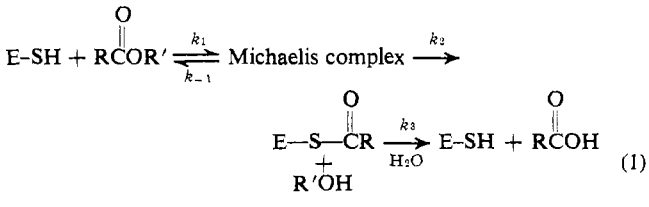
ABSTRACT: The acridine dye proflavine associates with papain or ficin at a hydrophobic binding site to produce a 1:1 dye–enzyme complex. Spectrophotometric titration of the dye by these enzymes at pH 6.6 yields dissociation constant values of 0.109 ± 0.039 and 1.33 ± 0.70 mM for these complexes with papain and ficin, respectively. This dye binding, which has previously been shown to result in enhancements of catalytic activity for papain and ficin, also enhances the rates at which these enzymes are inactivated by various *N*-alkylmaleimides. The kinetics of enzyme inactivation by *N*-ethylmaleimide in the presence and absence of proflavine indicate that the dye increases the noncovalent reversible binding affinity of papain or ficin toward the maleimides without affecting the nucleophilic reactivity of the catalytically essential thiol groups which are alkylated in the inactivation reaction. The implications of these findings as they concern the effects of proflavine on the catalytic activity of papain and ficin are discussed.

Previous investigations have established that proflavine (3,6-diaminoacridine bisulfate) interacts with papain (EC 3.4.4.10) and ficin (EC 3.4.22.3) in such a way as to give rise to apparent enhancements in the catalytic activity of these two sulfhydryl proteases toward certain synthetic ester substrates (Hall *et al.*, 1972; Skalski *et al.*, 1973; Hollaway, 1968). Although catalytic properties and the mechanism of action have been less extensively investigated for ficin than for papain, to the extent that comparisons have been made, it is clear that these two enzymes are very similar (Glazer and Smith, 1971). Thus it is not surprising that proflavine and other acridine dyes produce qualitatively similar effects with both enzymes. Consider for example the data of Table I where the effects of three acridine dyes upon papain- and ficin-catalyzed hydrolyses of the common ester substrate BzArgOEt[1] are compared. However, a comparison of the kinetics which characterize the effects of proflavine upon papain activity with those which characterize the effects of this dye upon ficin activity has led to the conclusion that the mechanisms accounting for dye-induced activity enhancement may be different for the two enzymes (Hall *et al.*, 1972).

More specifically, if it is assumed that papain or ficin action is characterized by a three-step "acyl-enzyme" scheme as indicated by eq 1 (Lowe, 1960; Glazer and Smith, 1971), the




(1)

conclusion can be reached that with ficin, proflavine binding results in an enhancement of active-site thiol reactivity, giving rise to an increase in the acylation rate constant $k_2$ (Hollaway, 1968). Yet with papain, the proflavine effect could be due instead to an enhancement of the initial binding interaction between the enzyme and the substrate; that is, to an increase in the ratio $k_1/k_{-1}$ (Hall *et al.*, 1972). For reference, the steady-state Michaelis–Menten parameters are defined in terms of the rate constants of eq 1 in eq 2 and 3.

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \tag{2}$$

$$K_{m(app)} = \left(\frac{k_{-1} + k_2}{k_1}\right)\left(\frac{k_3}{k_2 + k_3}\right) = \left(\frac{k_{cat}}{k_1}\right)\left(\frac{k_{-1}}{k_2} + 1\right) \tag{3}$$

In efforts to further elucidate this possible discrepancy between the catalytic properties of papain and ficin and to obtain clearer insight into the nature of the interactions of these enzymes with proflavine, we have investigated the binding of proflavine to both enzymes and the effects of this binding upon the nucleophilic reactivity of the active-site thiol group as reflected by rates of inactivation using *N*-alkylmaleimides as thiol-specific alkylating agents. The results of that investigation are reported in this paper. The accompanying paper (Hall and Anderson, 1974) reports studies of the effects of proflavine on competitive inhibition of papain- and ficin-catalyzed hydrolyses.

## Experimental Section

*Materials.* Twice crystallized papain (EC 3.4.4.10) as a suspension in 0.05 M acetate buffer (pH 4.5) and a crude ficin (EC 3.4.22.3) preparation from fig tree latex were obtained from Sigma Chemical Co. Sigma was also the supplier of EDTA (disodium salt) and the substrates BzArgOEt and Cbz-GlyONPh. L-Cysteine (free base) and proflavine sulfate were purchased from Mann Research Laboratories. *N*-Ethylmaleimide was obtained from Eastman Organic Chemicals and *N*-butylmaleimide from Nutritional Biochemical Corp. *N*-Hexyl-, *N*-heptyl-, *N*-octyl-, and *N*-decylmaleimides,

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[1] Abbreviations used are: BzArgOEt, α-*N*-benzoyl-L-arginine ethyl ester; Cbz-GlyONPh, *N*-carbobenzoxyglycine *p*-nitrophenyl ester.

TABLE 1: Effects of Saturating Concentrations of Acridine Dyes upon the Rates of Hydrolysis of BzArgOEt by Papain and Ficin.[a]

| Acridine Dye | % Activity $[(v_{dye}/v_0) \times 10^2]$ | |
|---|---|---|
| | Papain | Ficin |
| Proflavine | 145 | 325 |
| 10-Methylproflavine | 135 | 212 |
| 9-Aminoacridine | 122 | 150 |

[a] pH 6.6, ionic strength 0.3 M (added KCl), 5 mM cysteine, 1 mM EDTA, 25°. Per cent activity figures represent titrimetrically determined rate ratios in the presence and absence of 2 mM dye at constant substrate concentration (24 mM) and constant enzyme concentration ($1.2 \times 10^{-6}$ M papain or $1.0 \times 10^{-5}$ ficin).

prepared according to Heitz *et al.* (1968), were the generous gift of Dr. B. M. Anderson. CM-Cellulose (Cellex CM) was obtained from Bio-Rad Laboratories, and sodium tetrathionate from Pfaltz and Bauer, Inc. Buffer salts, KCl, NaCl, KCN, and NaCN, and organic solvents were reagent grade products meeting ACS standards of purity. Glass-distilled water was used exclusively.

*Methods.* Maleimide inactivation studies on papain and ficin were carried out essentially as described by Anderson and Vasini (1970). Papain at a concentration of 0.033 mg/ml ($1.4 \times 10^{-6}$ M based on a molecular weight for papain of 23,350 (Wolthers *et al.*, 1970)) was activated by incubation at 37° for 1 hr in a 0.22 M potassium phosphate buffer solution (pH 6.8) containing 5 mM KCN and 7 mM EDTA. This papain stock solution, chilled in ice, maintained constant activity toward the assay substrate Cbz-GlyONPh for a period of at least 6 hr. Ficin, isolated from the crude commercial fig latex preparation by CM-cellulose chromatography in the presence of sodium tetrathionate and further purified by rechromatography on CM-cellulose as described by Englund *et al.* (1968), was freed of excess sodium tetrathionate by dialysis overnight against 5 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA. The freshly dialyzed ficin was then activated in the same manner described for papain except that 2.5 hr of incubation at 37° was required before maximal activity was attained. The resulting active ficin stock solutions (enzyme concentration 0.061 mg/ml or $2.5 \times 10^{-6}$ M based on a molecular weight for ficin of 25,000 (Englund *et al.*, 1968)) maintained constant activity for well over 6 hr.

Maleimide stock solutions were prepared fresh daily in 2% aqueous ethanol and their concentrations were determined spectrophotometrically ($\epsilon_{300\ nm}$ 620 $M^{-1}$ $cm^{-1}$ (Gregory, 1955)). Maleimide inactivation experiments were conducted at 25° in 2-ml reaction mixtures containing 0.12 M potassium or sodium phosphate buffer (pH 6.8), 0.017 mg/ml of papain or 0.031 mg/ml of ficin, 0.5% ethanol, and *N*-alkylmaleimide in the presence or absence of $5 \times 10^{-5}$ M proflavine. (Proflavine stock solutions were prepared fresh daily and their concentrations determined spectrophotometrically using $\epsilon_{445\ nm}$ $3.5 \times 10^4$ $M^{-1}$ $cm^{-1}$ (Millich and Oster, 1959; Glazer, 1965).) Inactivations were initiated by introduction of the *N*-alkylmaleimide solution, and the time course of the inactivation was monitored by rate assays of papain or ficin activity remaining in 0.2-ml aliquots withdrawn at timed intervals from the reaction mixture. The spectrophotometric assay of Kirsch and Igelstrom (1966) using Cbz-GlyONPh as the substrate was employed for both papain and ficin. Assays were conducted at 25° on a Beckman Acta III spectrophotometer monitoring the release of *p*-nitrophenolate at 400 nm. Assay mixtures contained 0.012 M potassium phosphate buffer (pH 6.8), $1 \times 10^{-4}$ M substrate, 0.0017 mg/ml of total papain or 0.0031 mg/ml of total ficin, 0.05% ethanol, and 0.67% acetonitrile in a total volume of 3 ml.

Measurements of difference spectra were made essentially as described by Hollaway (1968) using split-compartment cells (Pyrocell Mfg. Co.) and a Cary 14 spectrophotometer. As a precaution against possible fluorescence errors the spectrophotometer was operated at a constant slit width and the reference cuvet contained solutions identical with the sample cuvet before mixing.

All pH measurements and pH-Stat titrations were made using a Radiometer PHM-26 pH meter equipped with a Radiometer GK2321-C combination electrode and interfaced with a Radiometer TTT-11 titrator, SBR2 recorder, and SBU1 syringe buret. The reaction vessel was thermostated at 25.0 ± 0.1° with water from a constant-temperature circulator (Haake).

## Results

*Effects of Proflavine on Maleimide Inactivation of Papain and Ficin.* Incubation of cyanide-activated papain or ficin at 25°, pH 6.8, in the presence or absence of $5 \times 10^{-5}$ M proflavine with *N*-alkylmaleimides at concentrations well in excess of the initial active enzyme concentrations results in losses of enzymic activity which follow straightforward pseudo-first-order kinetics. When the observed pseudo-first-order rate constants for the inactivation of papain or ficin with *N*-ethylmaleimide in the presence and absence of $5 \times 10^{-5}$ M proflavine are plotted as a function of maleimide concentration, it is apparent that proflavine enhances the rates of *N*-ethylmaleimide inactivation of both enzymes. But it is also apparent that at relatively high concentrations of the maleimide, the observed rate constants bear a distinctly nonlinear relationship to the maleimide concentration, implying that the inactivation is not a simple second-order kinetic process. Indeed, the data suggest that saturation of the enzyme by reversibly bound maleimide is approached at high concentrations of the inactivator. The simplest mechanism consistent with this behavior is represented by eq 4 whose rate law (under

$$E + Mal \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot Mal \xrightarrow{k_2} E_{inactive} \tag{4}$$

steady-state conditions) given in eq 5 takes the form of the

$$v = k_{obsd}[E]$$
$$k_{obsd} = \frac{k_2[Mal]}{[(k_{-1} + k_2)/k_1] + [Mal]} = \frac{k_2[Mal]}{K_{diss} + [Mal]} \tag{5}$$

familiar Michaelis–Menten expression. Double reciprocal plots of the data are shown in Figure 1. The slopes and intercepts of these plots (computer-generated least-squares lines) yield the values for the alkylation rate constants $k_2$, and the apparent protein–maleimide dissociation constants, $K_{diss}$, presented in Table II. It is especially to be noted that, within experimental error, $k_2$ values for either enzyme are identical in the presence and absence of proflavine whereas $K_{diss}$ values are increased by factors of about 2 and 2.6 (for papain and ficin, respectively) in the presence of the dye. If $k_2$ represents the rate of nucleophilic attack by the catalytically essential sulfhydryl group of papain or ficin upon bound maleimide

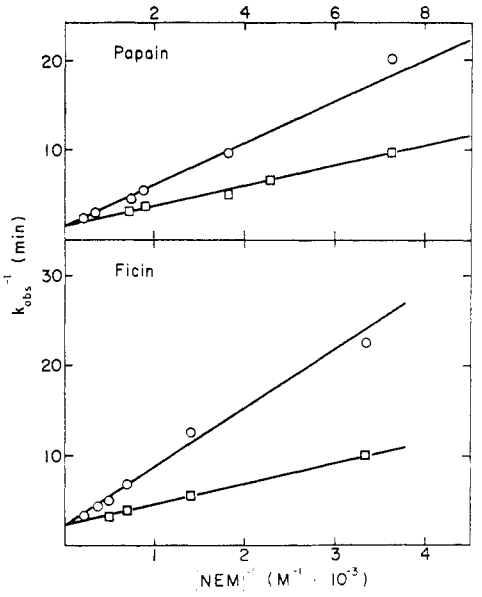


FIGURE 1: The effects of *N*-ethylmaleimide concentration on rates of inactivation of papain and ficin in the presence (□) and absence (○) of $5 \times 10^{-5}$ M proflavine.

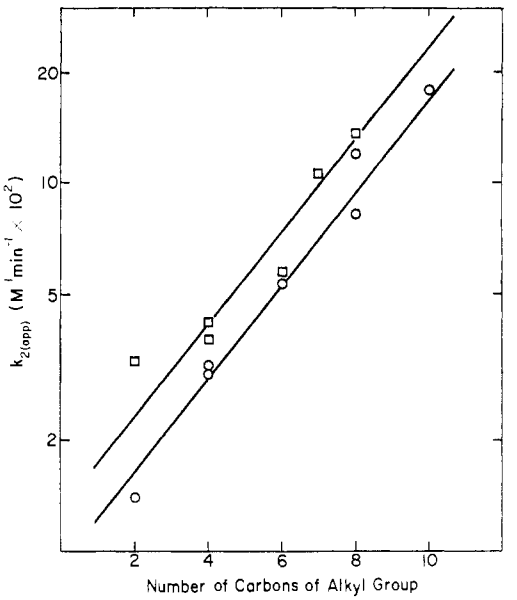


FIGURE 2: Alkyl group chain length effects upon the logarithms of apparent second-order rate constants for the inactivation by *N*-alkylmaleimides of papain (□) and ficin (○).

and $K_{diss}$ reflects the reversible binding interaction between the enzyme and the maleimide, the implication of these data is that proflavine enhances the binding interaction but has little, if any, effect upon the nucleophilic reactivity of the sulfhydryl group.

At maleimide concentrations below those needed to approach saturation of the enzyme in the reversible binding step of eq 4 (that is, at maleimide concentrations such that [E] $\ll$ [Mal] $\ll K_{diss}$) a plot of the observed pseudo-first-order inactivation rate constants as a function of maleimide concentration is linear. The slope of such a plot is an apparent second-order rate constant, $k_{2(app)}$, which characterizes the overall effectiveness of a given maleimide derivative as an inactivator of papain or ficin. In terms of eq 5, $k_{2(app)} = k_2/K_{diss}$.

Anderson and Vasini (1970), who were the first to observe saturation effects of the sort reported here in their investigation of the inactivation of papain by *N*-alkylmaleimides of varying alkyl chain length, found that the effectiveness of *N*-alkylmaleimides (in terms of $k_{2(app)}$ for inactivation of papain) increased dramatically with increasing alkyl chain length. We have found that this is also true in the case of ficin, as evidenced by the log $k_{2(app)}$ *vs.* maleimide alkyl chain length plots of Figure 2.

As expected from the accelerating effects noted earlier of proflavine upon the rates of *N*-ethylmaleimide inactivation of papain and ficin, the dye is also an effective accelerator of inactivations by other *N*-alkylmaleimides as indicated by the data of Table III. It is perhaps worth noting that the proflavine-induced enhancement in $k_{2(app)}$ does not change significantly from one maleimide derivative to another.

*Characterization of Proflavine Binding to Papain and Ficin.* Shifts of the visible spectrum of proflavine to longer wavelengths (red shifts) upon dye binding to papain or ficin have been exploited in determinations of dissociation constants of papain–proflavine and ficin–proflavine complexes by spectrophotometric titration (Skalski *et al.*, 1973; Hollaway, 1968). Using the procedure and rationale described by Hollaway (1968) for the determination of the overall dye dissociation constant $K_d$, and number of dye-binding sites per protein molecule $n$, we have determined binding parameters listed in Table IV from appropriate protein–dye difference spectra. To facilitate comparison with earlier investigations, Table IV also includes the results of Hollaway (1968) and of Skalski *et al.* (1973).

No shifts of the uv–visible spectra of proflavine or of the various *N*-alkylmaleimides used in this investigation are observed upon the mixing of proflavine with maleimide. This was taken to indicate that proflavine does not associate with the maleimides to a significant extent.

To check for the possibility that the ficin preparation used in our studies might be substantially different from that of Hollaway (1968), which was prepared by a somewhat different procedure, kinetic parameters were determined which allow a direct comparison between the two ficin preparations. The results are summarized in Table V. It is apparent that the two sets of data are in substantial agreement, particularly regarding the salient point that in the ficin-catalyzed hydrolysis of BzArgOEt, proflavine effects are manifested primarily in the variation of $k_{cat}$ rather than $K_{m(app)}$.

TABLE II: Kinetic Parameters for the Inactivation of Papain and Ficin with *N*-Ethylmaleimide in the Presence and Absence of Proflavine.

| Enzyme | Proflavine Concn (M $\times 10^5$) | $k_2$ ($min^{-1}$) | $K_{diss}$ (M $\times 10^3$) |
|---|---|---|---|
| Papain | | 0.778 ± 0.062 | 1.80 ± 0.18 |
| Papain | 5.0 | 0.830 ± 0.192 | 0.95 ± 0.26 |
| Ficin | | 0.444 ± 0.105 | 2.75 ± 0.80 |
| Ficin | 5.0 | 0.471 ± 0.032 | 1.07 ± 0.10 |

## Discussion

Spectrophotometric titrations (Table IV), equilibrium dialysis studies (Hollaway, 1968), and spectrofluorimetric titrations (Skalski *et al.*, 1973) have shown that the dye proflavine forms 1:1 complexes with papain and ficin. The

TABLE III: Rate Constants for the *N*-Alkylmaleimide Inactivation of Papain and Ficin in the Presence and Absence of Proflavine.

| Enzyme | Maleimide Derivative | Maleimide Concn (M × $10^4$) | Proflavine Concn (M × $10^5$) | $k_{obsd}$ ($min^{-1}$) | $k_{2(app)}$ ($M^{-1}$ $min^{-1}$) | Enhancement in $k_{2(app)}$ |
|---|---|---|---|---|---|---|
| Ficin | *N*-Ethyl | 2.98 | | 0.0420 | 141 | |
| | *N*-Ethyl | 2.98 | 5.0 | 0.1019 | 342 | 2.43 |
| Ficin | *N*-Butyl | 2.47 | | 0.0513 | 208 | |
| | *N*-Butyl | 2.47 | 5.0 | 0.147 | 597 | 2.86 |
| Papain | *N*-Ethyl | 1.38 | | 0.0495 | 360 | |
| | *N*-Ethyl | 1.38 | 5.0 | 0.105 | 764 | 2.12 |
| Papain | *N*-Butyl | 1.24 | | 0.0541 | 435 | |
| | *N*-Butyl | 1.24 | 5.0 | 0.107 | 857 | 1.97 |
| Papain | *N*-Hexyl | 1.12 | | 0.0578 | 516 | |
| | *N*-Hexyl | 1.12 | 5.0 | 0.124 | 1105 | 2.14 |

TABLE IV: Binding Parameters for the Complexes of Proflavine with Papain and Ficin as Determined by Spectrophotometric Titration.

| Enzyme | pH | Ionic Strength (M) | Wavelength Employed (nm) | Dye Concn ($\mu$M) | Enzyme Concn[a] ($\mu$M) | $K_d$ (M × $10^4$) | *n* |
|---|---|---|---|---|---|---|---|
| Papain[b] | 5.2 | 0.05 | 470 | 1280 | 24–193 | 1.09 ± 0.39 | 0.75 ± 0.14 |
| Papain[b] | 5.2 | 0.05 | 470 | 1280 | 19–150[c] | 1.08 ± 0.39 | 0.96 ± 0.11 |
| Alkylated papain[d] | 5.2 | 0.05 | 465 | 520 | 11–57 | 1.17 ± 0.35 | 1.11 ± 0.20 |
| Control papain[d] | 5.2 | 0.05 | 465 | 520 | 5–64 | 1.34 ± 0.29 | 0.73 ± 0.10 |
| Papain[e] | 6.0 | | 465 | 70 | 57–180 | 3.00 | |
| Hg–papain[e] | 6.0 | | 465 | 70 | | 1.75 | |
| Ficin[f] | 6.6 | 0.10 | 467.5 | 1280 | 23–123 | 13.3 ± 7.0 | 1.05 ± 0.27 |
| Ficin[g] | 6.6 | 0.10 | 467.5 | 600 | 23–350 | 3.5 ± 1.8 | 0.92 ± 0.40 |

[a] Except as otherwise noted, enzyme concentrations represent total protein and were determined spectrophotometrically from absorbance at 280 nm. [b] Mercury–papain (Worthington) in 0.05 M acetate buffer contained 5 mM cysteine and 1 mM EDTA. [c] Enzyme concentrations determined by rate assay (Hall *et al.*, 1972). [d] Papain (Sigma) activated by incubation with KCN (see Experimental Section), divided into two portions. One portion totally inactivated by alkylation with *N*-hexylmaleimide. Both portions then exhaustively dialyzed against 0.05 M acetate buffer (pH 5.2) containing 1 mM EDTA. After dialysis, control enzyme is about 10% active by rate assay (Hall *et al.*, 1972) and alkylated enzyme is totally inactive. [e] Data of Skalski *et al.* (1973) on papain purified by affinity chromatography. [f] Ficin prepared for this investigation by the method of Englund *et al.* (1968). Difference spectra determination in 0.05 M sodium phosphate buffer containing 1 mM cysteine and 1 mM EDTA. [g] Data of Hollaway (1968).

dissociation constant for the papain–proflavine complex is approximately $10^{-4}$ M, and that for the analogous ficin complex is somewhat larger. Since dye binding to papain is not significantly perturbed by alkylation of the active-site thiol group (Table IV), it would appear unlikely that the dye-binding site is in close proximity to the active site. This same conclusion was reached by Skalski *et al.* (1973) who observed that active papain, active papain in the presence of the tetrapeptide competitive inhibitor Gly-Gly-Tyr(Bzl)-Arg, nonactivatable papain, and Hg–papain all have similar affinities for proflavine. In the absence of evidence to the contrary, then, we will proceed on the assumption that proflavine binds to papain at a site remote from the active site which contains the catalytically essential thiol group. We will also assume an analogous remote binding site for proflavine binding to ficin, although direct evidence to support this assumption for ficin has yet to be sought.

Now since catalytic activity and the apparent reactivity of the catalytically essential thiol group toward alkylating agents must reflect properties of the active site itself, dye-induced enhancements of enzymic activity or apparent thiol reactivity must be attributed to relatively long-range conformational or allosteric effects of dye-binding at a remote site.[2] The likelihood that this indeed might be the case has already been recognized for both papain (Skalski *et al.*, 1973) and ficin (Hollaway, 1968). Conformational changes in the active sites of papain

[2] It is conceivable that the observed binary association of papain or ficin with proflavine might be incidental to the effects of the dye on enzymic activity or apparent thiol reactivity, these effects being due instead to modification of substrate or alkylating-agent properties by complexation with proflavine prior to association with the enzyme. That is, a dye–substrate complex or a dye–maleimide complex might interact more favorably with the enzyme active site than substrate or maleimide itself. Although α-*N*-benzoyl-L-arginine ethyl ester does associate with proflavine (Hollaway (1968) reports a dissociation constant of 0.204 M at pH 6.6 for this complex, a value which we have confirmed by spectrophotometric titration), the complexation is too weak to account for the observed effects of proflavine on the papain- or ficin-catalyzed hydrolysis of this substrate. We have found no evidence for any complexation between proflavine and *N*-alkylmaleimides under conditions employed in these studies.

TABLE V: Michaelis–Menten Parameters for the Ficin-Catalyzed Hydrolysis of BzArgOEt in the Presence and Absence of Proflavine.[a]

| Ficin Preparation | [Proflavine] (mM) | $K_{m(app)}$ (mM) | $k_{cat}$ ($sec^{-1}$) |
|---|---|---|---|
| This investigation[b] | 0 | 31.2 ± 3.6 | 1.93 ± 0.19 |
| | 1.0 | 22.0 ± 4.3 | 4.77 ± 0.73 |
| Hollaway (1968)[c] | 0 | 23.1 ± 3.4 | 2.40 ± 0.32 |
| | 4.0 | 19.0 ± 6.0 | 6.07 ± 1.91 |

[a] pH 6.6, 0.1 M ionic strength (added KCl), 25°. Initial rate data obtained by pH-Stat titration, and kinetic parameters calculated using a linear regression treatment of the Lineweaver–Burk form of the Michaelis–Menten equation. [b] Ficin concentration 8.8 μM in the presence of 5.0 mM cysteine activator. Substrate concentration range 4.0–50.0 mM. [c] Ficin concentration 12 μM in the presence of 1.0 mM cysteine activator. Substrate concentration range 2.0–100.0 mM.

and ficin have also been implicated to account for apparent increases in thiol-group reactivity which attend the binding of certain substrates and inhibitors to these enzymes (Whitaker, 1969; Whitaker and Lee, 1972).

The most important conclusion to be drawn from the results of this investigation is that although proflavine does indeed accelerate the inactivation of both papain and ficin by *N*-ethylmaleimide and other *N*-alkylmaleimides, for neither enzyme does this acceleration appear to be attributable to a dye-induced enhancement of the intrinsic reactivity of the active-site thiol group. Instead, the data of Figure 1 are most readily interpreted to imply that for both enzymes the dye effect may be attributed to the enhancement of a reversible association between the maleimide inactivators and a binding site for them in the active site region of the enzyme in question. The rate constant which characterizes the actual alkylation step of the inactivation is insensitive either to the nature of the alkyl substituent of the maleimide (Anderson and Vasini, 1970) or to the presence or absence of an activating dye.

This interpretation of these results parallels the observations mentioned in the introduction to this paper, and discussed in detail in an earlier communication (Hall *et al.*, 1972), concerning the activating effects of proflavine on the papain-catalyzed hydrolysis rates of ester substrates, which can also be rationalized in terms of substrate binding enhancements. However, the results reported here appear to conflict with the conclusion of Hollaway (1968) that proflavine binding does enhance the nucleophilicity of the catalytically essential thiol group of ficin. It might be noted that one of the pieces of evidence cited by Hollaway in support of this conclusion was his observation that the rate of reaction of *N*-methylmaleimide with ficin is enhanced in the presence of proflavine. It is likely, in light of the behavior of the *N*-ethyl derivative reported here, that the enhancement of *N*-methylmaleimide inactivation of ficin by proflavine can be attributed to a binding effect.

Although our results warrant the conclusion that the nucleophilicity of the essential thiol of papain or ficin remains unchanged in the presence of proflavine, it cannot be concluded that because proflavine enhancements of maleimide inactivation rates for both enzymes can be accounted for in terms of binding effects, the same must necessarily be true for the effects of the dye on the interactions of these enzymes with substrates. The presumed conformational change which accompanies proflavine binding and results in an increased affinity of the active sites of papain and ficin toward *N*-alkylmaleimides might leave the net binding affinity toward substrates unchanged. Yet without any effect on intrinsic thiol nucleophilicity, this conformational change could still alter the interactions between the bound substrate and the catalytically involved functional groups at the active site in such a way as to facilitate catalysis. Thus we are left with the possibility that proflavine could affect either the affinity of the enzyme for substrates, as we have suggested might be the case for papain (Hall *et al.*, 1972), or the rates of catalysis steps which follow substrate binding, which appears to be the case with ficin (Hollaway, 1968).

Apart from our central concern over apparent differences in the manner in which proflavine affects the interactions of papain and ficin with substrates, the results of this investigation may be taken to underscore the known similarities between these two enzymes. Indeed, proflavine binds to both enzymes to produce discrete 1:1 complexes with similar dissociation constants and similar effects on the electronic spectra of the bound dye.[3] With both enzymes the dye–enzyme complex is about twice as reactive toward alkylation by various *N*-alkylmaleimides as the free enzyme. Although the reactivity of ficin itself toward *N*-alkylmaleimides of various alkyl chain lengths is somewhat lower than that of papain, the variation of reactivity with alkyl chain length is quite similar for both enzymes. This can be taken to imply that ficin, as well as papain, has an extensive nonpolar active-site cleft which can interact through hydrophobic binding with the alkyl chains of the maleimides (Anderson and Vasini, 1970).

In view of the many close similarities between ficin and papain, a further investigation was undertaken concerning the apparent differences between these two enzymes in the effects of proflavine on catalysis kinetic parameters. That investigation is the subject of the accompanying paper (Hall and Anderson, 1974).

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[3] As has been noted previously (Bernhard *et al.*, 1966; Hollaway, 1968; Skalski *et al.*, 1973) the red shifts in dye spectra which accompany dye–protein binding imply a binding site with hydrophobic or very nonpolar character. Although the location of the proflavine binding site on papain is not known, it is certainly not inconceivable that it might be near an extremity of the extensive hydrophobic cleft of papain which also includes the catalytic active site with its essential thiol group (Drenth *et al.*, 1971; Berger and Schechter, 1970). Proflavine binding to ficin is presumably similar.

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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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