Mechanism of Association of *N*-Acetyl-L-phenylalanylglycinal to Papain†

Allen Frankfater* and Thresiamma Kuppy‡

ABSTRACT: Equilibrium and rate constants for the association to papain of a specific dipeptide substrate aldehyde analogue, *N*-acetyl-L-phenylalanylaminoacetaldehyde (Ac-Phe-glycinal), were obtained in the pH range 3.5–9.5 at 25 °C. The limiting values of K_I and the second-order association rate constant, k_2/K_s, corrected for hydration of Ac-Phe-glycinal in solution, are 9.08×10^{-10} M and 4.95×10^7 M^{-1} s^{-1}, respectively. Both K_I and k_2/K_s are found to depend on two groups in the free enzyme with $pK_1 \approx 4.2$ and $pK_2 \approx 8.6$. However, in the covalent hemithioacetal adduct the p*K*s of both groups assume values outside the range between 3 and 10. As a consequence, aldehyde binding suppresses the contributions made by both groups to the pH dependency of papain fluorescence between pH 3.5 and pH 9.5. The similarity in the fluorescence of the covalent adduct to that of the alkaline form of the free enzyme, together with the pH dependency of K_I, is consistent with a neutral hemithioacetal adduct in papain in which the pK_a of His-159 is abnormally low. In agreement, the value of 0.047 s^{-1} obtained for the first-order dissociation rate constant, k_{-2}, is at least 8 orders of magnitude smaller than calculated rate constants for decomposition of anionic hemithioacetals and more closely resembles those reported for the neutral adducts. The solvent deuterium isotope effect on the rate of association of Ac-Phe-glycinal with papain, $(k_2/K_s)_H/(k_2/K_s)_D$, is 0.77, indicative of the participation of the thiolate–imidazolium ion pair. Since addition of this form of papain to Ac-Phe-glycinal is expected to initially produce an anionic hemithioacetal in which His-159 is protonated, a rapid tautomerization must occur in a subsequent step or steps, resulting in the net transfer of a proton from His-159 to the oxyanion. The solvent deuterium isotope effect on the rate of dissociation of the papain–Ac-Phe-glycinal complex, $(k_{-2})_H/(k_{-2})_D$, is 1.5. The possible participation of His-159 as a base catalyst in the dissociation of the neutral hemithioacetal is discussed.

The pathway of papain-catalyzed hydrolysis reactions includes the formation and decomposition of a thiol ester acyl-enzyme intermediate (Bender & Brubacher, 1964; Lowe & Williams, 1964). Both the acylation and deacylation steps have been shown to be substitution reactions at the carbonyl carbon of the substrate, with the cysteine-25 sulfhydryl and water acting as nucleophiles. By analogy with nonenzymatic acyl substitution reactions, it was suggested that during acyl group transfer an additional intermediate is formed in which the carbonyl carbon assumes a tetrahedral configuration. Recently, Angelides & Fink (1979a,b) have obtained spectrophotometric evidence for a tetrahedral intermediate in low-temperature studies of the hydrolysis of N^{α}-benzyloxycarbonyl-L-lysine *p*-nitroanilide by papain. Other evidence for a tetrahedral intermediate comes from studies of structure–reactivity relationships in substrate catalysis (Lowe & Yuthavong, 1971; O'Leary et al., 1974), from X-ray diffraction studies of chloromethyl ketone substrate analogues bound to papain (Drenth et al., 1976), and from studies of the binding of aldehyde analogues of N^{α}-acylamino acids and dipeptides (Westerik & Wolfenden, 1972; Lewis & Wolfenden, 1977; Mattis et al., 1977; Bendall et al., 1977).

The reaction of aldehydes with papain occurs in two steps (Mattis et al., 1977):

$$E + A \underset{}{\overset{K_s}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EA' \qquad (1)$$

where EA is the Michaelis complex and EA′ is suggested to be a covalent, tetrahedral hemithioacetal with the cysteine-25 sulfhydryl. Evidence for hemithioacetal formation has been obtained from NMR studies of aldehyde binding (Bendall et al., 1977), from comparisons of binding constants of aldehydes with those of related amides, alcohols, and methyl ketones (Westerik & Wolfenden, 1972; Lewis & Wolfenden, 1977; Bendall et al., 1977), from observation of a measurable secondary isotope effect in aldehyde binding (Lewis & Wolfenden, 1977), from studies of aldehyde binding to cysteine-25 modified papain (Mattis et al., 1977), and by analogy to results obtained with serine proteinases (Kennedy & Schultz, 1979). The association of aldehydes to papain is suggested to be a model for the formation of a tetrahedral intermediate in substrate catalysis (Bendall et al., 1977).

In this present work, we have undertaken a detailed study of the binding of an aldehyde analogue of dipeptide substrate to papain. At the outset, we had hoped that the results might contribute to a resolution of some uncertainties in the papain mechanism by permitting a unique assignment of protonic forms to the amino acid residues involved in aldehyde binding. While an unambiguous assignment of pK_as to these residues was not possible from the pH dependency of this reaction, we believe our results are generally consistent with the participation of thiolate–imidazolium ion pair in aldehyde association. A more complete account of this work follows.

Experimental Procedures

Materials. Crystalline mercuripapain (Sigma) was further purified by chromatography on (*p*-aminophenyl)mercuri-Sepharose 4B (Sluyterman & Wijdenes, 1970), concentrated with an Amicon Diaflow apparatus, and stored frozen as the mercury derivative at −20 °C. Protein concentrations were determined from absorbance measurements at 278 nm (Glazer & Smith, 1961) and 210 nm (Tombs et al., 1959). Enzyme solutions were standardized by titration with DTNB[1] (Ellman,

† From the Department of Biochemistry and Biophysics, Loyola University Stritch School of Medicine, Maywood, Illinois 60153. *Received December 8, 1980; revised manuscript received April 7, 1981.* Supported in part by grants from the National Institutes of Health (RR-05368 and HD-10311).

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[1] Abbreviations used: Cbz, benzyloxycarbonyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CGN, *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester; CLN, N^{α}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester; Ac-Phe-glycinal, *N*-acetyl-L-phenylalanylaminoacetaldehyde; EDTA, (ethylenedinitrilo)tetraacetate; BAPA, N^{α}-benzoyl-L-arginine *p*-nitroanilide; Ac-Phe-aminopropanone, *N*-acetyl-L-phenylalanylaminopropanone; Ac-Phe-Gly-NA, *N*-acetyl-L-phenylalanylglycine *p*-nitroanilide; Me_2SO, dimethyl sulfoxide.

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1959). Papain activity was measured at 25 ± 0.2 °C in a GCA/McPherson double-beam recording spectrophotometer with either CGN or CLN as substrates (Bajkowski & Frankfater, 1975).

Ac-Phe-glycinal diethyl acetal was synthesized from *N*-acetyl-L-phenylalanine and aminoacetaldehyde diethyl acetal (Eastman) by the mixed anhydride procedure of Anderson et al. (1967). The product was recrystallized from ethyl acetate, mp 130–131 °C. Anal. Calcd for $C_{17}H_{26}N_2O_4$: C, 63.33; H, 8.13; N, 8.69. Found: C, 63.23; H, 8.16; N, 8.32. Prior to use, samples of the diethyl acetal were hydrolyzed to free aldehyde in dilute aqueous HCl for 24 h at room temperature (Westerik & Wolfenden, 1972).

Sodium tetrathionate (International Chemical and Nuclear) was recrystallized from 95% ethanol. Its sulfhydryl equivalency was determined by reaction with a small excess of either L-cysteine or dithiothreitol and titration of the unreacted thiol with DTNB (Ellman, 1959).

Spectra. The fluorescence spectra of (*p*-aminophenyl)-mercuri-Sepharose-purified papain and papain–aldehyde complex were measured at 25 °C in an Aminco-Bowman ratio recording spectrophotofluorometer essentially as described previously (Mattis et al., 1977). The fluorescence of free papain at 344 nm (excitation at 288 nm) was determined after preincubating a buffered enzyme solution, ionic strength 0.25 M, with dithiothreitol for 10 min in the fluorometer cell. The fluorescence of the complex was then measured after adding Ac-Phe-glycinal (1.3×10^{-4} M). This final concentration of aldehyde was much greater than the calculated value of K_I. Under these conditions, free aldehyde does not contribute significantly to the fluorescence.

Kinetic Measurements. Between pH 3.5 and pH 7.5, the equilibrium dissociation constant, K_I, for Ac-Phe-glycinal with papain was determined from the inhibition of hydrolysis of CGN under first-order conditions [$K_m \gg$ (S)] and enzyme concentrations on the order of $(K_I)_{obsd}$. This necessitated the use of 10-cm cells. Typically, preactivated papain and aldehyde were incubated in 25 mL of buffer (0.05 M buffer salt, 0.2 NaCl, and 0.5 mM EDTA) for 15 min to ensure equilibrium between hydrated and unhydrated aldehyde and complex. The reaction was then initiated by adding 25 μL of stock CGN solution in spectral grade acetonitrile. This reaction was monitored in Cary 15 spectrophotometer thermostated at 25 ± 0.2 °C. First-order rate constants were determined graphically by using data from at least 90% of the reaction. These were corrected for spontaneous hydrolysis of substrate when necessary. Above pH 7.5, inhibition constants were determined with BAPA as substrate [$K_m >$ (S)]. Initial rates were measured at 390 nm with a Perkin-Elmer Model 320 spectrophotometer in 1-cm cells on the 0.01 absorbance scale. Reactions were thermostated at 25 ± 0.1 °C with a Perkin-Elmer digital temperature controller.

The rate of dissociation of the enzyme–aldehyde complex was measured by following the decrease in fluorescence of the enzyme in the presence of sodium tetrathionate. This reagent reacts with free enzyme to form an inactive sulfenyl thiosulfate derivative (Boland & Hardman, 1972). In a typical experiment, (*p*-aminophenyl)mercuri-Sepharose-purified papain, Ac-Phe-glycinal, and mercaptoethanol were incubated for 10 min in buffer, ionic strength 0.25 M, 25 °C. Dissociation was then initiated by the rapid addition of an excess of sodium tetrathionate. Generally, the time of mixing was within 20 s. Fluorescence changes were monitored at the excitation and emission maxima. Pseudo-first-order rate constants for the trapping reaction were measured in the absence of aldehyde

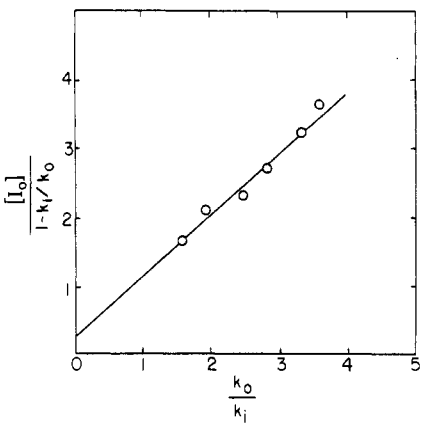


FIGURE 1: Inhibition of papain-catalyzed hydrolysis of CGN by Ac-Phe-glycinal at 25 °C, in 0.05 M sodium acetate, 0.2 M sodium chloride, and 0.5 mM Na_2 EDTA, pH 5.9, with $(E_3) = 1.92 \times 10^{-8}$ M, $(S_0) = 1.5 \times 10^{-6}$ M, and (I_0) varied from 6.31×10^{-9} to 5.23×10^{-8} M.

by following the time-dependent decrease in the rate of papain-catalyzed hydrolysis of BAPA [$K_m >$ (S)] after the addition of sodium tetrathionate. The final concentration of reactants were as follows: BAPA, 1×10^{-4} M; 1.6% Me_2SO; purified mercuripapain, 5×10^{-7}–1×10^{-6} M; mercaptoethanol, 1×10^{-4}–2.5×10^{-4} M; sodium tetrathionate, 5×10^{-6}–5×10^{-5} M. Calculated second-order rate constants were essentially independent of the concentrations of both tetrathionate and mercaptoethanol, indicating that tetrathionate reacted more rapidly with papain than with this mercaptan activator.

Rate constants for the dissociation of the papain–aldehyde complex were also measured by trapping free enzyme with CLN. Under conditions of (S) $\gg$ (I)K_m/K_I, free enzyme reacts in an essentially stoichiometric manner with substrate to form a Cbz-L-lysyl-enzyme intermediate which turns over at a rate proportional to the free enzyme concentration. From the increase in the rate of hydrolysis of CLN with time, the rate of dissociation of EA′ can be obtained. In a typical experiment, papain (6.7×10^{-8} M), L-cysteine or mercaptoethanol (5×10^{-3} M), and aldehyde (9.6×10^{-8} M) were preincubated in the spectrophotometer cell in buffer, ionic strength 0.25 M, 25 °C. After 15 min, dissociation was initiated by addition of CLN in Me_2SO (final concentration 4.3×10^{-4} M, 1.6% Me_2SO). Mixing was usually achieved within 12–15 s.

Results

Equilibrium Dissociation Constants for N-Acetyl-L-phenylalanylglycinal with Papain. K_I was calculated as described by Perlstein & Kézdy (1973) for a tightly binding competitive inhibitor according to eq 2. In this equation k_0

$$\frac{(A_T)}{1 - k_i/k_0} = \frac{K_I}{k_i/k_0} + (E_T) \qquad (2)$$

and k_i are either first-order rate constants or initial rates for substrate hydrolysis in the absence and presence of inhibitor, and (A_T) and (E_T) are total aldehyde and total enzyme concentration, respectively. Figure 1 shows a plot of these data at pH 5.0. The value of the enzyme concentration determined from the intercept is in good agreement with the value estimated by titration of the stock enzyme solution with DTNB. From the slope, K_I was calculated to be $(9.3 \pm 0.7) \times 10^{-9}$ M. The corresponding value at pH 6.5 was $(8.3 \pm 0.1) \times 10^{-9}$ M, in good agreement with the value of ~0.01 μM reported

Table I: Rate and Equilibrium Constants for Association of Ac-Phe-glycinal with Papain at 25 °C

pH[a]	K_I[b] × 10^{10} (M)	k_{-2} (s^{-1})	k_2/K_s[b,e] × 10^{-7} (M^{-1} s^{-1})
3.0		0.102 ± 0.012	
3.5	70.2 ± 4.6[c]	0.063 ± 0.008	0.88 ± 0.13
4.0	28.8 ± 2.1[c]	0.056 ± 0.008	1.96 ± 0.30
4.5	13.5 ± 0.6[c]	0.049 ± 0.011	3.63 ± 0.83
5.0	10.4 ± 0.7[c]	0.044 ± 0.006	4.22 ± 0.65
5.5	9.3 ± 0.8[c]	0.049 ± 0.010	5.44 ± 0.74
6.0	9.0 ± 0.2[c]	0.044 ± 0.010	4.88 ± 1.12
6.5	9.3 ± 1.1[c]	0.046 ± 0.004	4.97 ± 0.78
7.0	9.3 ± 1.2[c]	0.039 ± 0.007	4.21 ± 0.96
7.5	9.0 ± 0.7[c]		
7.5	10.1 ± 0.6[d]	0.047 ± 0.008	4.65 ± 0.84
7.8	12.1 ± 0.9[d]		
8.0		0.042 ± 0.010	3.49 ± 0.86
8.3	14.9 ± 0.5[d]		
8.5		0.052 ± 0.008	3.48 ± 0.55
8.6	17.3 ± 2.3[d]		3.02 ± 0.66
9.0	37.4 ± 4.1[d]	0.47 ± 0.009	1.27 ± 0.28
9.5	66.1 ± 14.6[d]		

[a] Buffer was 0.05 M buffer salt, 0.2 M NaCl, and 0.5 mM Na_2EDTA adjusted to the indicated pH. [b] Corrected for hydration according to $K_I = (K_I)_{obsd}/(1 + K_H)$. [c] Determined with CGN. [d] Determined with BAPA. [e] Calculated from the relationship $K_I^{-1} = k_2/(k_{-2}K_s)$.

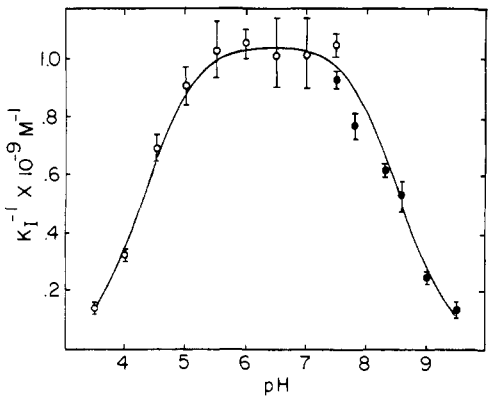


FIGURE 2: pH dependency of the equilibrium association of Ac-Phe-glycinal with papain at 25 °C and I = 0.25 M. Points are derived from inhibition data obtained with the substrates CGN (○) and BAPA (●). The solid line was calculated for a bell-shape curve with pK_1 = 4.3, pK_2 = 8.6, and $(K_I)_{lim}^{-1} = 1.10 \times 10^9$ M^{-1}.

earlier at this pH (Mattis et al., 1977).

If only the unhydrated aldehyde reacts with papain, K_I must be corrected for the hydration of the aldehyde in solution according to the relationship $(K_I)_{cor} = K_I/(1 + K_h)$ (Lewis & Wolfenden, 1977). The value of the hydration constant, K_h, for Ac-Phe-glycinal has been reported to be 7.9 (Lewis & Wolfenden, 1977). From the structure–reactivity relationships which have been shown to govern the acid dissociation behavior of hydrates of acetaldehyde and its derivatives (Bell, 1966), it is possible to determine that K_h for Ac-Phe-glycinal should be independent of pH between 3.5 and pH 9.5. Table I contains the corrected values for K_I in this pH range. The corresponding values for the association constant, K_I^{-1}, along with their standard errors, are plotted as a function of pH in Figure 2. The data points can be seen to lie along a bell-shaped curve which can be described by

$$1/K_I = \frac{(1/K_I)_{lim}}{1 + (H)/K_1 + K_2/(H)} \quad (3)$$

The solid line in Figure 2 was calculated for $(1/K_I)_{lim} = 1.10 \times 10^9$ M^{-1}, pK_1 = 4.3, and pK_2 = 8.6. The corrected value for $(K_I)_{lim}$, given in Table II, is 9.08×10^{-10} M.

Table II: pH-Independent Kinetic Parameters for the Association of Ac-Phe-glycinal with Papain

kinetic constant[a]	Ac-Phe-glycinal	pK_1	pK_2
$(K_I)_{lim} \times 10^{10}$ (M)	9.08[b]	4.3	8.6
$(k_{-2})_{lim}$ (s^{-1})	0.047 ± 0.005[c]		
$(k_2/K_s)_{lim} \times 10^{-7}$ (M^{-1} s^{-1})	4.95[b,d]	4.1	8.5
$(k_{-2})_{H_2O}/(k_{-2})_{D_2O}$	1.5		
$(k_2/K_s)_{H_2O}/(k_2/K_s)_{D_2O}$	0.77[e]		

[a] At 25 °C, I = 0.25 M. [b] Corrected for hydration of the aldehyde. [c] Average of 11 values obtained between pH 4.0 and 9.0. [d] Calculated from K_I and k_{-2} as described previously. [d] Determined from the slope of the lines in Figure 5 after correction for the solvent isotope effects on the rate of reaction of tetrathionate with papain, the rate of dissociation of the papain-aldehyde complex, and hydration of the aldehyde in water.

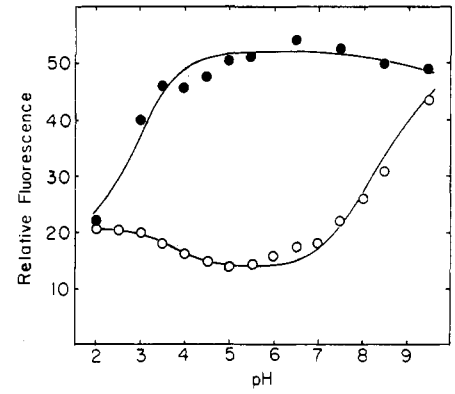


FIGURE 3: pH dependency of the relative fluorescence of papain at 25 °C, with I = 0.25 M, $(E_0) = 2.4 \times 10^{-7}$ M, excitation = 288 nm, emission = 344 nm, $(I_0) = 0$ (○), and $(I_0) = 1.3 \times 10^{-4}$ M (●).

Fluorescence of Papain and Papain–Aldehyde Complex. Figure 3 shows the pH dependency of the fluorescence of papain and papain–aldehyde complex. The decrease in the fluorescence of free papain between pH 9.5 and pH 5.0 has been attributed to the quenching of the fluorescence of Trp-177 on protonation of His-159 (Shinitsky & Goldman, 1967; Bendall & Lowe, 1976a). Below pH 5.0 the fluorescence of papain is observed to increase. The solid line was calculated by assuming two groups with pKs of 3.6 and 8.1 control the fluorescence the free enzyme. Similar results have previously been reported by Sluyterman & De Graaf (1970). These authors have obtained a pK_a of 8.6 for the alkaline limb of the fluorescence titration curve after correcting for the effects of ionization of tyrosyl residues on the fluorescence of the enzyme. The fluorescence of the complex, in contrast, shows a broad plateau in the region between pH 3.5 and pH 9.5. The data demonstrate that the group with $pK_a \approx 8.6$ in the free enzyme has little if any effect on fluorescence in the complex. The sharp decline in the fluorescence of the complex below pH 3.5 is not easily interpreted because the affinity of papain for the aldehyde decreases greatly in this region. Although the concentration of the aldehyde was always at least 100 times greater than its calculated dissociation constant, the validity of eq 3 below pH 3.5 could not be directly confirmed. The similarity of the fluorescence of the free enzyme and the complex at pH 2.0 suggests that complex could have been fully dissociated at this pH. Alternatively, the decrease in fluorescence of the complex below pH 3.5 could be due to the protonation of a residue having a pK_a of ~2.9. This latter interpretation is supported by the observation of a similar pH dependency for the fluorescence of the covalent derivative, trifluoroacetonylpapain (Bendall & Lowe, 1976a).

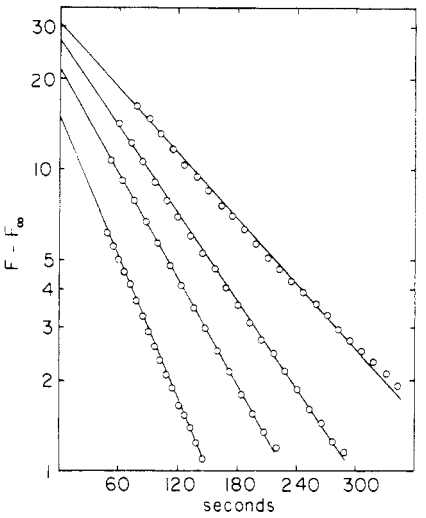


FIGURE 4: Dissociation of papain–Ac-Phe-glycinal complex at 25 °C, in 0.05 M sodium acetate, 0.2 M sodium chloride, and 0.5 mM Na_2EDTA, pH 5.5, with (mercaptoethanol) = 0.093 mM, $(E_0) = 8.1 \times 10^{-8}$ M, and $(I_0) = 7.51 \times 10^{-7}$ M. The sodium tetrathionate concentrations were from top to bottom: line 1, 1.88 mM; line 2, 2.79 mM; line 3, 3.69 mM; line 4, 5.4 mM.

Rates of Dissociation of Papain–Aldehyde Complex. The high affinity of papain for Ac-Phe-glycinal necessitated measuring dissociation rates in the presence of reagents which act to deplete either free enzyme or free aldehyde. The following equation describes the rate of disappearance of EA′, under conditions of $K_s \gg$ (A), in the presence of a reagent that traps the free enzyme

$$\frac{-d(EA')}{dt} = \frac{k_{-2}k_T(T)(EA')}{k_2[(A) - (EA')]/K_s + k_T(T)} \quad (4)$$

Ignoring (EA) at low (A), eq 4 can be derived from the relationship $-d(EA')/dt = k_{-2}(EA') - k_2(E)[(A) - (EA')]/K_s$ and the steady-state assumption $d(E)/dt = 0 = k_{-2}(EA') - [k_2[(A) - (EA')]/K_s + k_T(T)](E)$. In these equations k_{-2} is the first-order rate constant for the decomposition of the complex, k_2/K_s and k_T are second-order rate constants for the reaction of free enzyme with unhydrated aldehyde and trapping reagent, respectively, and (A) is the initial concentration of unhydrated aldehyde. According to eq 4, the disappearance of EA′ is first order when (A) $\gg$ (EA′). Under these conditions the observed first-order rate constant obeys the relationship

$$\frac{1}{k_{obsd}} = \frac{k_2}{k_{-2}k_TK_s}\frac{(A)}{(T)} + \frac{1}{k_{-2}} \quad (5)$$

In eq 4 and 5, we have ignored the rehydration of the aldehyde following its dissociation from the enzyme. Since rehydration is calculated to be more rapid than dissociation (Kennedy & Schultz, 1979), its effect was to decrease the contribution made by dissociation of EA′ to the concentration A. Consequently, even at lower aldehyde concentrations employed in some experiments, the dissociation reaction appeared reasonably first order. Figure 4 shows first-order plots of the decrease in the fluorescence of the papain–aldehyde complex at pH 4.5 and 25 °C on addition of various concentrations of sodium tetrathionate. In Figure 5, observed first-order rate constants, obtained at pH (pD) 5.5 over a 20-fold range in the ratio (A)/(T), are graphed according to eq 5. The solid lines were drawn by the method of least squares. From the intercept, k_{-2} was found to be 0.049 ± 0.010 s^{-1} in H_2O.

According to eq 5, the slopes of the lines in Figure 5 are equal to $k_2/(K_sk_{-2}k_T)$. It can be shown in turn that this is essentially equal to $1/(K_1k_T)$ (see below). The value obtained

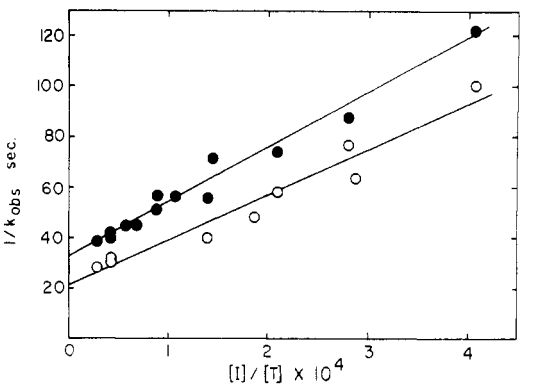


FIGURE 5: Reciprocal first-order rate constants for the dissociation of papain–aldehyde complex at different initial concentrations of Ac-Phe-glycinal and sodium tetrathionate at 25 °C, in 0.05 M sodium acetate, 0.2 M sodium chloride, and 0.5 mM Na_2EDTA, with (mercaptoethanol) = 0.092 mM and $(E_0) = 0.92 \times 10^{-7}$ M: in H_2O, pH 5.5 (○); in D_2O, pD 5.48 (●).

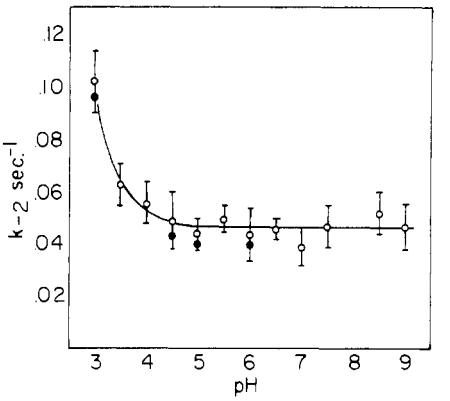


FIGURE 6: pH dependency of the dissociation of papain–Ac-Phe-glycinal complex at 25 °C and $I = 0.25$ M with sodium tetrathionate (○) and CLN (●) as trapping reagents for the free enzyme.

for the slope in H_2O was (1.80 ± 0.16) × 10^{-5} s. The value for k_T at pH 5.5 was found to be 541 ± 65 M^{-1} s^{-1}. After correction for the effect hydration on the concentration of free aldehyde, these yielded a value for K_I of (1.15 ± 0.20) × 10^{-9} M, in good agreement with the value of (0.93 ± 0.08) × 10^{-9} M obtained independently at this pH (Table I). This result affirms the validity of this approach for determining k_{-2}. Values for k_{-2} at several pHs are listed in Table I and are shown in Figure 6. Between pH 4.0 and pH 9.0, the average value for k_{-2} is 0.047 ± 0.005 s^{-1} (Table II).

Alternatively, the rate of dissociation of the complex was studied with CLN as the trapping reagent for free enzyme, under conditions where the rate of hydrolysis of CLN was zero order in (S). The rate of increase in enzyme activity with time was first order, with the final rate of hydrolysis of CLN being equal to the control value in the absence of inhibitor. The first-order rate constants were independent of substrate concentration over the range studied, indicating that in eq 4, $k_T(T) \gg k_2(A - EA')/K_s$ and $k_{obsd} \approx k_{-2}$. These values for k_{-2} are shown in Figure 6. The mean value for k_{-2} obtained between pH 4.5 and pH 6.0 was 0.041 ± 0.005 s^{-1}, in good agreement with results obtained with sodium tetrathionate. In several experiments, hydroxylamine was employed as a trapping reagent for free aldehyde. For these reactions the rate of dissociation of EA′ is given by eq 6. This equation predicts

$$\frac{-d(EA')}{dt} = \frac{k_{-2}k_T(T)(EA')}{k_2[(E) - (EA')]/K_s + k_T(T)} \quad (6)$$

that the dissociation reaction in the presence of NH_2OH will be first order only for relatively high values of $k_T(T)$. This

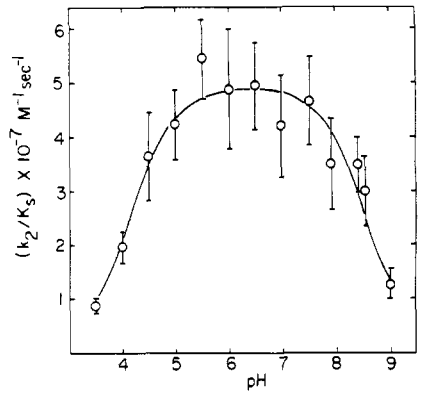


FIGURE 7: pH dependency of the association of Ac-Phe-glycinal with papain at 25 °C and $I = 0.25$ M. The points were calculated from the experimentally determined values of K_I and k_{-2} as described in the text, and the solid line was calculated for a bell-shaped curve with $pK_1 = 4.1$, $pK_2 = 8.5$, and $(k_2/K_s)_{lim} = 4.95 \times 10^7$ M^{-1} s^{-1}.

condition could not be achieved with hydroxylamine. However, at a given (NH_2OH), it was possible to estimate the time required for 50% dissociation of the complex. When these values for $t_{0.5}$ at pH 5.0 were extrapolated to infinite (H_2N-OH), an estimate of ≥ 0.01 s^{-1} was obtained for k_{-2}.

The values reported here for k_{-2}, determined by three independent methods, are ~10-fold greater than the value of 0.0014 s^{-1} reported earlier (Mattis et al., 1977). At present, the reason for the discrepancy is unclear. However, it should be noted that in the earlier report k_{-2} was calculated from experimentally determined values for K_I, k_2, and K_s with eq 7 (see below), whereas in this work we have measured k_{-2} directly.

Calculation of the Second-Order Rate Constant for Association of Ac-Phe-glycinal with Papain. For a two-step association reaction (eq 1), the equilibrium dissociation constant, K_I, is related to the individual kinetic constants according to eq 7 (Mattis et al., 1977).

$$\frac{1}{K_I} = \frac{k_2 + k_{-2}}{k_{-2}} \frac{1}{K_s} \tag{7}$$

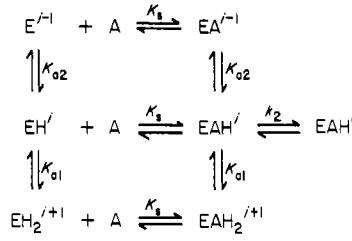
For Ac-Phe-glycinal, $k_2 \gg k_{-2}$ (Mattis et al., 1977). Therefore, to an excellent approximation $k_2/K_s = k_{-2}/K_I$. With this relationship, k_2/K_s was calculated from the corrected values of K_I and k_{-2}. These are listed in Table I and are plotted as a function of pH in Figure 7. The solid line in Figure 7 was calculated according to eq 3 for $pK_1 = 4.1$, $pK_2 = 8.5$, and $(k_2/K_s)_{lim} = 4.95 \times 10^7$ M^{-1} s^{-1}.

Rate Constants in the Presence of Deuterium Oxide. Values of pD for deuterium oxide solutions were obtained by adding 0.4 to the reading on the pH meter (Glascoe & Long, 1960). From the results shown in Figure 5, k_{-2} in D_2O was found to be 0.031 ± 0.002 s^{-1}. Using the average value of 0.047 s^{-1} for k_{-2} in H_2O (Table II) yields an isotope effect on k_{-2} of ~1.5.

The data in Figure 5 may also be used to determine the effect of D_2O on k_2/K_s. As indicated previously, the slopes of the lines are equal to $k_2/(K_s k_{-2} k_T)$. Therefore, one can obtain the isotope effect $(k_2/K_s)_H/(k_2K_s)_D$ from the ratio of the slopes after correcting for the effects of D_2O on k_{-2}, k_T, and the concentration of unhydration aldehyde in solution. At pH (pD) 5.5, the ratio $(k_T)_H/(k_T)_D$ $(k_T)_D$ was found to be ~0.72. A similar inverse isotope effect has been observed for the alkylation of papain by chloroacetate (Creighton et al., 1980). The deuterium solvent isotope effect on the hydration of aliphatic aldehydes has previously been determined to be 0.84 (Gruen & McTique, 1963). From the ratio of the slopes of the lines in Figure 5 (0.83), $(k_2/K_s)_H/k_20K_s)_D$ can be calculated to be ~0.77.

Discussion

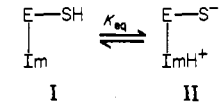
Kinetic studies of substrate hydrolysis and inhibitor binding by papain indicate that K_s in eq 1 is essentially independent of pH between 3.5 and 9.5 (Lowe & Yuthavong, 1971; Mole & Horton, 1973; Lucas & Williams, 1969). The pH dependency observed for the association of Ac-Phe-glycinal with papain, shown in Figure 2, may therefore be largely attributed to the effect of pH on the equilibrium between the Michaelis complex, EA, and the covalent adduct, EA′, in eq 1. Application of the rules of Dixon (1953) to the data in Figure 2 shows that groups, with pK_as of 4.3 and 8.6 in the free enzyme, become perturbed when the aldehyde binds so that their pK_as in EA′ fall outside the range between pH 3 and pH 10. This conclusion is further supported by the observations that both the fluorescence of the complex (Figure 3) and its rate of decomposition (Figure 6) are essentially independent of pH from pH 3.5 to 9.5. The data in Figure 2 also indicate that Δi, the change in charge in the enzyme in going from EA′ to EA, is +1 below pH 4.3 and −1 above pH 8.6 (Dixon, 1953). The pH dependency of k_2/K_s (Figure 7) suggests that the state of ionization of two residues in papain is important for aldehyde association with one of the two residues being protonated in the active enzyme. Collectively, these results provide good evidence for the following minimal scheme for aldehyde association to papain between pH 3.5 and pH 9.5.



(8)

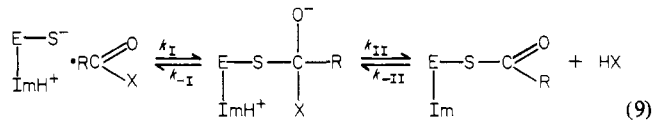
One of two ionizable groups which become perturbed in the hemithioacetal may be identified as the active-site nucleophile, the Cys-25 sulfhydryl, since it becomes covalently linked to the aldehyde (Lewis & Wolfenden, 1977; Bendall et al., 1977). The second group is presumed to be the imidazole of His-159 because of its proximity to this cysteine residue (Husain & Lowe, 1968; Drenth et al., 1971) and because of other evidence which indicates that the pK_a of His-159 is strongly influenced by the chemical state of Cys-25 (Lewis et al., 1976, 1981; Johnson et al., 1981). However, our results do not locate the position of the proton at the active site in EH and EAH′.

It has been suggested that the cysteine–histidine catalytic pair in EH may exist as a mixture of two tautomeric forms as shown



I II

Evidence for presence of significant levels of II between pH 5 and pH 8 has been obtained (Polgar, 1974; Lewis et al., 1976, 1981; Creighton & Schamp, 1980; Creighton et al., 1980; Johnson et al., 1981). It has been further argued that II is the catalytically active form of papain (Jolley & Yankeelov, 1972; Angelides & Fink, 1979a,b; Polgar, 1973, 1974; Lewis et al., 1981). Evidence for this is derived from the lack of a significant deuterium solvent isotope effect on k_2/K_s for substrate hydrolysis (Polgar, 1979). On this basis, the following scheme can be proposed for acylation of papain by substrates (Polgar, 1973)

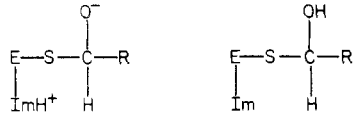


(9)

According to eq 9, formation of the tetrahedral intermediate from the Michaelis complex (k_1) and its retrogression of the Michaelis complex (k_{-1}) is not assisted by proton transfer to or from histidine. In contrast, decomposition of the tetrahedral intermediate (k_{II}) and its re-formation from the acyl-enzyme (k_{-II}) is aided by histidine acting as a general acid–general base catalyst. The analogous mechanism for formation and decomposition of a tetrahedral hemithioacetal between papain and Ac-Phe-glycinal would involve the uncatalyzed addition and elimination of a Cys-25 thiolate anion is steps k_2 and k_{-2}, respectively. The mechanism described by eq 9 is supported by results obtained for model reactions with thiol esters, hemithioacetals, and mixed oxygen–sulfur acetals which seem to show that protonation and deprotonation of the sulfur is generally not concurrent with the cleavage or formation of the carbon–sulfur bond (Fedor & Bruice, 1964; Bender & Heck, 1967; Lienhard & Jencks, 1966; Jensen & Jencks, 1979).

The mechanism depicted by eq 9 may not be entirely adequate to describe the reaction of papain with substrates and aldehydes. Thus, this scheme does not require that the rate of formation of the tetrahedral adduct be dependent on the state of ionization of the histidine residue, since the thiolate anion is expected to be as good a nucleophile in E as in EH. However, a bell-shaped pH dependency is observed both for the formation of the tetrahedral adduct in acylation (Angelides & Fink, 1979a,b) and in aldehyde association (Figure 7), suggesting that the histidine residue may participate in both processes.[2] This conclusion for aldehydes might also be implied in the observation that hemithioacetal formation is accompanied by a marked change in the apparent proton affinity of the histidine residue.

There are two possible structures for the singly protonated form of the hemithioacetal, EAH′, between pH 3 and 10. These are represented by structures III and IV. Structure

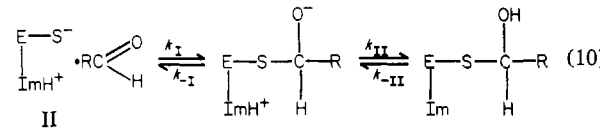


III IV

III resembles the presumptive, transient anionic tetrahedral intermediate in substrate hydrolysis. If structure III is the predominant form of the hemithioacetal between pH 3.5 and 9.5, then the apparent pK_a of the hydroxyl group must be ≤ 3.0 and the pK_a of His-159 must be ≥ 10 in this adduct. In IV, the carbonyl oxygen is protonated and its pK_a must be ≥ 10, while the pK_a of His-159 would be ≤ 3.0. On the basis of reasonable estimates for the pK_a of a hemithioacetal in solution (Bell, 1966; Hupe & Jencks, 1977), IV is expected to be ~9 kcal/mol more stable than III. The observation that the fluorescence of the hemithioacetal adduct between pH 3.5 and pH 9.5 resembles that of the native enzyme above pH 9.0, where His-159 is fully unprotonated, also favors structure IV (Figure 3). In addition, studies of aldehyde association to chymotrypsin (Kennedy & Schultz, 1979) indicate that the neutral form of the hemiacetal is the product in that reaction. The assignment of $pK_a \leq 3$ to His-159 in the covalent complex is supported by the results of ^{19}F NMR and fluorescence measurements of *S*-(trifluoroacetonyl)papain, which show the pK_a of His-159 in this derivative to be ~2.9 at an ionic strength of 0.25 M (Bendall & Lowe, 1976a). In the *S*-trifluoroacetonyl derivative the carbonyl group is extensively hydrated, resembling the tetrahedral hemithioacetal adduct in this present study.

Protonation of the carbonyl oxygen is expected to significantly stabilize the hemithioacetal adduct. From the data of Lienhard & Jencks (1966) and estimates for the pK_a of the hemithioacetal, it is possible to calculate that the rate constants for decomposition of the anionic hemithioacetals between acetaldehyde and ethanethiol (pK_a = 10.25) and methyl mercaptoacetate (pK_a = 7.83) are about 5×10^6 s^{-1} and 4×10^8 s^{-1}, respectively. In contrast, the value of 0.047 s^{-1} obtained for k_{-2} in this work more closely resembles the measured rate constants for decomposition of neutral hemithioacetals (Lienhard & Jencks, 1966).

A central question, relevant to the mechanism of aldehyde association, is whether formation and decomposition of the hemithioacetal in papain is subject to acid–base catalysis by the enzyme. The finding, in this present work, of an inverse isotope effect of 0.77 on the rate of addition of Ac-Phe-glycinal to papain provides evidence that tautomer II is the form of papain that adds to the carbonyl and that proton transfer is not rate determining for this reaction (Creighton & Schamp, 1980; Creighton et al., 1980; Polgar, 1979). This conclusion is supported by model studies which show that the unassisted addition of a mercaptide anion to aliphatic aldehydes is very rapid ($k \approx 1 \times 10^5$–5×10^5 M^{-1} s^{-1}) and represents the dominant pathway for hemithioacetal formation above pH 3 for even relatively basic mercaptans such as ethanethiol (Lienhard & Jencks, 1966). Since it is likely that structure IV is the predominant form of the hemithioacetal in solution, a proton transfer must occur in subsequent steps following formation of the initial anionic adduct. This mechanism is depicted by eq 10.



II III IV

The question remains as to whether the interconversion of species III and IV in eq 10 occurs directly in a single step or in several steps through the intermediacy of solvent molecules. If this proton transfer occurs in a single step, then decomposition of the neutral hemithioacetal would be facilitated by His-159 acting as a base.

The present data do not permit us to distinguish between catalytic and noncatalytic pathways for hemithioacetal decomposition. However, we believe the following observations may point toward catalysis by His-159. First, the value of <3 for the pK_a of His-159 in the papain–aldehyde adduct, relative to usual values of ~4 in acylpapains and *S*-alkylthiopapains (Brubacher & Bender, 1966; Bendall & Lowe, 1976a,b; Johnson et al., 1981), may reflect a direct interaction between the N^{δ}-1 nitrogen of His-159 and the hydroxyl group of the hemithioacetal (Bendall & Lowe, 1976a). Second, a comparison of the rate of decomposition of the hemithioacetal in papain with the corresponding uncatalyzed rates for model hemithioacetals (Lienhard & Jencks, 1966), and mixed oxygen–sulfur acetals of benzaldehyde (Jensen & Jencks, 1979), suggests that k_{-2} in our study may be greater than expected for an uncatalyzed process. Finally, the presence of a D_2O solvent isotope effect of ~1.5 on k_{-2} may be consistent with

[2] For a discussion of how the imidazolium cation of His-159 might participate in the formation of the tetrahedral intermediate in acylation, see Lewis et al. (1981).

proton transfer being partially rate determining in papain–hemithioacetal decomposition. However, it should be noted that there are other explanations which might account for this modest isotope effect.

In mechanisms for aldehyde association involving direct proton transfer between His-159 and the carbonyl oxygen, it may be necessary to assume that the carbonyl oxygen of the aldehyde is oriented toward the region of the active site usually thought to be occupied by the leaving group of substrates. During substrate hydrolysis, protonation of the heteroatom of the leaving group by His-159 would facilitate the cleavage of the carbonyl carbon–heteroatom bond in the acylation step. In contrast, protonation of the carbonyl oxygen might lead to a nonproductive stabilization of the tetrahedral intermediate. For substrates, this may be prevented by a specific interaction between the carbonyl oxygen and a distinct oxygen binding site in the enzyme (Drenth et al., 1976). However, it might also be argued that it is the steric requirement of the bulky leaving group in substrates which prevents an orientation in which the carbonyl oxygen is directed toward His-159. Since an aldehydic hydrogen could be accommodated in the carbonyl oxygen binding site, aldehydes could bind in the "nonproductive mode" with the carbonyl oxygen toward the histidine.

This interpretation of the role of His-159 in aldehyde association can account for the observation that the rate of dissociation of the covalent complex is independent of pH between pH 3.5 and 9.0 since the histidine in the complex appears to be in its fully unprotonated form in this pH range and may also explain the pH dependency of the association rate. It does not readily account for the increase in k_{-2} seen below pH 3.5. However, it is possible that the increase in k_{-2} between pH 3.5 and 3.0 reflects a change in the state of ionization of Asp-158 in the complex (Bendall & Lowe, 1976a,b).

From the data in Table II and knowledge of K_s, it is possible to calculate the equilibrium constant, k_2/k_{-2}, for the interconversion of Michaelis complex (EAH) and the covalent adduct (EAH'). The equilibrium dissociation constants for Ac-Phe-Gly-NA (Lowe & Yuthavong, 1971) and Ac-Phe-aminopropanone (Bendall et al., 1977) are about 1.0 and 1.5 mM, respectively. Utilizing a value of 1.0 mM for the K_s for Ac-Phe-glycinal yields an estimate for k_2/k_{-2} of $\sim 10^6$. From the data of Lienhard & Jencks (1966) and structure reactivity relationships, the equilibrium constant for the addition of a thiol to Ac-Phe-glycinal in solution can be estimated to be about 300–600 M^{-1}. The hemithioacetal may thus be $\sim 10^3$ times more stable in papain than in solution. Analogous stabilization factors for hemiacetals in serine proteinases vary from about 10 to 10^4 and are correlated with the known kinetic specificity of these enzymes in substrate hydrolysis (Thompson & Bauer, 1979; Kennedy & Schultz, 1979). Similar specificity correlations exit for hemithioacetal formation with papain (Westerik & Wolfenden, 1972). These stabilization factors have been interpreted as reflecting a decrease in the entropy of activation for formation of the covalent adduct by virtue of the initial noncovalent association of the aldehyde in the Michaelis complex (Thompson & Bauer, 1979).

Acknowledgments

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Binding of 2,2-Diphenylpropylamine at the Aldehyde Site of Bacterial Luciferase Increases the Affinity of the Reduced Riboflavin 5′-Phosphate Site†

Thomas F. Holzman and Thomas O. Baldwin*

ABSTRACT: We have found a new class of inhibitors of the bacterial bioluminescence reaction, the *N*,*N*-diphenylalkylamines and acids. We have studied the action of one of these compounds, 2,2-diphenylpropylamine. The amine was competitive with the long-chain aliphatic aldehyde substrate ($K_i \simeq 0.1$ mM) but caused an increase in the affinity of the enzyme for reduced riboflavin 5′-phosphate ($FMNH_2$). The inhibitor was attached to Sepharose 6B by a bis(oxirane) spacer, and the interactions of bacterial luciferase with the immobilized ligand were analyzed. The binding of luciferase to the immobilized inhibitor was enhanced by $FMNH_2$ and was decreased by decanal. The results of these studies showed that the 2,2-diphenylpropylamine–luciferase complex has an increased affinity for $FMNH_2$. Likewise, the $FMNH_2$–luciferase complex has an increased affinity for 2,2-diphenylpropylamine. The inhibitor also binds to the enzyme–4a-peroxydihydroflavin complex to block the binding of the aldehyde substrate, while binding of the aldehyde substrate to either the free enzyme or the enzyme–4a-peroxydihydroflavin complex blocks binding of 2,2-diphenylpropylamine.

Investigation of the inhibition of enzymes by the reversible binding of small molecules has been used extensively in the analysis of enzyme structure and function. Inhibitors that have been used include biologically important allosteric modifiers and synthetic substrate analogues, as well as molecules that bear little structural similarity to either substrate(s) or allosteric ligands. The substrate specificity of the bacterial luciferase reaction has been studied by using analogues of the flavin substrate (Mitchell & Hastings, 1969; Meighen & MacKenzie, 1973; Watanabe et al., 1980; Tu et al., 1977) and analogues of the aldehyde substrate (Spudich & Hastings, 1963; Hastings et al., 1966); compounds that bear little structural similarity to either the flavin or the aldehyde have also been used in competitive binding studies (Tu & Hastings, 1975; Makemson & Hastings, 1979).

The enzyme bacterial luciferase is an $\alpha\beta$ dimer that catalyzes the oxidation by O_2 of $FMNH_2$[1] and a long-chain saturated aldehyde to yield FMN, the carboxylic acid, and blue-green light (Figure 1). The enzyme has a single active center located primarily, if not exclusively, on the α subunit (Meighen et al., 1971a,b; Cline & Hastings, 1972). A single flavin is required per $\alpha\beta$ during the light-emitting reaction (Becvar & Hastings, 1975). The stoichiometry of the aldehyde substrate with respect to enzyme is generally assumed to be one as well, but it has not been determined.

Nealson & Hastings (1972) demonstrated that several compounds known to be inhibitors of the P-450 mixed-function oxidase system from liver microsomes (McMahon et al., 1969) are also inhibitors of luciferase. The compounds used by Nealson and Hastings were 2-(2,3-dichloro-6-phenylphenoxy)ethylamine (DPEA), 2-(2,3-dichloro-6-phenylphenoxy)-*N*,*N*-diethylamine (DPDA), and 2-(*N*,*N*-diethylamino)ethyl 2,2-diphenyl-*n*-pentanoate (SKF 525A) (see Figure 2). Both DPEA and DPDA are competitive with aldehyde (Nealson & Hastings, 1972). SKF 525A, on the other hand, blocks the formation of intermediate II, probably by competing with $FMNH_2$ for binding to the enzyme. The fluorescent dye 8-anilino-1-naphthalenesulfonate (Ans) is an inhibitor of luciferase competitive with $FMNH_2$ but has little effect on the binding of FMN (Tu & Hastings, 1975). Makemson & Hastings (1979) showed that *N*-benzyl-*N*-methyl-2-propynylamine (pargyline), known to inhibit monoamine oxidase by the formation of a covalent adduct with the flavin (Chuang et al., 1974), is a reversible inhibitor of luciferase, apparently competitive with aldehyde. Structural analogues of the aldehyde substrate also inhibit the bioluminescence reaction. Long-chain aliphatic alcohols and acids as well as long-chain aldehydes with an unsaturated bond close to the aldehyde functional group are inhibitors of the luciferase reaction (Spudich & Hastings, 1963; Hastings et al., 1966).

† From the Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843. *Received March 17, 1981.* This publication comprises a portion of the Ph.D. thesis submitted by T.F.H. to the Graduate College of the University of Illinois, Urbana, IL. This work was supported by grants from the National Science Foundation (PCM 79-25335) and the National Institutes of Health (AG-00884).

[1] Abbreviations used: Ph_2PA, 2,2-diphenylpropylamine (DϕPA in figures); Ph_2PA–Sepharose, 2,2-diphenylpropylamine–bis(oxirane)–Sepharose; FMN, riboflavin 5′-phosphate; $FMNH_2$, reduced FMN; DTE, dithioerythritol; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

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