Conformational Changes in Papain During Catalysis and Ligand Binding†

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ABSTRACT: Concentrations of 2-butanol as low as 1×10^{-4} M were found to cause a time-dependent, essentially irreversible inactivation of papain. The inactivation appears to be specific for derivatives of *n*-butane (such as 2-butylamine, 2-butanone, 1-butanol). The kinetics of the inactivation reaction were first order in 2-butanol and first order in active enzyme. The pseudo-first-order rate constant for inactivation at 25° and pH 5.2 is 24.3 ± 2.3 M^{-1} min^{-1}, and the energy of activation is 10.1 ± 1.0 kcal/mol. From the effect of substrate on the inactivation process it is concluded that papain undergoes a conformational change during the catalyzed hydrolysis of ester substrates. The inactivation process is dependent on a group with pK_{app} = 4.0 which could be the same group which is involved in the catalytic reaction. Sulfhydryl group titration indicates that the inactivation is associated with a loss of the free thiol. The effects of 2-butanol on the uv absorption, circular dichroic, and fluorescent emission spectra of papain are consistent with a small conformational change being associated with the inactivation. The following mechanism for the inactivation process is most consistent with the experimental observations. 2-Butanol binds to papain in a rapid, reversible step to form a complex which undergoes a subsequent rate-limiting change to an inactive conformation.

Papain is a sulfhydryl protease which catalyzes the hydrolysis of *N*-acylamino acid esters according to Scheme I,

Scheme I

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P_2$$
$$\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\quad P_1$$

where ES = Michaelis complex and EA = acyl-enzyme (Glazer and Smith, 1971). One of the distinguishing features of papain is its unusual stability toward denaturing reagents, *e.g.*, urea (Sluyterman, 1967) or high concentrations of organic solvents (Drenth *et al.*, 1968; Barel and Glazer, 1969). This characteristic may be due to its compact structure (Drenth *et al.*, 1968). Several previous studies have shown that the presence of alcohols during papain-catalyzed hydrolyses results in transesterification (Mycek and Fruton, 1957; Glazer, 1966; Brubacher and Bender, 1966; Fink and Bender, 1969).

During an investigation of the effects of small ligands on papain-catalyzed reactions we wished to determine whether asymmetric synthesis would occur during the transesterification reaction with 2-butanol (A. L. Fink, in preparation). The observed results, however, were rather unusual and unexpected. Rather than acting as a nucleophile to compete with water in partitioning the acyl-enzyme in the transesterification reaction (Scheme II), the 2-butanol caused the inactivation of

Scheme II

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \begin{cases} \xrightarrow{k_3H_2O} E + P_2 \\ + \\ P_1 \xrightarrow{k_3ROH} E + P_3 \end{cases}$$

papain. Since the effect is observed with concentrations of 2-butanol as low as 1×10^{-4} M it seems unlikely to be the usual kind of solvent-induced inactivation (denaturation) of proteins in the presence of organic solvents. A few reports suggesting solvent-induced conformational effects have been made, although for much higher solvent concentrations. For example, several alcohols have been found to induce a conformational change in pepsinogen rendering it inactivatable by acid (Neumann and Shinitzky, 1971), and the increase in the rate of deacylation of acyl-chymotrypsins in the presence of isopropyl alcohol and dioxane has been attributed to such an effect (Faller and Sturtevant, 1966).

Materials and Methods

Materials. Cbz-glycine *p*-nitrophenyl[1] ester was obtained from Cyclo (lot N 1629) and recrystallized several times from ethyl acetate–hexane, mp 126.5–127.5°. Cbz-L-lysine *p*-nitrophenyl ester, also from Cyclo (lot N 1645), was recrystallized from acetonitrile, mp 152–154°. Cbz-glycine, Cyclo (lot N 1781A), mp 118–120°, and *N*-benzoyl-L-arginine ethyl ester, Mann (lot N 4143), mp 131–132°, were used without further purification.

Chromatoquality 2-butanol (MCB), Spectroquality 2-propanol (MCB), reagent grade 1-butanol (MCB), 2-pentanol (Baker), 2-butylamine (Aldrich), and 2-butanone (Aldrich) were distilled prior to use (the central 60% of the distillate being used). Acetonitrile was Spectroquality (MCB) and used without further purification. Doubly distilled water was used and all buffer materials and EDTA (Baker) were reagent grade.

2-Butyl and 2-propyl Cbz-glycinate were synthesized by acid-catalyzed reactions according to the method of Greenstein and Winitz (1961). The 2-propyl ester formed white crystals, mp 50.5–53.0°, and showed the expected nuclear magnetic resonance (nmr) and mass spectra. The 2-butyl ester was obtained as a pale yellow oil which was homogeneous on paper chromatography (in ethyl acetate–hexane 36:64; and absolute methanol). Nmr and mass spectral evidence were consistent with the desired structure.

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[1] Abbreviations used are: Cbz, carbobenzoxy; Cbz-GlyONph, Cbz-glycine *p*-nitrophenyl ester; Cbz-LysONph, Cbz-L-lysine *p*-nitrophenyl ester; Cbz-GlyOBu and Cbz-GlyOPr, 2-butyl and 2-propyl ester of Cbz-Gly, respectively; Cbz-Gly, Cbz-glycine; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

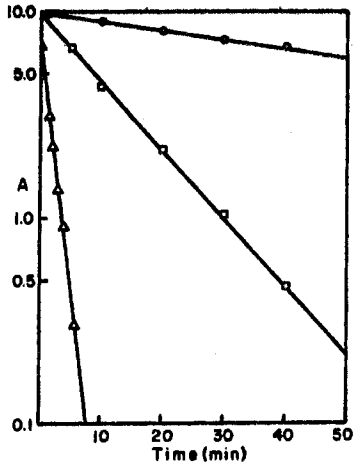

FIGURE 1: Typical first-order plots for the 2-butanol-induced inactivation of papain. 2-Butanol concentrations: (○) 1.62×10^{-4} M; (□) 4.42×10^{-3} M; (△) 1.0×10^{-2} M. pH 5.2, μ = 0.05 M, 25 ± 0.1°, 2.5×10^{-4} M EDTA.

Papain, as a crystalline suspension, was obtained from Worthington, activated with mercaptoethanol and purified by affinity chromatography (Blumberg *et al.*, 1970). The active enzyme was stored at 4°. Enzymatic activity was determined by a kinetic assay using BzArgOEt (Bender *et al.*, 1966; Mole and Horton, 1973).

Kinetic Measurements. Measurements of the rate of release of *p*-nitrophenol were made with either a Cary 14 or Beckman Kintrac spectrophotometer at 347.6 nm. The hydrolysis of the 2-butyl and 2-propyl esters was determined using a pH-Stat (Radiometer). All kinetic measurements were made at 25.0 ± 0.1°, pH 5.2, μ = 0.05 M, and [EDTA] = 2.5×10^{-4} M. Stock solutions of substrate were prepared in acetonitrile such that the final acetonitrile concentration was 3.2%.

The effect of pH and temperature on the rate of inactivation was measured as follows. The enzyme was incubated in buffer (citrate, acetate, phosphate, or borate) of appropriate pH and temperature, containing 2-butanol, 2.5×10^{-4} M EDTA, 1×10^{-4} M cysteine, and μ = 0.05 M. Aliquots were removed at appropriate time intervals and immediately assayed with Cbz-LysONph under the standard assay conditions (pH 5.2, 2.5×10^{-4} M EDTA, 25.0 ± 0.1°). Controls were run to determine the rate of inactivation in the absence of 2-butanol. Typical enzyme concentrations were 1.5×10^{-5} M.

Effect of Substrate on Inactivation. In these experiments 2-butanol was added during the papain-catalyzed hydrolysis of Cbz-LysONph under zero-order conditions. The rate of inactivation was obtained by comparison of the curve resulting from addition of 2-butanol to the identical reaction with the omission of 2-butanol. The rate of inactivation was also determined under identical conditions but with the substrate omitted. Typical concentrations used were $E_0 = 3.6 \times 10^{-7}$ M, 2-butanol = 8.7×10^{-3} M, Cbz-LysONph = 3.2×10^{-4} M.

Spectral Effects. The uv absorption difference spectrum was obtained using the method of Herskovits (1967). Fluorescent measurements were made on an Hitachi-Perkin-Elmer Model MPF-2A fluorescence spectrophotometer with excitation at 290 nm. CD measurements were made on a Jasco Model J20 spectropolarimeter.

Results

Initial Observations of the Effect of 2-Butanol on the Rate of Papain-Catalyzed Hydrolyses. When the papain-catalyzed hydrolysis of Cbz-glycine *p*-nitrophenyl ester was studied in the presence of 1×10^{-2} M 2-butanol, the reaction was found to be inhibited in a rather peculiar manner. The reaction began at a reduced rate and within a few seconds the rate of formation of *p*-nitrophenol decreased very rapidly and the reaction appeared to stop. Very similar results were obtained using Cbz-L-lysine *p*-nitrophenyl ester as substrate, and with 2-butanol over a range of concentrations from 1×10^{-3} to 5×10^{-2} M.

TABLE I: Pseudo-First-Order Rate Constants for the 2-Butanol-Induced Inactivation of Papain.

[2-Butanol] (M)	[Papain] (M)	k_{obsd}[a] (min^{-1})	k_{obsd}/[2-Butanol]
1.80×10^{-2}	5.83×10^{-8}	5.1×10^{-1}	28.2
1.00×10^{-2}	1.43×10^{-7}	2.3×10^{-1}	22.5
8.70×10^{-3}	3.62×10^{-7}	2.4×10^{-1}	21.7
4.42×10^{-3}	1.87×10^{-7}	1.0×10^{-1}	22.6
3.13×10^{-3}	5.83×10^{-8}	7.2×10^{-2}	23.0
3.39×10^{-3}	4.73×10^{-8}	9.4×10^{-2}	27.7
1.62×10^{-4}	5.83×10^{-8}	4.0×10^{-3}	25.0
			Av 24.3

[a] k_{obsd} is the observed first-order rate constant for the inactivation reaction, corrected for non-2-butanol loss of activity occurring under the same conditions. pH 5.2, μ = 0.05 M, 25.0 ± 0.1°, 2.5×10^{-4} M EDTA.

In order to determine whether the 2-butyl ester of Cbz-glycine was being formed by transesterification and acting as a competitive inhibitor we examined the effect of Cbz-GlyOBu and Cbz-GlyOPr on papain-catalyzed hydrolyses. Cbz-GlyOBu was found to have an inhibitory effect on the papain-catalyzed hydrolysis of Cbz-LysONph; however, the effect was much less than that observed with the corresponding concentration of 2-butanol, and the kinetics were inconsistent with competitive and noncompetitive inhibition.

By studying the effect of papain on Cbz-GlyOBu using the pH-Stat it was observed that Cbz-GlyOBu was a substrate for papain. The reaction did not follow Michaelis–Menten kinetics, presumably because the 2-butanol liberated by the reaction was exerting its inhibitory effect. The limited solubility of Cbz-GlyOPr precluded precise determination of K_I. Using Cbz-GlyONph as substrate, K_I was estimated to be approximately 1×10^{-2} M ([Cbz-GlyOPr] = 1.0×10^{-4} M). Cbz-GlyOPr was hydrolyzed slowly by papain.

Time-Dependence of 2-Butanol-Induced Inactivation. The effect of various concentrations of 2-butanol at zero time on the papain-catalyzed hydrolysis of Cbz-LysONph was investigated. At concentrations below 1×10^{-2} M there was no detectable inhibitory effect at $t = 0$. At higher concentrations such an effect was noted, *e.g.*, at 1.0 M 2-butanol inactivation was complete in less than 1 sec.

The effect of incubating papain with 2-butanol for various time intervals prior to adding substrate was studied. The data, Figure 1, show that the inactivation of papain is time dependent and that the rate of inactivation increases with increasing concentration of 2-butanol.

Examination of the rates of inactivation of papain showed them to be first order in enzyme concentration for at least 90% of the reaction (Figure 1).

The observed first-order rate constants for inactivation as a function of 2-butanol concentration (corrected for non-2-butanol inactivation) are shown in Table I.

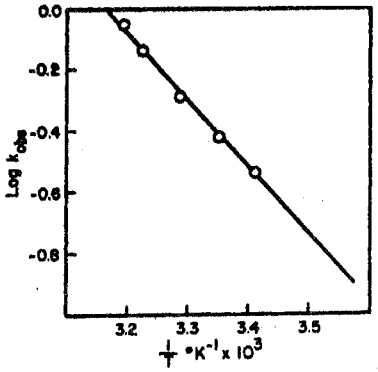

FIGURE 2: Arrhenius plot for the 2-butanol-induced inactivation of papain. Conditions: 1.5×10^{-2} M 2-butanol, 5.0×10^{-6} M papain, 2.5×10^{-4} M EDTA, 1×10^{-4} M cysteine.

At concentrations of 2-butanol higher than 5×10^{-2} M (and enzyme concentrations $<1 \times 10^{-6}$ M) the rate of inactivation was too rapid to determine by the procedure used. At much higher enzyme and 2-butanol concentrations the inactivation was slower although still first order for 90% of the reaction. For 4.3×10^{-1} M 2-butanol and 7.5×10^{-5} M papain $k_{obsd} = 0.28$ min^{-1}. From Table I one would calculate a value of 10.2 min^{-1}. At 1×10^{-6} M 2-butanol there was no detectable 2-butanol-induced inactivation over a 100-min period.

Irreversibility of the Inactivation. In order to determine whether the inactivation was reversible, the effect of diluting the 2-butanol concentration to a negligible value and of removing the 2-butanol from the enzyme by gel filtration was examined. In a typical experiment 2-butanol (6.4×10^{-3} M) was incubated with papain (1.5×10^{-5} M) at 25.0°, pH 5.2, until inactivation was complete. The solution was then diluted to a 2-butanol concentration of 2.1×10^{-5} M and papain = 4.9×10^{-8} M. The solution was then assayed at intervals over a 60-min period, but no activity was detected. Since control experiments indicated that the final concentration of 2-butanol did not cause detectable inactivation over the time period involved, and a control experiment omitting the 2-butanol showed retention of >90% enzymatic activity, the results indicate the inactivation is irreversible. This result was corroborated by the gel filtration experiments in which Sephadex G-25 was used to separate the inactivated enzyme from 2-butanol. The eluted enzyme fraction showed no activity over a 40-min period, whereas activity was present in the control experiment in which the 2-butanol was omitted. However, after a 20-hr period at 0° the 2-butanol-treated enzyme fraction showed the return of 15% of the activity of the control at that time.

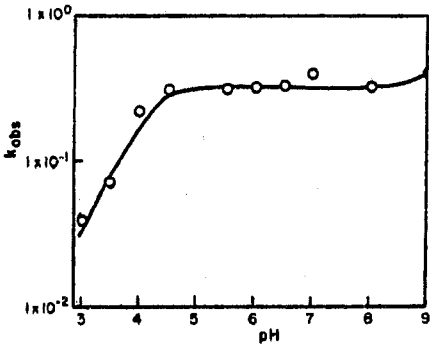

FIGURE 3: The pH dependence of 2-butanol-induced inactivation of papain. The points are experimental, the curve is calculated for $pK_{app} = 4.0$. 2-Butanol = 1.5×10^{-2} M, papain = 3.0×10^{-6} M, EDTA = 2.5×10^{-4} M, 25.0 ± 0.1°.

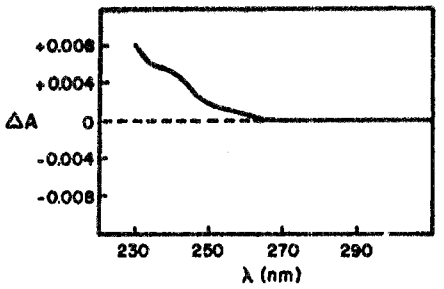

FIGURE 4: Uv absorption difference spectrum of 2-butanol-inactivated papain. 2-Butanol = 1.25×10^{-1} M, papain = 8.33×10^{-6} M, pH 5.2, 25.0°.

Effect of Substrate on Inactivation. In order to determine the effect of the presence of substrate on the 2-butanol-induced inactivation, the 2-butanol was added during the course of the catalytic reaction under substrate-saturating conditions ($S \gg K_m$, zero-order kinetics). The rate of inactivation under these conditions was then compared with that obtained under identical conditions, but without substrate. For Cbz-LysONph as substrate k_{obsd} for inactivation was found to be 3.5 min^{-1} while under identical conditions in the absence of Cbz-LysONph k_{obsd} was 0.19 min^{-1}. The substrate therefore caused a 20-fold increase in the rate of inactivation. In the presence of the less specific substrate Cbz-GlyONph a threefold increase in the rate of inactivation was found.

Effect of Temperature and pH. Figure 2 shows the effect of temperature on the first-order rate constant for inactivation. The values calculated for $\Delta H^{\pm}$ and $\Delta S^{\pm}$ were found to be 10.1 ± 1.0 kcal/mol and −29 eu, respectively, at 25°. The effect of pH on the rate of inactivation is shown in Figure 3 where the solid line is drawn for a pK of 4.0. These experiments were carried out in both the presence and absence of cysteine and no differences were observed.

Effect of 2-Butanol on the Uv, Fluorescence, and CD Spectra of Papain. The effect of 2-butanol on the uv absorption properties of papain was examined using difference spectra. No significant difference spectrum was observed although a small positive deviation occurred at wavelengths below 250 nm (Figure 4).

The addition of 2-butanol (final concentration 5.0×10^{-2} M) to papain (2.7×10^{-6} M) caused time-dependent changes in the fluorescence emission spectrum at pH 5.2. The changes paralleled the rate of inactivation and ceased when inactivation was complete. The addition of another aliquot of 2-butanol caused no further change. The spectrum of the fully inactivated enzyme showed a 7.5% increase in intensity and a red shift in the λ_{max} from 336.5 to 337.8 nm compared to the native enzyme (uncorrected values).

No significant effects of 2-butanol on the CD spectrum of papain were observed in the 310–250-nm range. At lower wavelengths a decrease in ellipticity was observed (Figure 5).

Effect of Related Compounds on Papain. Several compounds of related structure to 2-butanol were examined for a similar effect. The results are shown in Table II and indicate that the inactivating effect is peculiar to four-carbon chain compounds, the nature and position of the substituent having only minor effect.

Varying the ionic strength (0.003–0.3 M) and EDTA concentration (0–2.5×10^{-4} M) had no significant effect on the rate of inactivation. No uptake or release of protons was observed when 2-butanol was added to papain at pH 4.5.

Various tests were performed in order to make sure that the inactivating effect was due to 2-butanol and not to some

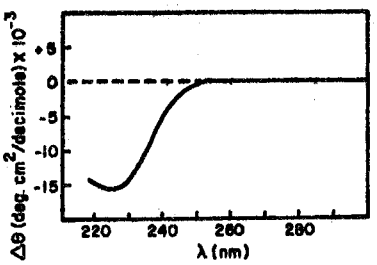

FIGURE 5: Circular dichroism difference spectrum for 2-butanol-inactivated papain. [E] = 1.0×10^{-5} M, 2-butanol = 1.5×10^{-1} M, pH 5.2, 25.0°; path length; 1.0 cm for 250–310 nm, 0.1 cm for 215–250 nm.

impurity. For example, several experiments were carried out in the presence of cysteine in which the concentration was varied from 0–5 $\times 10^{-4}$ M. The presence of cysteine had a negligible effect on the rate of inactivation. Different lots of 2-butanol were also used and gave identical results.

Sulfhydryl Group Titration. The effect of 2-butanol-induced inactivation on the concentration of titratable thiol groups in papain was determined using Nbs_2 as the titrant (Ellman, 1959). The control reaction in which 2-butanol was omitted showed 0.9 SH group per mole of active enzyme whereas the 2-butanol-inactivated enzyme showed no titratable thiol. The 2-butanol solutions in the absence of enzyme had no effect on Nbs_2.

Discussion

Several previous studies have shown that the presence of alcohols during papain-catalyzed hydrolysis results in some transesterification (Scheme II) (*e.g.*, Hinkle and Kirsch, 1971a,b; Fink and Bender, 1969; Lake and Lowe, 1966; Glazer, 1966). Thus the initial explanation for the inhibitory effect of 2-butanol on the papain-catalyzed hydrolysis of Cbz-GlyONph and Cbz-LysONph was that nucleophilic attack by the alcohol on the acyl-enzyme resulted in the formation of the corresponding 2-butyl ester. The 2-butyl ester then acted as a competitive inhibitor by binding strongly to the enzyme (a reasonable expectation in view of the analogous structure but much poorer leaving group of the 2-butyl ester compared to the *p*-nitrophenyl ester).

If this interpretation were correct, then the addition of Cbz-GlyOBu to the papain-catalyzed reaction should have a similar effect. However, since the addition of higher concentrations of Cbz-GlyOBu than those of 2-butanol had a much smaller effect, the inhibition must have been due to 2-butanol itself. This conclusion is further supported by the papain-catalyzed hydrolysis of Cbz-GlyOBu and by the time dependence of the inhibition.

The latter observation rules out the possibility that the 2-butanol was acting simply as a competitive or noncompetitive inhibitor, and indicates that the action of 2-butanol is directly on the enzyme. As shown in Table I the presence of 2-butanol causes complete inactivation of papain at a rate proportional to the concentration of 2-butanol.

Possible Mechanisms of Inactivation. Four possibilities suggest themselves to account for these observations. (1) The 2-butanol denatures the enzyme. (2) The 2-butanol reacts covalently with papain to form an irreversibly inhibited enzyme. (3) Papain exists as two conformers in equilibrium with each other, one catalytically active, the other inactive. The 2-butanol perturbs the equilibrium in favor of the inactive form. (4) 2-Butanol binds to papain and induces a conformational change resulting in an inactive product. A fifth possibility, that of some impurity in the 2-butanol being responsible for the inactivation, may be discounted on the basis of (a) the lack of protective effect of cysteine, (b) identical rates of inactivation for the same 2-butanol concentrations when 2-butanol from different sources was used, and (c) the inactivating effect of related derivatives of butane and pentane.

TABLE II: Rates of Inactivation of Papain by Related Compounds.[a]

Compound	Concn (M)	k_{obsd} (min^{-1})	Relative Rate of Inactivation[b]
2-Butanol	3.2×10^{-3}	7.5×10^{-3}	1.0
1-Butanol	3.2×10^{-3}	2.6×10^{-3}	0.35
2-Butanone	5.0×10^{-3}	0.43	0.34
2-Butylamine	3.2×10^{-3}	2.1×10^{-3}	0.28
2-Propanol	5.0×10^{-2}	0	0
2-Pentanol	8.3×10^{-3}	5.1×10^{-4}	0.025
	0.5	7×10^{-3}	$\ll 1$

[a] Conditions: pH 5.2, 25.0 ± 0.1°, $E_0 \sim 3 \times 10^{-7}$ M. k_{obsd} = observed first-order rate of inactivation. [b] Relative to 2-butanol = 1.0 at that concentration.

It seems unlikely that 2-butanol denatures the enzyme for several reasons: the energy of activation for the inactivation is rather low for such a reaction; no precedent exists to indicate that such low concentrations (*e.g.*, 10^{-4} M) of 2-butanol or similar compounds cause protein denaturation (in fact papain is stable in much higher concentrations of other aliphatic alcohols); the uv difference spectra do not show the changes expected for denaturation; and no protection by substrate was observed (Bernhard and Rossi, 1968; Hinkle and Kirsch, 1970).

Since the inactivation reaction seems to be specific to four-carbon chain compounds (Table II) with such diverse functional groups as hydroxyl, keto, and amino, it seems unlikely that a covalent modification is involved.[2] It can also be shown that a scheme involving equilibrium between an active and inactive conformation of papain (analogous to the case of α-chymotrypsin (Fersht, 1972)) in which 2-butanol drives the equilibrium to the inactive form is inconsistent with the experimental observations.

The existence of a zymogen of papain, propapain, has recently been reported (Brocklehurst and Kierstan, 1973) in which activation occurs by thiol–disulfide interchange. Could the inactive papain resulting from treatment with 2-butanol result from thiol–disulfide interchange? It seems unlikely since low concentrations of cysteine convert propapain into active papain (Brocklehurst and Kierstan, 1973) yet 2-butanol will still cause the inactivation of active papain even in the presence of cysteine.

A simple explanation for the inactivation effect of 2-butanol on papain which is consistent with our observations is shown in Scheme III. The 2-butanol (B) initially binds to the active

SCHEME III

$$E_a + B \underset{}{\overset{K_1}{\rightleftharpoons}} E \cdot B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_i + B$$

enzyme (E_a) to form a noncovalent complex (E·B) with dissociation constant K_1. The complex then undergoes reaction

[2] Butadione is known to react covalently with arginine residues and hence is an example of a covalent modification which could result in inactivation (Yang and Schwert, 1972).

to form E_i, an inactive form of papain. E_i may or may not contain bound 2-butanol; further experiments utilizing labeled 2-butanol should answer this question. Since the inactivation is essentially irreversible, $k_{-1} \approx 0$. If the formation of EB is assumed rapid relative to its subsequent transformation, the rate of inactivation is given by eq 1 which reduces

$$V = \frac{k_2 B_0 [E_a]}{K_1 + [E_a]} \quad (1)$$

to eq 2 if $K_1 \gg [E_a]$, where E_a = active enzyme, B_0 = total

$$V \approx \frac{k_2 B_0 [E_a]}{K_1} \quad (2)$$

2-butanol concentration, and k_2 and K_1 refer to Scheme III. Thus the observed pseudo-first-order rate constant, $k_{obsd} = (k_2/K_1)B_0$, and from Table I, the value of $k_2/K_1 = 24.3 \pm 2.3$ M^{-1} min^{-1}. According to eq 1, if $[E_a] \cong K_1$ a non-first-order rate of inactivation should be observed. At a papain concentration of 7.5×10^{-5} M no deviation from first order was observed in the inactivation. Therefore $K_1 \gg 7.5 \times 10^{-5}$ M. The value of K_1 would not be expected to be very small; in fact, the value for the dissociation constant of 1-butanol to papain has been estimated at 0.75 M (Fink and Bender, 1969). A value of this order of magnitude for K_1 would make $k_2 \sim 1.8 \times 10^3$ sec^{-1}. This is a very reasonable value for the rate of a conformational change in a protein (Hammes, 1968).

Additional support for a rate-determining conformational change is found in the spectral data. For example, since the rate for the change in fluorescence of the enzyme paralleled the rate of inactivation the fluorescence change must be ascribed directly to the inactivation process. Steiner (1971) has proposed that most of the fluorescence emission in papain is due to one or possibly two tryptophans. Thus the change in λ_{max} and intensity of the fluorescence emission on inactivation suggests perturbations involving one of the tryptophans in the vicinity of the active site. The shift in λ_{max} suggests a more polar environment for the tryptophan in the inactive enzyme. The effect cannot be due to the binding of 2-butanol directly adjacent to the tryptophan since this would decrease the polarity. Therefore the tryptophan must be in a different environment in the inactive enzyme due to either a shift in the position of tryptophan and/or its neighboring groups. The uv absorption and circular dichroic difference spectra show no tryptophan perturbation, but this is not surprising since the fluorescence is much more sensitive and showed only a small effect. The difference in the uv spectra of active and inactive papain at wavelengths below 250 nm is consistent with a conformational change involving the peptide backbone. The magnitude of the spectral differences suggests that the conformational changes are relatively small.

The loss of titratable thiol groups in the 2-butanol-treated enzyme can account for the loss of catalytic activity since the one free thiol in papain, that of Cys-25, is essential for activity. The lack of thiol in the inactivated enzyme implies that either the -SH is sterically inaccessible to the Nbs_2 or it is tied up in a chemical bond. The latter seems highly unlikely as far as the inactivating reagent is concerned. Steric inaccessibility could result from either the 2-butanol being tightly bound in the vicinity of the thiol blocking attack by Nbs_2 or substrate, or to an induced conformational change in which the approach to the thiol becomes blocked by part of the enzyme.

Effect of Substrate on Inactivation. If the 2-butanol bound in the active site the presence of the substrate (under saturation conditions) would be expected to prevent or decrease the inactivation reaction, unless the 2-butanol bound equally well or better than substrate. The latter possibility may be ruled out since K_m for the substrate (Cbz-LysONph) is 2×10^{-6} M and K_s has been estimated to be approximately 3.3×10^{-5} M (Brubacher and Bender, 1966), whereas K_1 for 2-butanol is much greater than 7.5×10^{-5} M and probably close to 1 M. Since the presence of the substrate increases the rate of inactivation (Table II) the 2-butanol must bind at a site distinct from the active site. Furthermore, the substrate, during the reaction, must cause some change in the enzyme structure which facilitates the inactivation reaction. This observation is strong evidence for the existence of a conformational change either on binding of substrate and/or during the subsequent steps in the catalytic reaction.

pH Dependence. Papain-catalyzed hydrolyses have been shown to be dependent on groups in the enzyme with $pK_a \sim 4.3$ (3.5 → 4.7) and 8.5 which appear to be best assigned to the thiol of Cys-25 and the imidazole of His-159 (Lowe, 1970; Jolley and Yankeelov, 1972). Since the 2-butanol-induced inactivation shows similar dependence on a group with $pK_{app} = 4.0$ (Figure 3) it is tempting to associate the group with $pK = 4.0$ in inactivation with that group of the same pK in the catalytic reaction.

The pH dependence of inactivation may be explained in at least two ways. (1) The 2-butanol binds in close proximity to a group of $pK = 4.0$ whose ionic state affects the binding and subsequent conformational change. (2) The 2-butanol binds to a site distant from the group with $pK = 4.0$. However, the ionic state of this group determines which of two pH dependent conformations of the enzyme will exist and the inactivation is faster with the form existing under basic conditions. If this were the case then it is possible that the pH dependence of the catalytic reaction arises from the same source, *i.e.*, two pH dependent conformations of the enzyme.

Conclusions

We have provided experimental evidence to indicate that some four-carbon chain ligands, such as 2-butanol, bind to papain in a manner which results in a conformational response (Citri, 1973) yielding an essentially irreversibly inactivated papain, in which the thiol group of Cys-25 is no longer accessible to Nbs_2.

Since saturating concentrations of specific substrates enhance the rate of inactivation rather than protecting against it, we can conclude that the 2-butanol binds at a site distinct from the substrate binding site. Furthermore the presence of the substrate causes a change in the conformation of the enzyme such that it is more susceptible to the inactivation process. The available data suggest that the conformational changes induced by substrate and 2-butanol are small and probably localized. Since both inactivation and catalytic processes have similar dependencies on pH it is possible that the observed pK reflects an ionization controlling a conformational change which results in a catalytically active and 2-butanol-susceptible form at higher pH.

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Mechanism of Calcium Induction of *Renilla* Bioluminescence. Involvement of a Calcium-Triggered Luciferin Binding Protein†

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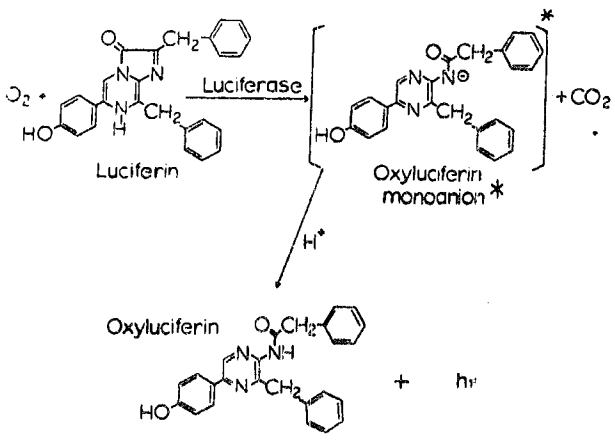
ABSTRACT: When extracts of bioluminescent Anthozoans, such as *Renilla*, are prepared in the presence of metal chelators they can be induced to bioluminesce upon the addition of calcium ions. In this sense such bioluminescence emissions resemble those of the calcium-dependent photoprotein isolated from *Aequorea*. However, in the case of *Renilla* such calcium-induced bioluminescence involves a calcium-triggered release of luciferin from a binding protein prior to catalysis by luciferase. The luciferin binding protein can be completely separated from luciferase and the presence of luciferin bound to the binding protein has been demonstrated by spectroscopy. Light production requires the presence of the charged luciferin binding protein, luciferase, calcium, and oxygen. Luciferin can be removed from the binding protein and the resulting discharged binding protein can be recharged in the presence of excess luciferin. The properties of the recharged binding protein are the same as those of the native one. The calcium triggering of the luciferin binding protein of *Renilla* is the logical choice of mechanism for linking the nerve impulse to the bioluminescent system in this animal. The evidence suggests that a luciferin binding protein exists among the bioluminescent Anthozoans generally but not among the bioluminescent Hydrozoans. Thus, in the Anthozoans the calcium-induced *in vitro* bioluminescence is due to two proteins. One is a calcium-triggered luciferin binding protein and the other is luciferase. In the Hydrozoan, *Aequorea*, the photoprotein is viewed as playing a dual role. That is, it acts as a luciferin binding protein on the one hand while in the presence of calcium it acts like a luciferase.

A brilliant green luminescence is produced by *Renilla reniformis*, an Anthozoan coelenterate, upon appropriate stimulation. In recent years there has been considerable progress in elucidating the biochemical components involved in this bioluminescence. The identity of several of the proteins involved (Cormier and Totter, 1968; Cormier *et al.*, 1970a,b; Karkhanis and Cormier, 1971; Wampler *et al.*, 1971; DeLuca *et al.*, 1971; Hori *et al.*, 1972) as well as the structure of a fully active analog of the light producing substrate, or luciferin (Hori *et al.*, 1973; Hori and Cormier, 1973), have been determined. The light-producing reaction involves the oxidation of the fully active luciferin analog as shown in Scheme I. The above reactions produce a blue emission (λ_{max}

SCHEME I



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