Cryoenzymology of Papain: Reaction Mechanism with an Ester Substrate†

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ABSTRACT: The reaction between papain and N^{α}-carbobenzoxy-L-lysine methyl ester has been studied at subzero temperatures in fluid aqueous dimethyl sulfoxide. We have previously shown that this cryosolvent has no adverse effects on the catalytic or structural properties of papain (Fink, A. L., & Angelides, K. J. (1976) *Biochemistry 15,* 5287). Monitoring the reaction by absorbance spectroscopy revealed maximal changes in the vicinity of 276 nm. When the reaction was initiated by mixing enzyme and substrate at very low temperatures, a series of three reactions, prior to rate-limiting deacylation, was observed. Reaction 1 had a pseudo-first-order rate constant $> 1\ s^{-1}$ at −65 °C and is interpreted to correspond to the binding of substrate. Reaction 2 was pH* dependent, with an estimated pK* of ≤ 3.4 and $k_{obsd}^{lim} = 8 \times 10^{-2}\ s^{-1}$ at −40 °C, and an energy of activation (E_a) of 7.4 kcal mol^{-1}. (pH* and pK* refer to the apparent protonic activity and pK in the cryosolvent.) Reaction 2 is attributed to a repositioning of the active-site imidazole. Reaction 3 had $E_a = 10.2$ kcal mol^{-1} and was also pH* dependent, with pK* = 4.8 and $k_{obsd}^{lim} = 8.9 \times 10^{-4}\ s^{-1}$ at −16.5 °C. Reaction 3 is ascribed to formation of the acyl-enzyme. If papain inactivated by alkylation with N^{α}-tosyl-L-lysine chloromethyl ketone was used in place of active papain, none of these reactions was observed. On the other hand if *S*-methylthio-Cys-25-papain was used, reactions 1 and 2 were still present but reaction 3 was absent. Reactions 1 and 2 could also be detected by changes in the fluorescence emission of the enzyme. Although the rate of reaction 2 was pH* independent above pH* 4.0, the magnitude of the corresponding decrease in fluorescence emission was pH* dependent with a pK* of 5.1 at −36 °C. Reactions 2 and 3 were absent with mercuripapain. A detailed mechanism involving movement of the active-site imidazole, which accounts for many of the apparently contradictory previously reported pK assignments, is proposed. A key feature of this mechanism is the existence of two conformational states of the enzyme, one with the imidazole of His-159 hydrogen bonded to Asn-175 ($pK_{Im} \sim 4$; $pK_{SH} \sim 8$), and the other involving the imidazole protonated, and electrostatically interacting with the carboxylate of Asp-158 and the thiolate of Cys-25 ($pK_{Im} \sim 8$; $pK_{SH} \sim 4$).

Cryoenzymology, the use of very low temperatures and fluid solutions, is a novel method that provides the potential to detect, accumulate, and characterize intermediates in enzyme-catalyzed reactions of specific substrates. The subject has recently been reviewed (Fink, 1977; Makinen & Fink, 1977; Fink, 1976a; Douzou, 1977; Douzou, 1974). For papain catalysis we have previously shown that the reaction pathway in 60% aqueous dimethyl sulfoxide at subzero temperatures is essentially the same as in the absence of the cosolvent at ambient temperatures (Fink & Angelides, 1976). In particular, the kinetic parameters for catalysis and the intrinsic spectral properties of the enzyme show no evidence of any solvent- or temperature-induced structural changes or other adverse effects as a function of increasing Me_2SO[1] concentrations.[2] Furthermore, when the reaction between papain and N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester was initiated at −70 °C, pH* 7.0, the amount of *p*-nitrophenol ester liberated was essentially stoichiometric with the enzyme concentration and indicated the formation and trapping of ≥85% of the corresponding acyl-enzyme (Fink & Angelides, 1976). No further release of *p*-nitrophenol was noted over a period of hours. The technique, then, is admirably suited for producing high concentrations of stabilized enzyme–substrate intermediates. In fact if crystalline enzyme is used, crystalline intermediates sufficiently stable for x-ray crystallographic studies may be obtained (Fink & Ahmed, 1976; Alber et al., 1976).

Information concerning enzyme–substrate interactions and structural changes within the enzyme or substrate during the catalytic reaction can be obtained from physicochemical studies of the intermediates trapped at these low temperatures. One such approach, as has been demonstrated with chymotrypsin (Fink, 1976b), is to incorporate a chromophoric group as a part of the substrate. Alternately, as in the work reported here, the intrinsic absorption and fluorescence properties of the enzyme can be utilized to monitor the catalytic reaction (cf. Fink & Wildi, 1974). N^{α}-Carbobenzoxy-L-lysine methyl ester was chosen as the specific substrate for this study since it is essentially transparent in the region of tryptophan absorption in the enzyme. It has previously been shown that the majority of the fluorescence emission of papain is due to just one tryptophan residue, namely, Trp-177, which is located, fortuitously, in the active site (Lowe & Whitworth, 1974). For this substrate the overall rate-limiting step is deacylation (Bender & Brubacher, 1966).

Considerable evidence has accumulated in support of a minimum scheme for the catalytic reaction of papain of the sort shown in Scheme I where ES is the Michaelis complex, EA the acyl-enzyme, and P_1 and P_2 the alcohol (or amine) and acid products, respectively (Lowe, 1976; Glazer & Smith, 1971). Several studies have suggested that papain-catalyzed hydrolyses may involve additional intermediates to those in

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[1] Abbreviations used: Tos-LysCH_2Cl, N^{α}-tosyl-L-lysine chloromethyl ketone; Me_2SO, dimethyl sulfoxide; UV, ultraviolet; papain-S-S-CH_3, the S-methanethio derivative of Cys-25 of papain; EDTA, ethylenedinitrilotetraacetic acid.

[2] As in most enzyme-catalyzed reactions the value of K_m increases with increasing cosolvent concentration. We believe this corresponds to a less favorable partitioning of the substrate to the active site, involving the hydrophobicity of the cryosolvent.

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Scheme I (Lake & Lowe, 1966; Henry & Kirsch, 1967; Holloway & Hardman, 1973; Fink & Gwyn, 1974; Mattis & Fruton, 1976). However, with the exception of the formation of a thiol ester acyl-enzyme intermediate, the details of the catalytic steps in papain reactions continue to remain elusive. In particular controversy has revolved around the groups responsible for the pH dependence of the reactions.

SCHEME I

$$E + S \rightleftarrows ES \rightleftarrows EA \rightarrow E + P_2$$
$$+$$
$$P_1$$

The present investigation was undertaken to provide further and more conclusive evidence regarding the nature of the intermediates in papain-catalyzed reactions. In addition cryoenzymology affords the opportunity to study individual steps in the overall reaction and thereby provides a means of achieving a better understanding of the underlying groups responsible for pH dependence.

We report here evidence for three intermediates on the productive catalytic pathway and present kinetic, thermodynamic, and structural information regarding these intermediates and their transformations. In addition a detailed mechanism, consistent with our observations, is proposed, which accounts for many of the apparently contradictory pK assignments which have been previously reported.

Experimental Procedures

Materials. N^{α}-Carbobenzoxy-L-lysine methyl ester and N^{α}-carbobenzoxy-L-lysine p-nitrophenyl ester were obtained from Vega-Fox lots no. F7158 and F7713, respectively. N^{α}-Tosyl-L-lysine chloromethyl ketone was a product of Sigma lot no. T-1710. Reagent grade sodium acetate, sodium formate, boric acid, EDTA, β-mercaptoethanol, trichloroacetic acid, and dimethyl sulfoxide were from Mallinckrodt. The dimethyl sulfoxide was further purified by distillation from calcium hydride under vacuum at 37 °C, recrystallized at 2 °C, and stored at or below 4 °C. Methyl methanethiosulfonate and its [^{14}C]methyl form were generously supplied by Dr. George Kenyon, Department of Pharmaceutical Chemistry, University of California, San Francisco. The specific activity of the ^{14}C-labeled compound was 12 mCi/mmol. [^{14}C]Iodoacetic acid was purchased from Amersham/Searle and had a specific activity of 57 mCi/mmol.

Papain (2× crystallized) was obtained from Worthington Biochemical Corp. (lots no. 36D184 and 36B828), activated with 30 mM β-mercaptoethanol, 25 mM EDTA, and further purified by affinity chromatography using the method of Blumberg et al. (1970). The purified enzyme was then concentrated by ultrafiltration to approximately 8 mg/mL in a 20-mL Amicon Diaflo apparatus equipped with a UM2 membrane. The purified enzyme was stored in a sealed vial under nitrogen at 4 °C.

Tos-LysCH_2Cl-inactivated papain was prepared by a modification of the method of Whitaker & Perez-Villaseñor (1968). A solution of 0.5 mM purified papain was mixed with a 16-fold excess of Tos-LysCH_2Cl at pH 4.5 in 0.1 M sodium acetate buffer containing 0.5 mM EDTA. After 10 min at room temperature, the residual enzymatic activity was less than 1% of the initial activity (as measured in an assay using N^{α}-carbobenzoxy-L-lysine p-nitrophenyl ester).

Papain-S-S-CH_3, the S-methylthio derivative of Cys-25 of papain, was prepared by the method of Smith et al. (1975). The absence of catalytic activity (less than 1%) after addition of methyl methanethiosulfonate indicated that the reaction was complete. In order to separate papain-S-S-CH_3 from the other components in the reaction mixture, the mixture was passed through a 1 × 25 cm column of Sephadex G-25. The solution was then concentrated to 2.5×10^{-4} M. The specific catalytic activity of the papain reactivated from the papain-S-S-CH_3 derivative after treatment with 20 mM β-mercaptoethanol was within experimental error of the activity of the initial enzyme.

Methods. The concentration of papain and derivatives of papain was determined from the absorbance at 280 nm using $\epsilon = 5.77 \times 10^4$ cm^{-1} M^{-1} (Skalski et al., 1973). The catalytic activity of affinity-chromatographed papain was checked daily either by burst titration with N^{α}-carbobenzoxy-L-tyrosine p-nitrophenyl ester (Bender et al., 1966) or a kinetic assay with N^{α}-carbobenzoxy-L-lysine p-nitrophenyl ester (Brubacher & Bender, 1966). Both gave results in good agreement with each other.

Aqueous organic buffers used for the kinetic measurements were made up on a volume/volume basis at 0 °C as previously described (Fink, 1973). Buffer systems used were: 0.1 M formate (pH* 2.8–5), 0.2 M acetate (pH* 5–7), and 1 mM borate (pH* 7–9.2). All aqueous organic solvents were made up to 0.1 M ionic strength using KCl. All buffers contained 250 μM EDTA. The apparent protonic activity (pH*) was measured using a Radiometer glass electrode and calomel reference electrode at either 0 or 25 °C. Values of pH* at subzero temperatures were obtained by extrapolation using the data of Hui Bon Hoa & Douzou (1973; Douzou, 1974). All pH*s reported here have been corrected to the specified temperature of the experiment.

Spectral measurements at subzero temperature were performed with a Cary 118C spectrophotometer equipped with a repetitive scanning attachment, and with a Perkin-Elmer MPF-4 spectrofluorometer, either at fixed wavelength or by repetitive spectral scans. The experiments were carried out in the following manner. Enzyme and substrate solutions of the appropriate concentrations were prepared in the aqueous organic buffer at 0 °C, taken up into precooled syringes, mounted on a six-jet tangential mixing block, and cooled to the specified temperature of the experiment. The contents were then injected into a precooled cell; in the case of the UV absorption experiments, a double-walled vacuum-jacketed quartz cell with 1-cm pathlength was used; in the fluorescence measurements the contents were injected into a cell that was maintained at the desired temperature by a specially constructed, thermostated, brass cell block holder. Mixing was both rapid and homogeneous as determined using dye solutions. Additional mixing at the low temperature was achieved by the use of a "vibrating reed" mixer with a frequency of 60 Hz. The temperature was maintained using Neslab LT-9 or Heto Ultra Cryotherm low-temperature baths in which circulating ethanol was used as a coolant. In all experiments the temperature was constant within ±0.1 °C and was continuously monitored with a thermocouple or temperature-sensitive diode probe.

To determine the amount of enzyme-substrate intermediate accumulated as the product of reaction 2 or 3 (see Results), the following methods were used. Papain and N^{α}-carbobenzoxy-L-lysine methyl ester were mixed as described above and the spectral changes corresponding to the formation of the intermediate (product of reaction 2 or 3) were monitored. An appropriate aliquot of either N^{α}-carbobenzoxy-L-lysine p-nitrophenyl ester, [^{14}C]iodoacetic acid, or [^{14}C]methyl methanethiosulfonate (tenfold excess over enzyme) was added to the reaction mixture at a selected time. With the former a "burst" of p-nitrophenol was liberated in the reaction between free papain and N^{α}-carbobenzoxy-L-lysine p-nitrophenyl ester

TABLE I: Representative Kinetic Data for the Reaction of Papain with N^{α}-Carbobenzoxy-L-lysine Methyl Ester.[a]

Reaction	pH*	Temp (°C)	k_{obsd} (s^{-1})
2	3.4	−38.5	5.2×10^{-3}
	4.1	−27.0	4.3×10^{-2}
	4.1	−38.5	2.0×10^{-2}
	4.1	−48.5	1.3×10^{-2}
	4.1	−60.0	5.5×10^{-3}
	4.4	−60.0	5.5×10^{-3}
	5.8	−48.9	1.2×10^{-2}
	6.8[b]	−40.0	1.2×10^{-2}
	6.8[b,c]	−40.0	1.2×10^{-2}
	7.0	−65.0	1.3×10^{-3}
	8.9[a]	−42.7	1.2×10^{-2}
3	3.8	−16.9	2.6×10^{-5}
	4.3	−56.0	1.1×10^{-5}
	5.7	−17.0	2.0×10^{-4}
	6.0	−59.0	1.3×10^{-5}
	6.5	−16.9	3.7×10^{-4}
	6.9	−47.7	2.0×10^{-5}
	9.0	−27.0	2.8×10^{-5}

[a] Conditions: $E_0 = 1.0 \times 10^{-5}$ M; $S_0 = 1.0 \times 10^{-3}$ M except as noted. Typical average deviations were ± 10%. [b] $E_0 = 3.0 \times 10^{-5}$ M; $S_0 = 1.0 \times 10^{-3}$ M. [c] For papain-S-S-CH_3.

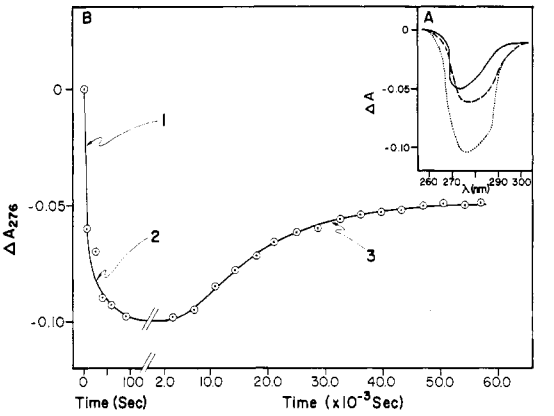

FIGURE 1: (A) UV-difference spectra of the products of reaction 1 (—), reaction 2 (····), and reaction 3 (- - - -) in the reaction of papain with N^{α}-carbobenzoxy-L-lysine methyl ester at −40 °C, pH* 6.8, $E_0 = 1.0 \times 10^{-5}$ M, $S_0 = 1.0 \times 10^{-3}$ M, 60% aqueous dimethyl sulfoxide. The difference spectra were obtained by subtracting the sum of the individual absorbance contributions of the enzyme and substrate under identical conditions from that of the reaction product. (B) Time course of the reaction at −40 °C under the same conditions as in A; the solid curve is a calculated one based on $k_{obsd}^2 = 2.0 \times 10^{-2}$ s^{-1} and $k_{obsd}^3 = 5.7 \times 10^{-5}$ s^{-1}; the points (⊙) are experimental values.

which gave a measure of the active-site molarity of the free thiol. In the case of the ^{14}C-labeled compounds, the aliquot was followed by a portion of 20% trichloroacetic acid in 60% dimethyl sulfoxide. After the enzyme precipitated, it was washed successively with several portions of anhydrous petroleum ether and acetone to remove the excess reagent, and then counted on a Beckman liquid scintillation counter Model CPM 100. The basis of these experiments in which free papain was inhibited by [^{14}C]methyl methanethiosulfonate or [^{14}C]iodoacetate, and precipitated by trichloroacetic acid, is that the reverse reaction (k_{-2}) and deacylation (k_4) (see Scheme II) be slower than the rate of alkylation of free thiol and subsequent enzyme denaturation. In control experiments alkylation of the free thiol in papain (to >99.0%) and denaturation were complete in less than 10 min at −40 °C pH* 6.1 in 60% dimethyl sulfoxide. Under these same conditions of temperature, solvent, and pH*, the deacylation rate, k_4 (estimated $k_{obsd} = 1 \times 10^6$ s^{-1}), is very much slower than the rate of alkylation of the free sulfhydryl group and the rate of enzyme denaturation by trichloroacetic acid. Both the "burst" titration and ^{14}C-labeled isotopic methods gave results in excellent agreement with each other.

All experimental measurements were done at least in duplicate.

Computer Methodology. The differential equations for the time dependence of the intermediates are given in Fink (1976a) and were solved numerically by the Runge-Kutta-Gill method on an IBM 360/40 computer (cycle time, 1 μs) using Fortran IV, or a PDP-11 using Basic-Plus. Plotting was done on an IBM 1620 plotter in either Basic or Fortran IV languages. The equation is a kind of "stiff" differential (Garfinkel & Marbach, 1977) and requires much computer time. However, the following techniques were used to speed up calculations: (1) the integration step size is variable and automatically controlled so that the error is kept below 0.01% and (2) the values used for the rate constants for reactions 1-3 were multiplied by 10^3. This in effect shortens the required computer time by 10^3. A copy of the program is available from the authors on request.

Results

Ultraviolet Absorption Experiments. When the reaction between N^{α}-carbobenzoxy-L-lysine methyl ester and papain was initiated under non-turnover conditions and monitored in the UV region, the maximal spectral changes were observed in the vicinity of 276 nm (Figure 1A). Under conditions of excess substrate, pH* 6.8 and −40 °C, three consecutive reactions were observed (Figure 1B). The first reaction (reaction 1) appeared as a very rapid decrease in absorbance with λ_{max} for the difference spectrum of the product at 276 nm (Figure 1A). Even at −65 °C reaction 1 was complete within the dead-time of the system and a $k_{obsd} > 1$ s^{-1} was estimated ($S_0 \gg E_0$). The rate of the subsequent decrease in A_{276} (reaction 2) was found to be independent of pH* over the range 4.1 to 8.2. However, below pH* 4.1 the rates decreased considerably (Table I). A pK* of approximately 3.4 ± 0.3 at −40 °C was estimated from a plot of k_{obsd} vs. $1/[H^+]$ for four experiments carried out in the range pH* 3.4 to 4.1. A more accurate value of this pK* was not obtained due to the anticipated instability of the enzyme at lower pH* values. The limited data also prevented unambiguous determination of whether one or two ionizations are involved; however, the data do give a good fit to a single ionization. No irreversible denaturation occurred at pH* 3.4 (Fink & Angelides, 1976). The Arrhenius plot for reaction 2 was linear over the temperature range investigated (−30 to −65 °C). From extrapolation of the Arrhenius plot to 25 °C and correction for the effect of the solvent on K_m (Fink & Angelides, 1976), values of $\Delta G^{\ddagger} = 13.2$ kcal mol^{-1}, $\Delta S^{\ddagger} = -22$ eu, and $\Delta H^{\ddagger} = 6.8$ kcal mol^{-1} were calculated for 25 °C, 0% cosolvent.

Reaction 3 was observed as a slow increase at A_{276} (Figure 1B) and was found to be pH* dependent with a pK* = 4.8 ± 0.2 at −16.5 °C. The Arrhenius plot for reaction 3 was linear over the range −16 to −50 °C and provided values of $\Delta G^{\ddagger} = 16.1$ kcal mol^{-1}, $\Delta S^{\ddagger} = -21.7$ eu, and $\Delta H^{\ddagger} = 9.6$ kcal mol^{-1} at 25 °C, 0% cosolvent. The temperature dependence of the pK* for reaction 3 was measured to provide an estimate of the heat of ionization of the residue(s) associated with this pK*. A value of $\Delta H_{ion} = 8.0 \pm 2.0$ kcal mol^{-1} was estimated from the following data: pK* = 4.0 ± 0.2 at −5 °C, pK* = 4.8 ± 0.2

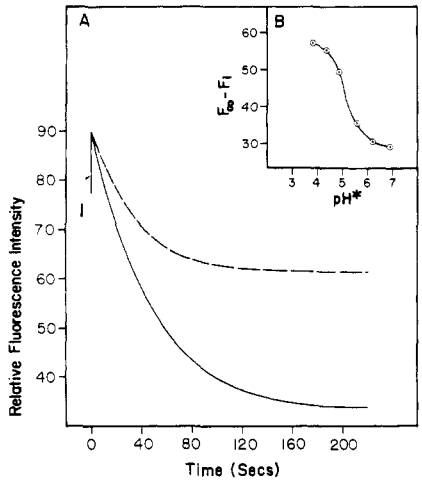

FIGURE 2: (A) Reactions 1 and 2 as monitored by fluorescence emission spectroscopy. Excitation at 290 nm, emission at 333 nm. Conditions were pH* 3.9 (—), pH* 6.7 (- - - -), 60% aqueous dimethyl sulfoxide, −35.8 °C, $E_0 = 2.0 \times 10^{-5}$ M, $S_0 = 1.0 \times 10^{-3}$ M. The solid line labeled 1 corresponds to reaction 1. (B) Magnitude of the fluorescence quenching for reaction 2 as a function of pH*. Conditions were the same as in A. $F_0 - F_1$ = the magnitude of the decrease in fluorescence emission in arbitrary units. The solid curve is calculated for pK^* = 5.1.

at −16.5 °C; and pK^* = 5.8 ± 0.3 at −48.9 °C. The pK^* of 5.1 at −36 °C observed for the amplitude of the fluorescence change associated with reaction 2 (see below) was found to fall on the same line as these three values.

Representative kinetic data for these reactions are given in Table I. No changes occurred when either enzyme or substrate were omitted from the reaction mixture. None of the reactions were observed with papain previously inactivated with Tos-LysCH_2Cl. With papain-S-S-CH_3 both reactions 1 and 2 were observed; however, reaction 3 was absent. With mercuripapain a small initial absorbance increase was observed. Reactions 2 and 3 were absent.

Two procedures, based on the ability to selectively react with the thiol of Cys-25 in the free enzyme but not in the intermediates in which the reverse reaction for their formation, i.e., k_{-i}, was slow, were used to determine the concentration of the intermediates formed in reactions 2 and 3. Because the reaction of N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester is much faster than that of the corresponding methyl ester, it could be used as an acylating reagent (Fink & Angelides, 1976). At −40 °C the turnover reaction for the nitrophenyl ester substrate is negligible, and the amount of active enzyme (from E and ES) can be calculated from the "burst" of *p*-nitrophenol produced. In the alternate method [^{14}C]iodoacetate or [^{14}C]methyl methanethiosulfonate were used to titrate the free thiol. At the completion of reaction 2 greater than 90% of the -SH groups reacted with the alkylating reagents, indicating that in their presence the reverse reaction, $I_2 \rightleftarrows I_1 \rightleftarrows E + S$, was sufficiently rapid that all, or essentially all, of I_2 was reconverted back to free enzyme and alkylated. On the other hand the rate of formation of I_3 determined by alkylation was found to be identical to that obtained spectrophotometrically under similar conditions. After 4.5 half-lives the concentration of I_3 obtained by both alkylation and acylation procedures was 84 ± 4% of the total enzyme concentration. In control experiments in which mercuripapain or Tos-LysCH_2Cl-inactivated papain were used 0% active enzyme was detected by these methods. If the substrate was omitted, 100% active enzyme was present according to [^{14}C]iodoacetate titrations.

To facilitate the kinetic analysis and aid in characterization of the nature of the covalent attachment in I_3 (i.e., tetrahedral intermediate or acyl-enzyme), reactions were carried out in the presence of excess added methanol (1.0×10^{-3} to 1.7 M). The added methanol had no apparent effect on the magnitude of the absorbance change or the rate of reactions 1 or 2. However, both the magnitude and rate of reaction 3 were depressed at high concentrations of methanol, Table II. Furthermore, titrations with [^{14}C]iodoacetate revealed that in the presence of excess P_i the concentration of covalent intermediates was also decreased (Table II).

TABLE II: The Effect of Added Methanol on Reaction 3.[a]

[Methanol] (M)	k_{obsd}^3 (s^{-1})	$[I_3]$[b] (%)[c]
0	5×10^{-5}	83
1.0×10^{-3}	3×10^{-5}	80
7.0×10^{-3}	4×10^{-5} [d]	30
1.0×10^{-2}	1×10^{-5}	33
1.7	[e]	5

[a] Conditions: $E_0 = 1.0 \times 10^{-5}$ M, $S_0 = 1.0 \times 10^{-3}$ M, pH* 6.8, −40 °C, 60% aqueous dimethyl sulfoxide. [b] From titration with [^{14}C]iodoacetate at completion of reaction 3. [c] Covalently modified enzyme as a percent of total enzyme concentration. [d] $S_0 = 3.0 \times 10^{-3}$ M. [e] Rate was too slow to measure readily.

All the experiments carried out with the methyl ester substrate at subzero temperatures were under conditions at which turnover was negligibly slow. Confirmation that turnover did not occur was based on the following: extrapolation of the known kinetics in aqueous solution, 25 °C, to those for 60% aqueous dimethyl sulfoxide, subzero temperatures (Fink & Angelides, 1976); pH-stat experiments to measure the rate at 0 °C; observation that the rates of reactions 2 and 3 were proportional to substrate concentration but not enzyme concentration when $S_0 \gg E_0$ and that the amplitude was proportional to the enzyme concentration (Fink, 1976a) (under conditions of $E_0 \ll S_0 \ll K_m$, the turnover reaction rate will be proportional to E_0).

Fluorescence Experiments. Since the substrate is nonfluorescent, the intrinsic fluorescence of papain, due mostly to the active-site Trp-177 (Lowe & Whitworth, 1974), was also used to monitor the reaction at subzero temperatures. Time-dependent changes in the fluorescence emission were detected. A rapid increase followed by a slower decrease was observed, corresponding to reactions 1 and 2 seen in the UV absorption experiments (Figure 2A). Reaction 3 was not detected by fluorescence changes. The rate of reaction 2 was again found to be pH* independent from pH* 4.1 to 6.8, with a value of $k_{obsd} = 2.2 \times 10^{-2}$ s^{-1} being obtained at −36 °C, $S_0 = 1.0 \times 10^{-3}$ M. The magnitude of the decrease in fluorescence emission, however, was found to be pH* dependent. From pH* 3.8 to 6.8 the relative fluorescence intensity change for reaction 2 decreased by 50%. From a plot of relative fluorescence quenching vs. pH* a pK^* of 5.1 ± 0.2 at −36 °C was determined for this process (Figure 2B). The origin of this fluorescence quenching is considered in the Discussion section.

Kinetic and Thermodynamic Analysis. The simplest model to explain the results is given in Scheme II.

SCHEME II

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} I_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EA \xrightarrow{k_4} E + P_2$$
$$+$$
$$P_i$$

The model assumes in the forward direction that there is a very rapid association of enzyme with substrate to produce the Michaelix complex, ES_1 (reaction 1). This is subsequently converted in a first-order process to I_2 (reaction 2) which

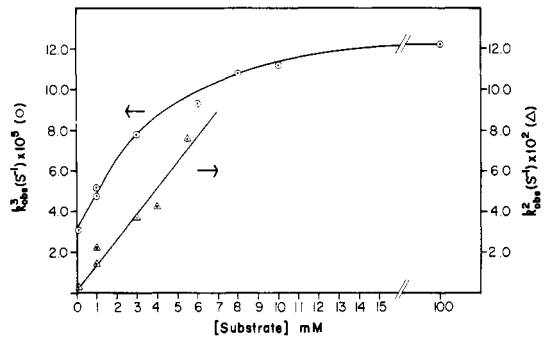

FIGURE 3: The rate of reactions 2 (△) and 3 (○) as a function of substrate concentration, pH* 6.8, −40 °C, 60% aqueous dimethyl sulfoxide, E_0 = 1.0×10^{-5} M.

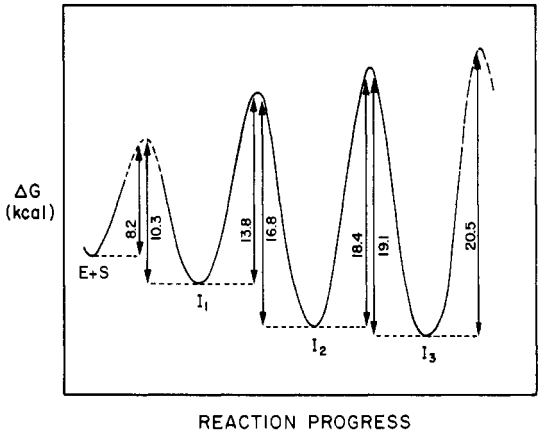

FIGURE 4: Free-energy diagram for the reaction of papain with N^{α}-carbobenzoxy-L-lysine methyl ester at −40 °C, 60% aqueous dimethyl sulfoxide. A scheme of the form $E + S \rightleftarrows ES_1 \rightleftarrows I_2 \rightleftarrows I_3'$ is assumed where I_3' corresponds to either EA + P_1 or I_3 + EA + P_1 (see Schemes II and III). The broken lines represent uncertainties in the values of $\Delta G^{\ddagger}$ due to necessary assumptions in ascertaining the values for the rates of reactions 1 and 4 under these conditions.

undergoes conversion to the covalent acyl-enzyme intermediate and release of the alcohol moiety, P_1 (reaction 3). For the non-turnover, non-steady-state, $S_0 \gg E_0$, conditions used, the expressions for the first-order rate constant, k_{obsd}^2, for reaction 2, and the maximum concentration of I_2 are given in eq 1-2 (Fink, 1976a). Thus plots of k_{obsd}^2 vs. S_0 may provide

$$k_{obsd}^2 = k_{-2} + \frac{k_2}{1 + (K_1/S_0)} \quad (1)$$

$$[I_2]_{max} = \frac{k_2 E_0/(1 + (K_1/S_0))}{k_{-2} + [k_2/(1 + (K_1/S_0))]} \quad (2)$$

values of k_2, k_{-2}, and K_1. Unfortunately it was not experimentally feasible to work at sufficiently high substrate concentrations to determine the value of k_2 or K_1 in this manner, Figure 3. The values which could be obtained were $k_{-2} = (2.4 \pm 2) \times 10^{-3}$ s^{-1} at −40 °C, and $k_2/K_1 = (10 \pm 4)$ M^{-1} s^{-1}. The rapidity of reaction 1 prevented direct determination of the values of k_1 and k_{-1}. From the data of Figure 3, K_1 must be $>1 \times 10^{-3}$ M. A reasonable estimate would be 10^{-2} M under these particular experimental conditions. This would mean that $k_2 = 1 \times 10^{-1}$ s^{-1} and $K_2 = 2 \times 10^{-2}$ M, i.e., step 2 significantly favors the product of reaction 2.

A comparable kinetic analysis of reaction 3 is complicated by the necessity of knowing whether the rate of this reaction corresponds to the actual formation of EA + P_1 (Scheme II), or whether the rate may correspond to the formation of a preceding intermediate in which P_1 is still attached to the

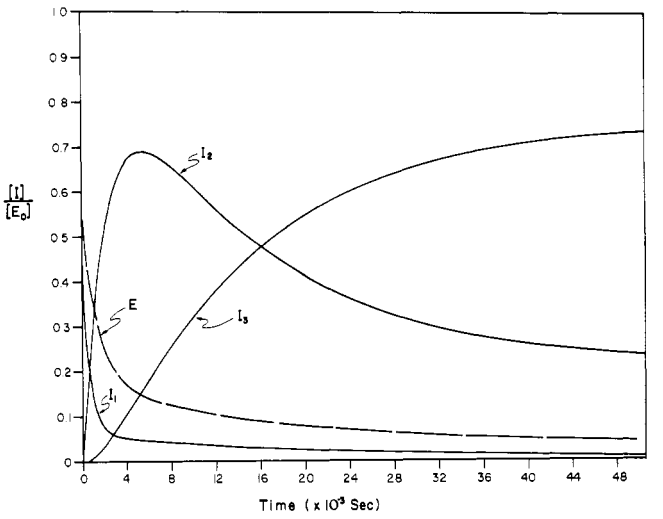

FIGURE 5: Computer simulation of the time dependence of the concentrations of intermediates I_1, I_2, and I_3 in the reaction of papain with N^{α}-carbobenzoxy-L-lysine methyl ester at −40 °C, pH* 6.8, 60% aqueous dimethyl sulfoxide. The ordinate for I_1 is ×10. The values of the microscopic rate constants used were $k_1 = 10^5$ M s^{-1}, $k_{-1} = 10^4$ s^{-1}, $k_2 = 0.6$ s^{-1}, $k_{-2} = 4 \times 10^{-4}$ s^{-1}, $k_3 = 1.2 \times 10^{-4}$ s^{-1}, and $k_{-3} = 3.3 \times 10^{-5}$ s^{-1}.

substrate, followed by a faster reaction involving the release of P_1, Scheme III. If the rate-limiting process in reaction 3 corresponds to the formation of acyl-enzyme plus methanol (Scheme II), then no simple expression relating k_{obsd}^3 to the microscopic rate constants exists, because P_1 is a time-dependent variable. This problem can be overcome by using a large excess of P_1 over enzyme concentration. The kinetic expressions for k_{obsd}^3 are of the same general form for both Schemes II and III as far as the dependence of rate on the concentrations of P_1 ($P_1 \gg E_0$) is concerned. Both schemes also yield expressions for the maximum product concentration which predict a decrease in the concentration of the product of reaction 3 as the concentration of excess P_1 is increased, as is observed. The nonzero intercept in Figure 3 for reaction 3 is more consistent with Scheme III; however, the data are insufficient to be able to make an unambiguous choice between these two schemes. For Scheme III, k_3 and k_{-3} correspond to first-order rate constants and values of 1.2×10^{-4} s^{-1} and 3.3×10^{-5} s^{-1}, respectively, are obtained for pH* 6.8 and −40 °C.

SCHEME III

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{\substack{k_{-2} \\ \text{fast}}}{\overset{k_2}{\rightleftharpoons}} I_2 \underset{\substack{k_{-3} \\ \text{slow}}}{\overset{k_3}{\rightleftharpoons}} I_3 \underset{\substack{k_{-4} \\ \text{fast}}}{\overset{k_4}{\rightleftharpoons}} EA + P_1$$

Having obtained the values for the microscopic rate constants it is now possible to construct an energy diagram (Figure 4) for the reaction as well as to simulate the time dependence of the concentration of the intermediates (Figure 5). By extrapolation of the Arrhenius plots and correction for the effect of cosolvent on substrate binding (Fink & Angelides, 1976), it is possible to calculate the values for the observed intermediate transformations under normal conditions. In this manner we estimate for aqueous solution, 25 °C, pH 6.1, $E_0 = 1 \times 10^{-5}$ M, $S_0 = 1 \times 10^{-3}$ M, and the values of k_{obsd} to be 1×10^3 s^{-1} and 10 s^{-1} for reactions 2 and 3, respectively.

Discussion

The results of this investigation of the reaction of papain with a specific ester substrate indicate that at suitably low

temperatures a series of time-dependent changes in the spectrum of the enzyme can be detected, reflecting events at the active site. For the low temperature technique to provide mechanistically significant information it is important that the reaction at such low temperatures be analogous to that under normal conditions. Previous studies have shown that 60% aqueous Me_2SO and subzero temperatures do not perturb the overall catalytic and structural properties of papain (Fink & Angelides, 1976).

The three reactions observed at subzero temperatures in the reaction of papain with N^{α}-carbobenzoxy-L-lysine methyl ester are interpreted, as discussed in detail below, to represent binding, a substrate-induced conformation change in the enzyme, and formation of the covalent acyl-enzyme.

Reaction 1. The available data for reaction 1 suggest that it represents the initial binding of substrate. For example we estimate that the observed pseudo-first-order rate constant for the binding process under our non-turnover conditions would be $>1\ s^{-1}$ at $-65\ °C$, as was found for reaction 1 (Fink, 1976a,b). That reaction 1 is not observed when either enzyme or substrate was omitted from the reaction mixture, or when papain inactivated with Tos-LysCH_2Cl was used, lends support to this interpretation since it would not be expected that substrate could bind productively to this derivative. The observed increase in initial absorbance on addition of the substrate to mercuripapain suggests that the substrate binds to this derivative in a manner in which the environment about Trp-177 is different than for native papain. The fact that reaction 1 is detectable by changes in the fluorescence emission of the enzyme indicates that the environment about Trp-177 is changed, as would be anticipated for substrate binding (Drenth et al., 1975, 1976). The possibility that reaction 1 also involves binding in a nonproductive mode, and/or additional steps cannot be ruled out.

Since both reactions 1 and 2 are observed with papain-S-S-CH_3 and the rate constant for reaction 2 is the same with papain and papain-S-S-CH_3 (Table I), the thiol of Cys-25 cannot be intimately involved in the underlying processes. That substrate binding and subsequent intermediate transformation take place in the presence of the modified active-site thiol may seem rather surprising at first. However, in a careful examination of a space-filling model (CPK) of the active-site region of papain, it was found that with the preferred conformer[3] of the *S*-methanethio derivative the $-CH_3$ of the mixed disulfide was tucked in a pocket close to the β-methylene of Cys-25 and that there are no severe steric interactions between the $-SCH_3$ group and any residue in the active-site area.

Reaction 2. A few previous investigations of papain catalysis under normal conditions have indicated the presence of intermediates additional to those shown in Scheme I. In particular, evidence to support a kinetically significant conformational change has been presented by Lake & Lowe (1966), Holloway & Hardman (1973), Fink & Gwyn (1974) and Mattis & Fruton (1976). For example, Mattis & Fruton (1976), using fluorescent mansyl-peptide substrates, observed biphasic kinetics which they interpreted to reflect the presence of an additional intermediate prior to amide bond scission and acyl-enzyme formation.

There are a number of interesting features concerning the data on reaction 2. These include: the low pK^* (≤ 3.4); the fact that the rate is pH* independent above pH* 4.4 and yet the magnitude of the fluorescence emission change corresponding to the reaction reflects a pK^* of 5.1; that the reaction is apparently unaffected by conversion of the active-site thiol to the *S*-methanethio derivative; that the kinetic analysis indicates the reaction is effectively irreversible; and that the calculated rate for normal conditions is of the order of $10^3\ s^{-1}$. The observation that the reaction is manifested as a fluorescence change indicates that the environment about Trp-177 is changed during this process.

SCHEME IV

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EA + P_1$$
$$k_2 \updownarrow k_{-2}$$
$$ES_2$$

The possibility that the product of reaction 2 is not on the productive catalytic pathway but is instead a "dead-end" complex, e.g., ES_2 in Scheme IV, can be eliminated on the following grounds. Since the observed spectral changes are absent when papain inactivated by Tos-LysCH_2Cl, or mercuri-papain are used, the observed changes must involve substrate bound to the active site. Upon warming, intermediate 2 is transformed to acyl-enzyme. Thus intermediate 2 must either be on the productive catalytic pathway or in readily reversible equilibrium with it. If Scheme IV did obtain one would expect reaction 3 to show up in fluorescence studies unless the intrinsic fluorescence change associated with reaction 3 exactly off-set that due to the reversal of reaction 2. Even in the unlikely event that such a coincidence did occur and Scheme IV was in fact correct, the Arrhenius plots for reactions 2 and 3 indicate that the dead-end complex would still be a kinetically significant intermediate under normal conditions. The possibility that reaction 2 could be related to nonproductive binding of the substrate can be eliminated on the basis of arguments previously presented for a similar case (Fink, 1976b).

The observed changes in the intrinsic absorption and fluorescence properties of the enzyme during reaction 2 suggest that a substrate-induced conformational change takes place upon substrate binding, or at least that changes in the relative positions of bound substrate and enzyme groups in the active site occur. We propose that there is a movement of the imidazole of His-159 from its crystallographically determined position, i.e., hydrogen-bonded to Asn-175, to one involving electrostatic interaction with the carboxylate of Asp-158 (see Figure 6). This movement could be achieved by a rotation of the C^{α}-C^{β} bond of His-159 by 79°. Small movements of the side chains Trp-177, Asp-158, and Cys-25 may also occur (Angelides & Fink, 1978a).

Drenth et al. (1976) have recently shown from a high-resolution x-ray crystallographic structure analysis of a papain-inhibitor complex that significant changes at the active site do occur upon inhibitor binding. A rotation of the C^{α}-C^{β} bond of His-159 will facilitate proton transfer of the $N^{\epsilon}2$ proton to the leaving group of the substrate. Additional support for a conformational change subsequent to substrate binding comes from the observation that the active-site sulfhydryl group is more reactive toward alkylation in the presence of substrate or competitive inhibitor (Sluyterman, 1968). In contrast, however, the pK of the active-site sulfhydryl of ficin apparently is not perturbed in the presence of a competitive inhibitor (Whitaker, 1969). The proposed structural changes are considered in more detail subsequently.

Reaction 3 is the slowest reaction observed under the non-

[3] It is known that simple disulfide bond angles are between 103 and 107° and that restriction to freedom of rotation of the S-S bond is between 10 and 20 kcal mol^{-1} (Steinrauf et al., 1958; Scott et al., 1952). Because of this rotational restriction there is a preferred dihedral angle between the two S-C axes in simple disulfides of 90° (Torchinskii, 1974).

turnover, low-temperature conditions and is ascribed to formation of the acyl-enzyme. Reaction 3 was absent when the essential sulfhydryl of Cys-25 was blocked in the *S*-methanethio or mercuripapain derivatives, demonstrating the importance of a free thiol for reaction 3. Confirmation that the product of reaction 3 involves the covalent attachment of the substrate to Cys-25 may be found in the alkylation and acylation titration experiments which indicate 83 and 85% respectively of covalently modified product. The computer simulation of the time dependence of the concentration of intermediates (Figure 5) indicates that significant amounts of I_2 and E will be present at the completion of reaction 3, and no doubt account for the fact that only 85% of the -SH groups were unavailable for titration.

The potent effect of relatively low methanol concentrations on reaction 3 is rather surprising. The simplest interpretation is that the relatively large second-order rate constant for the reverse reaction (i.e., acyl-enzyme + alcohol → preceding enzyme–substrate intermediate) is solely responsible. Methanol is well known as a powerful nucleophilic competitor for acyl-papains (Brubacher & Bender, 1966; Henry & Kirsch, 1967; Fink & Bender, 1969) under normal conditions. For example the partitioning ratio of *N*-acetyl-L-tryptophanyl-papain between water and methanol favors methanolysis by a factor of 79 at 25 °C, pH 7.0 (Fink & Bender, 1969). In 60% dimethyl sulfoxide this would become 200. It is also possible that some specific interaction occurs between methanol and papain. Complex effects of methanol on the papain-catalyzed hydrolysis of alkyl esters have been reported previously in the concentration range to 2 M (Lake & Lowe, 1966; Henry & Kirsch, 1967; Fink & Bender, 1969). In addition the enzyme is catalytically inactive in the presence of high concentrations of methanol (Fink & Angelides, 1976; Sluyterman & De Graaf, 1969).

The possibility that the product of reaction 3 is a tight EA·P_1 complex, and that the overall rate-limiting step in turnover is P_1 dissociation rather than deacylation, may be discarded since, contrary to observation, no effect of added P_1 on the rate would be expected in such a situation. Also the fact that added nucleophile ligands can be used to increase the rate of the slowest step with ester substrates (Hinkle & Kirsch, 1971; Fink & Bender, 1969; Brubacher & Bender, 1966) is inconsistent with a common rate-limiting step involving dissociation of the acyl-enzyme–P_1 complex.

Proposed Mechanism. The results of the present investigation, in conjunction with those from previous studies, have led to the development of the following model of the mechanism of papain catalysis, represented in Figure 6. The model is consistent with the majority of data concerning papain catalysis, and in particular with the apparently contradictory reported p*K* assignments in papain, modified papain, papain–inhibitor complexes, and papain catalyses. A more detailed discussion of this aspect is given in the next section.

We propose that free papain, i.e., unliganded, exists in two forms. One, which we will call UP, corresponds to the crystallographically determined structure in which the $N^{\epsilon}2$-hydrogen of the imidazole of His-159 is hydrogen bonded to the amide H of the side chain of Asn-175 (E in Figure 6). It should be noted that the x-ray crystallographic studies were done at high pH (9.3) where the imidazole, regardless of its p*K* (i.e., 4 or 8) exists primarily as the free base. In the other form of the enzyme the imidazole has moved to a position that we will call DOWN (E′ in Figure 6), in which it is protonated, and forms an ionic bond to the carboxylate side chain of Asp-158 (see Discussion of reaction 2).

In the UP conformation the p*K* value for the imidazole is

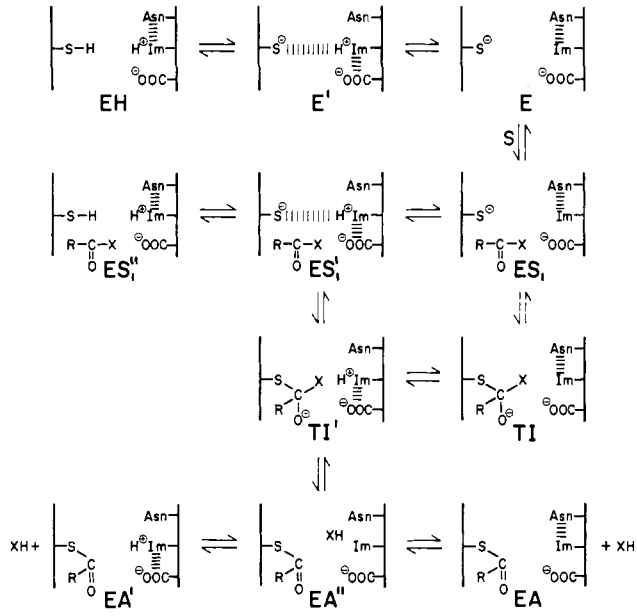

FIGURE 6: Proposed mechanism of action for papain. E′ represents the DOWN isomer (see text) in which the position of the imidazolium of His-159 is fixed by electrostatic interactions with the thiolate of Cys-25 and carboxylate of Asp-158. E represents the form in which His-159 is in its crystallographically determined locus, i.e., hydrogen bonded to the side chain carbonyl of Asn-175. See text for discussion.

lower than normal for the following reasons. The formation of the hydrogen bond and shielding of the hydrogen bond from the solvent by the tryptophan place the imidazole in a rather hydrophobic microenvironment. The proton which dissociates from the imidazolium ion is released into the bulk water, where it is highly solvated. Both Lewis et al. (1976) and Polgar (1973, 1976a,b) have indicated that the medium in the active-site area in papain has a low dielectric constant and is rather hydrophobic, with considerable ordered water (Drenth et al., 1968, 1971). Both the thiol of Cys-25 and carboxyl of Asp-158 bear normal p*K*s of approximately 8 and 3, respectively, in the UP conformation.

In the DOWN conformer the imidazolium forms ion pairs to both thiolate and carboxylate. This conformation predominates in the intermediate pH region (4–8). For the DOWN form the p*K* of the thiol is 4, that of the imidazole is 8; hence at neutral pH the imidazole bears a net positive charge. In this conformation the p*K*s of the thiol and carboxyl are lowered due to the electrostatic interactions with imidazolium, whereas the imidazole p*K* is elevated for the same reason. Interconversion between E and E′ (Figure 6) involves two transient species, resulting from either proton transfer preceding the conformational change, or vice versa, neither of which would be sufficiently stable to exist in significant concentrations. The position of the equilibrium between the UP and DOWN conformers will be affected by both pH and active-site-bound ligand.

Polgar (1974a,b) was the first to present spectroscopic evidence that the thiolate–imidazole ion pair predominates between pH 4.2 and 8.2. He observed ultraviolet absorption at 335 nm assignable to the thiolate at pH values below 8.0. Perhaps the best information bearing on this question are the data that have emerged from fluorescence and potentiometric difference titrations of free papain, papain-S-cysteine, and papain-S-S-CH_3. Using the natural spectroscopic probe of Trp-177, the fluorescence of the native enzyme shows a pH dependence with p*K* = 8.6. The quenching interaction arises

only from a charge-transfer complex of the imidazolium and indole in the active site (Shinitzky & Goldman, 1967). When the thiol group is blocked by either forming the disulfide with cysteine (Sluyterman & de Graaf, 1970), the sulfinic acid, or papain-S-S-CH_3 (Lewis et al., 1976; Johnson & Shafer, private communication), the pH dependence of the fluorescence reveals a group(s) with pK = 4. The fluorescence quenching for the enzyme-inhibitor complex, papain-alanyl-L-phenylalanylaminoacetonitrile also depends on the pK of a group(s) around 4 (Lowe, 1976). Drenth et al. (1975, 1976), Polgar (1974, 1976a,b), Shipton et al. (1975), Lewis et al. (1976), and Sluyterman & Wijdenes (1976) have interpreted these results by proposing that the thiol and imidazole ionizations are coupled. The pK of the thiol group changes from 4.0 to 8.0 on deprotonation of the imidazole. Similarly the pK of the imidazole shifts from 4.3 to 8.5 when the active site thiol is deprotonated. The microscopic ionization constants calculated in the potentiometric difference study of papain and papain-S-S-CH_3 (Lewis et al., 1976) indicated that in the intermediate pH range 90% of the papain exists in a form wherein the active site contains a thiol anion and an imidazolium cation. The remaining 10% is represented by the free base form of the imidazole and a thiol group. Thus in the neutral pH region, according to our proposed model, 90% will be in the DOWN and 10% in the UP form, for the free enzyme.

Based on examination of a space-filling active-site model the substrate will preferentially bind to the UP form, since access to the active-site will be sterically hindered in the DOWN position. The catalytic reaction, therefore, initially involves binding of substrate to the UP conformation (E) to form complex ES_1 (Figure 6). The removal of E by conversion to ES_1 will consequently result in the conversion of E′ to E. Additional experimental support for a scheme of the form E′ $\rightleftarrows$ E $\rightleftarrows$ ES_1 will be reported shortly (Angelides & Fink, 1978a). Two routes to the next intermediate along the reaction pathway are open to ES_1. One, which involves general-base catalysis by the imidazole of the nucleophilic attack of the thiol of Cys-25 on the substrate carbonyl, we believe to be an unlikely path (ES_1 → TI in Figure 6). No precedent exists in nonenzymatic systems for such concerted general base-nucleophilic catalysis. The other route involves movement of the imidazole of His-159 to the DOWN position. Concomitant with this conformational change, the pKs of the imidazole and thiol change so that in the new complex, ES_1′, they exist predominantly as the ion pair (imidazole pK ~ 8; thiol pK ~ 4).

One would expect the product of this reaction, ES_1′, to be relatively unstable and rapidly form a tetrahedral intermediate by nucleophilic attack of the thiolate anion of Cys-25. In the resulting tetrahedral intermediate (TI′ in Figure 6) the oxyanion is expected to be stabilized by hydrogen bonding to the peptide amide hydrogen of Cys-25 and ϵ-NH_2 of Gln-19 (Drenth et al., 1976). This tetrahedral intermediate would exist in equilibrium with a corresponding one, TI (Figure 6), in which the imidazole was in the UP conformation. High pH would be expected to favor the latter form. At neutral pH, as in the analogous case of the free enzyme, a substantial fraction would be expected to exist in the TI′ form. At low pH protonation of the thiolate occurs leading to the formation of ES_1″. This form cannot react further to produce a tetrahedral intermediate.

In addition to indirect support for the presence of a tetrahedral intermediate in the acylation reaction we have recently been able to make direct observation of the tetrahedral intermediate in the reaction of papain with N^{α}-carbobenzoxy-L-lysine p-nitroanilide (Angelides & Fink, 1978b). We have observed in this case that tetrahedral intermediate formation is rate limiting at low pH and breakdown is rate limiting at high pH. A similar circumstance appears to exist with the methyl ester (vide infra). For substituted phenyl esters and anilides of hippuric acid (Lowe & Williams, 1965; Lowe & Yuthavong, 1971), Hammet ρ values were obtained which suggested that formation of the tetrahedral intermediate was rate determining for the aryl esters and breakdown was rate limiting for the anilides (Lowe & Williams, 1965; Lowe & Yuthavong, 1971). This is supported by an observed nitrogen isotope effect ($^{14}N/^{15}N$) close to the theoretical limit, for amide hydrolysis, indicating that the rate-limiting step involves carbon-nitrogen bond scission (O'Leary et al., 1974).

The next stage of the catalytic reaction is collapse of the tetrahedral intermediate into the acyl-enzyme, EA″ (Figure 6). This initially formed acyl-enzyme species would isomerize very rapidly to the EA form (UP) or pick up a proton to form EA″ (DOWN). Protonation of the leaving group is achieved by the $N^{\epsilon}2$ proton of the imidazole. In the DOWN position the imidazole is in a prime orientation to facilitate this proton transfer since the orbital of the lone pair electrons of the leaving group of a nitrogen or oxygen and the $N^{\epsilon}2$ are periplanar and a direct proton transfer is possible, in accord with expectations based on the stereoelectronic theory of Deslongchamps et al. (1975).

The deacylation reaction, which is not shown, corresponds essentially to the reverse of acylation. The first step involves the imidazole in EA(UP) acting as a general base catalyst. Kinetic isotope studies suggest general base catalysis in the deacylation step (Whitaker & Bender, 1965; Brubacher & Bender, 1966; Hinkle & Kirsch, 1970) and the pH dependence of deacylation is sigmoidal with a pK of approximately 4 (Williams & Whitaker, 1967; Whitaker & Bender, 1965; Bender & Brubacher, 1966) consistent with the rate-limiting step being the general-base catalyzed attack by imidazole in the UP position (where its pK is 4). The resulting tetrahedral intermediate will exist in two, pH-dependent forms, analogous to TI and TI′ in Figure 6 where X = OH. Collapse of the tetrahedral intermediate will involve the pathway TI $\rightleftarrows$ TI′ → ES_1′ → ES_1 → E $\rightleftarrows$ E′.

Application of the Model to the Reaction with N^{α}-Carbobenzoxy-L-lysine Methyl Ester. Reaction 1 corresponds to substrate binding to E to form ES_1 and the concurrent conversion of E′ to E to ES_1 (Figure 6). The fluorescence changes observed for reaction 1 thus reflect changes in the Trp-177 environment in going from E′, i.e., the DOWN conformation with imadazolium, to ES_1, the UP state with imidazole free base. The enhanced fluorescence emission observed for reaction 1 is consistent with loss of imidazolium (which is known to be an effective quenching agent) (Shinitzky et al., 1966; Shinitzky & Goldman, 1967).

In the intermediate pH range reaction 2 corresponds to the transformation of ES_1 to TI′ via ES_1′. At low pH the reaction involves the conversion of ES_1 into ES_1″, again via ES_1′. In both cases the rate-limiting step is the ES_1 to ES_1′ transformation, i.e., the rate of reaction 2 reflects ES_1 → ES_1′. The observed pK* of ≤3.4 at −40 °C for the rate of reaction 2 most probably corresponds to that of the carboxylate of Asp-158; it cannot reflect the imidazole since protonation of the imidazole should facilitate the reaction. It is also possible that the observed pK* of 3.4 corresponds to a non-active-site group controlling the structural stability of the enzyme at low pH.

Reaction 2 involves protonation of the imidazole and would therefore be expected to result in quenching of the fluorescence emission of Trp-177, as observed. The observed pH dependence of the fluorescence emission decrease for reaction 2 may be

accounted for as follows. At low pH (pK* = 5.1 at −36 °C) the product of the reaction is ES_1'' in which the thiol of Cys-25 is protonated. The loss of thiolate weakens the electrostatic interactions maintaining the imidazolium in the DOWN position and consequently in ES_1'' the imidazolium is predominantly in the UP position. In this state the imidazolium is closer to Trp-177 and consequently greater quenching is observed. The pK* of 5.1 at −36 °C thus reflects that of the thiolate in ES_1'. Confirmation that this pK* reflects the same group as that responsible for pK_1* for reaction 3 comes from the observation that its value falls exactly on the line for a plot of pK_1* vs. $1/T$ for reaction 3.

Reaction 2 was observed to be unaffected when papain–S–S–CH_3 was used. Since the rate of reaction 2 is postulated not to involve the thiol group, and since the -S-methyl group does not hinder substrate binding, one would expect that the reaction would proceed as far as the ES_1'' stage for this derivative.

In the intermediate pH range reaction 3 involves transformation of the tetrahedral species TI′ to the acyl-enzyme EA′ with the intermediacy of EA″. As previously discussed the kinetic analysis of reaction 3 suggests the presence of an intermediate whose breakdown to acyl-enzyme (EA′) is more rapid than its formation. This is exactly the behavior expected for EA″. The observed pK* of 4.8 (−16.5 °C) for reaction 3 reflects the protonation of the thiolate in ES_1', the same ionization responsible for the pH* dependence of fluorescence emission in the product of reaction 2. The resulting species, ES_1'', is unable to form a tetrahedral intermediate. At high pH the species TI will be formed in reaction 2 and will accumulate, since general-acid catalyzed assistance by imidazolium is required in the transformation to acyl-enzyme. No fluorescence changes are observed for reaction 3 because the ratio of imidazolium/imidazole in the tetrahedral intermediate (TI′/TI) will be similar to that in the product (EA′/EA).

Origin of the pH Dependence. According to our model (Figure 6) the pKs of the ionizable active-site groups are different in the UP and DOWN conformations. The model predicts pK's of 4 and 8 for acylation and of 4 for deacylation under turnover conditions, as are observed (Stockell & Smith, 1957; Smith & Parker, 1958; Whitaker & Bender, 1965; Bender & Brubacher, 1966).

The fluorescence and potentiometric titration data for papain and *S*-methylthio-Cys-25-papain of Shafer and coworkers (Lewis et al., 1976) are entirely in accord with our proposed model and demonstrate the different pK values for the imidazole and thiol in the two conformers. For example, one previously puzzling aspect was the observation that fluorescence titrations of native papain show a pK of 8, whereas for papain–S–S–CH_3 the pK is 4 (Sluyterman & deGraaf, 1970; Lewis et al., 1976). Our model predicts such behavior since the free enzyme will be predominantly in the DOWN (E′) form in which the imidazole pK is 8, whereas modification to the -SCH_3 derivative prevents formation of the thiolate anion to stabilize the imidazolium in the DOWN form and only the UP conformation, with imidazole pK of 4, is present. Similarly the observations that blocking the carboxyl of Asp-158 abolishes catalytic activity (Perfetti et al., 1976; Löffler & Schneider, 1974) are now readily explained by the necessity for free carboxylate to permit formation of the catalytically essential DOWN conformation. The proposed model also explains the pH dependence, and enhanced reactivity compared with cysteine, of alkylation (i.e., lack of reaction below pH 4 where thiolate is absent; charge repulsion of negatively charged reagents such as chloroacetate at high pH (Wallenfels & Eisele, 1968; Chaiken & Smith, 1969) and the pH dependence of zinc and mercury binding (Drenth et al., 1975; Sluyterman & Wijdenes, 1976)).

Several studies suggest cooperative ionizations of active-site groups with pK values in the vicinity of 3–4 (Drenth et al., 1975; Bendall & Lowe, 1976a,b; Lewis et al., 1976; Sluyterman & Wijdenes, 1976). Such a phenomenon is predicted by the model for enzyme in the DOWN conformation. For example, for the free enzyme, E′, protonation of the thiolate (pK = 4) will result in isomerization to the UP state (E″, analogous to ES_1'' in Figure 6) which will change the pK of the carboxyl.

As noted in a recent review pointing out the inherent dangers in assignment of pK values (Knowles, 1976), one of the optimal approaches to the assignment of a pK in the catalytic reaction is to determine the pK of an elementary step. This is more feasible in a cryoenzymological investigation such as the present one and we believe that our assignment of pK* = 4.8 at −40 °C (which extrapolates to 3.8 at 25 °C) to the thiolate in the DOWN conformation is eminently reasonable. The observed heat of ionization, 8 kcal mol^{-1}, is entirely consistent with that for a thiol. Other thiol groups for which similar heats of ionization have been reported include those of ficin (Holloway et al., 1971) and glyceraldehyde-3-phosphate dehydrogenase (MacQuarrie & Bernhard, 1971). The reason that the heat of ionization is observed to be 0 under steady-state conditions (Smith & Parker, 1958; Smith et al., 1958; Hinkle & Kirsch, 1971) is not clear.

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