Mechanism of Action of Papain with a Specific Anilide Substrate†

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ABSTRACT: The reaction between papain and N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide has been studied at subzero temperatures in fluid aqueous dimethyl sulfoxide solvents. When the reaction was initiated by mixing the enzyme and the substrate at temperatures in the 0 to −65 °C region, a series of four reactions (reactions 1–4) prior to turnover were detected by changes in the substrate spectrum. Three of the intermediates had spectra similar to that of the substrate, and one had a spectrum that closely resembled that of the product *p*-nitroaniline. The slowest of these preturnover reactions, reaction 4, was also detected in aqueous solution at ambient temperatures by using rapid reaction techniques. Reaction 2 has pK*s (pK* = apparent pK in the cryosolvent) of 4.4 and ≥8.0 at −25 °C. Reaction 3 has a pK* of 4.0 at −25 °C and reaction 4 has pK*s of 4.3 and ≥8.3 at −3 °C. Both reactions 2 and 4 were absent with the Cys-25 *S*-methylthio derivative of papain. The concentrations of the products of reactions 2 and 4 were pH* dependent. In the former, the yield of the intermediate decreased with increasing pH*, whereas in the latter the amount of intermediate accumulated increased with increasing pH*. The experimental observations have led to the following interpretation of these reactions. Reaction 1 corresponds to the binding of the substrate yielding the initial Michaelis complex. Reactions 2 and 3 are both associated with conformational changes of the active-site groups and, in particular, with rotation of the imidazole of His-159 so as to break and re-form a salt bridge with the carboxylate of Asp-158. Kinetic evidence is presented for reaction 2 to show that the conformation change precedes substrate binding; i.e., it reflects an isomerization of the free enzyme, whereas reaction 3 represents an isomerization of the enzyme–substrate complex. From the value of the equilibrium constant for reaction 2, it is found that papain is present predominantly in the ion-pair state in the absence of ligand at neutral pH. Reaction 4 involves the thiol of Cys-25 and is ascribed to the formation of a tetrahedral intermediate.

Although there is considerable evidence indicating that papain-catalyzed hydrolyses of acylamino derivatives involve an acyl-enzyme intermediate (Lowe & Williams, 1965; Kirsch & Igelström, 1966; Brubacher & Bender, 1966; Lowe, 1976), relatively little information is available concerning the dynamic processes occurring during the catalysis. Recent reports have suggested the existence of one or more intermediates between the Michaelis complex and the acyl-enzyme in the papain-catalyzed hydrolyis of specific substrates (Henry & Kirsch, 1967; Holloway & Hardman, 1973; Fink & Gwyn, 1974; Mattis & Fruton, 1976; Angelides & Fink, 1978). The delineation of the mechanism of action of papain requires a knowledge of all the intermediates on the reaction pathway before specific details of the mechanism may be successfully resolved. We have sought to obtain this information by the technique of cryoenzymology (Fink, 1976b).

The utility of subzero temperatures in enzyme mechanism studies has been recently reviewed (Fink, 1977). Briefly, three main advantages are afforded by the technique. The most (kinetically) specific substrates can be used since the overall catalytic reaction can be slowed essentially to zero, drastic reductions in rates of intermediates can be obtained, and each intermediate can potentially be successfully accumulated at high concentrations.

Previous studies with papain have shown that neither the catalytic nor structural properties of the enzyme are adversely affected by the presence of cryosolvents and subzero temperatures (Fink & Angelides, 1976). In addition, it has been possible to accumulate and stabilize detectable amounts of intermediates on the catalytic reaction pathway by using specific substrates. For example, the acyl-enzyme found from reaction with N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester could be trapped in >80% yield at temperatures below −50 °C (Fink & Angelides, 1976). By using the corresponding methyl ester substrate, two intermediates intervening between the Michaelis complex and the acyl-enzyme were detected by monitoring the intrinsic UV of the enzyme (Angelides & Fink, 1978). From this latter study, a detailed model for the mechanism of papain catalysis of esters was formulated (Angelides & Fink, 1978).

We have now investigated the reaction of papain with the specific substrate, N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide, at subzero temperatures with the idea that the chromophoric *p*-nitroaniline moiety would function as a sensitive probe of the events occurring during the actual bond-breaking/making step(s) as well as possible changes in the environment of the leaving group site in the enzyme during the catalytic process (Fink, 1976a).

In this article, we present evidence for the existence of two intermediates in addition to the initial Michaelis complex and the acyl-enzyme in the papain-catalyzed hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide at subzero temperatures. A particularly interesting finding was that of a kinetic process which reflects an underlying conformational isomerization of the enzyme prior to substrate binding in the pH-optimum region.

Experimental Procedures

Materials. N^{α}-Carbobenzoxy-L-lysine *p*-nitrophenyl ester, N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide, and L-tryptophanamide were obtained from Vega-Fox Biochemicals, lots F7713 (mp 136–138 °C) and F7877 (mp 86–89 °C), respectively.

Twice recrystallized papain from Worthington was further purified by affinity chromatography as described by Blumberg et al. (1970). The enzyme was stored under nitrogen at 4 °C. The activity and concentration were checked daily either by burst titration with N^{α}-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester or kinetic assay with N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester (Bender et al., 1966; Bender & Brubacher, 1966).

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Papain-S-S-CH_3,[1] in which the thiol of Cys-25 was modified as the *S*-methylthio derivative, was prepared by the method of Smith et al. (1975). Methyl methanethiosulfonate was generously supplied by Dr. G. Kenyon, University of California, San Francisco. Papain, inactivated by Tos-LysCH_2Cl, was prepared by a modification of the procedure described by Whitaker & Perez-Villaseñor (1968). The specific activity of the [^{14}C]methyl methanethiosulfonate was 1.5×10^{-2} Ci/mol. [^{14}C]Iodoacetate was purchased from Amersham/Searle and had a specific activity of 5.7×10^{-2} Ci/mmol.

Reagent grade dimethyl sulfoxide, ethanol, sodium formate, acetate, and borate were all from Mallinckrodt. The dimethyl sulfoxide was distilled from calcium hydride under vacuum at 37 °C, recrystallized at 2 °C, and stored at or below 4 °C. Cryosolvents used for this investigation were prepared on a volume/volume basis at 0 °C as previously described (Fink, 1973, 1976a; Angelides & Fink, 1978).

Methods. Steady-state and low-temperature kinetics were determined with a Cary 118C recording spectrophotometer equipped with a repetitive scanning attachment and a thermostated brass cell holder so as to maintain the temperature within ±0.1 °C. In the experiments in which the solution temperature was below −30 °C the experiments were carried out by using a specially constructed, vacuum-jacketed, quartz cell, as described previously (Angelides & Fink, 1978). A Heto UltraCryoterm or Neslab LT-9 constant-temperature bath with ethanol as circulant was used to maintain the sample temperature constant within ±0.2 °C for the duration of the experiment. In all spectral experiments, the temperature was monitored throughout the course of the experiment with a thermocouple or temperature sensitive diode probe.

Values of pH* (the apparent pH in aqueous organic solution) at subzero temperatures were obtained from the known temperature dependence for these cryosolvents (Hui Bon Hoa & Douzou, 1973; Douzou, 1974). To ensure that no structural or catalytic perturbations occurred to the enzyme at high pH* (9.3) in 60% dimethyl sulfoxide, we incubated the enzyme in the solvent at 4 °C and assayed over a period of time. The activity remained constant relative to that of a control in aqueous media for several days.

Enzyme and substrate solutions were mixed at subzero temperatures either by using the low-temperature mixing device previously described (Angelides & Fink, 1978), or as follows. A small aliquot of enzyme solution, diluted 1:3 with aqueous 60% dimethyl sulfoxide at 0 °C, was added to a cooled solution of substrate in buffer in the spectrophotometer cell, thermostatted at the appropriate temperature, and mixed with a vibrating reed stirrer. Enzyme preparations in aqueous organic solutions were kept at 0 °C or below to prevent denaturation, since the midpoint of the reversible transition between native and denatured enzyme in 60% dimethyl sulfoxide occurs at +12 °C at pH* 3.5 (Fink & Angelides, 1976). The progress of the reaction was monitored either by repetitive spectral scans (500–300 nm) (at low substrate concentrations) or by following the spectral changes at a fixed wavelength as a function of time. Since neither product, substrate, nor enzyme absorbs at 500 nm, this wavelength provided a means of monitoring the base-line stability. If changes occurred at 500 nm, the experiment was discarded. Control experiments in which either enzyme or substrate was omitted showed no time-dependent spectral changes. Kinetic analyses of experiments in which successive reactions had similar rates were done with the aid of a modified computerized iteration program after Wiberg (1965).

The experiments to determine the amount of enzyme–substrate complex accumulated as the product of each reaction were performed with the isotopic alkylation method previously described (Angelides & Fink, 1978).

A number of spectral characteristics were determined for N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide to facilitate interpretation of the observed spectra. They are as follows: in 60% dimethyl sulfoxide the position of the λ_{max} of the substrate's absorption is 322 nm at −40 °C. An approximately linear correlation was found between ϵ_{322} and the temperature, increasing with decreasing temperature (−3.0 °C, ϵ 9270 M^{-1} cm^{-1}; −11.0 °C, 10 700; −20.1 °C, 13 400; −30.7 °C, 17 500; −40.0 °C, 17 330; −51.6 °C, 19 100; −58.7 °C, 22 100). *p*-Nitroaniline, the product of the overall catalytic reaction, has a similar dependence on temperature; the position of its λ_{max}, however, was 380 nm in 60% dimethyl sulfoxide. In 80% ethanol the spectral characteristics of the substrate and product were slightly blue-shifted from those in 60% dimethyl sulfoxide. The values of λ_{max} were 317 and 378 nm, respectively. The λ_{max} of the spectrum of *p*-nitroaniline also was not significantly perturbed in solvents of high and low polarity (Angelides & Fink, 1979). The substrate showed very minor changes in λ_{max} (<2 nm) with change in pH* in the 2.9–9.3 region.

The steady-state parameters k_{cat} and K_m for the hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitroaniline and N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester were determined from complete reaction curves using least-mean-squares plots of v vs. v/S (Bender et al., 1964). Substrate concentrations used were $\geq 3K_m$. Corrections were applied for spontaneous hydrolysis of the substrates.

Fluorescence lifetime measurements were made on an Ortec Model 9200 nanosecond fluorescence spectrophotometer interfaced with a PDP 11/20 computer. Excitation light was passed through a Ditric interference filter with excitation at 290 nm. A Ditric interference filter with a transmission window at 334 nm was used to isolate the desired wavelength of emitted light. Spectra were collected with identical instrument settings and for equal time periods. Decay spectra were deconvoluted and analyzed in terms of two fluorescence lifetimes by the method of moments (Isenberg & Dyson, 1969).

Results

Steady-State Kinetics of Anilide Hydrolysis. The hydrolysis of N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide catalyzed by papain at several pH values followed Michaelis–Menten kinetics throughout the concentration range employed. Representative values at 25 °C are $k_{cat} = 0.25$ s^{-1} and $K_m = 1.0 \times 10^{-5}$ M at pH 6.0 and $k_{cat} = 0.09$ s^{-1} and $K_m = 3.5 \times 10^{-5}$ M at pH 9.2.

At 0 °C, in 60% dimethyl sulfoxide, the release of *p*-nitroaniline corresponding to the turnover was monitored by fixed wavelength measurements at 400 nm or above, where the substrate has negligible absorbance. In the cryosolvent, the turnover reaction was followed both under pseudo-first-order conditions ($K_m \gg S$) and by the initial velocity technique, and with substrate in excess over enzyme. The value of k_{cat}/K_m obtained from pseudo-first-order experiments at 0 °C, pH* 6.0, in the cryosolvent was 3.0 ± 0.2 M^{-1} s^{-1}. The decrease in k_{cat}/K_m from 2.5×10^{-4} to 3.0 M^{-1} s^{-1} is due to the combined effects of temperature and cryosolvent on k_{cat} and K_m. Excellent agreement between the observed absorbance increases at 410 or 440 nm and those calculated for the substrate concentration employed was obtained in the organic

[1] Abbreviations used: Tos-LysCH_2Cl, N^{α}-tosyl-L-lysine chloromethyl ketone; UV, ultraviolet; papain-S-S-CH_3, the *S*-methylthio derivative of Cys-25; *A*, absorbance.

Table I: Kinetic and Thermodynamic Data for Reactions 2–4

parameter	reaction 2	reaction 3	reaction 4
pK^*	$pK_1^* = 4.4 \pm 0.3$ $pK_2^* \geq 8.0$ at −25 °C	$pK_1^* = 4.0 \pm 0.3$ at −25 °C	$pK_1^* = 4.3 \pm 0.3$ $pK_1^* = 8.6 \pm 0.3$ at −3 °C
k^{lim}_{obsd} (s^{-1}) at −25 °C	$4.0 \pm 0.5 \times 10^{-3}$	$9.9 \pm 0.8 \times 10^{-4}$	$9.3 \pm 0.5 \times 10^{-5}$
$\Delta G^{\ddagger a}$ (kcal mol^{-1})	17.7 ± 1.0^b	18.5 ± 1.0^b	20.1 ± 1.1^c
$\Delta S^{\ddagger}$ (eu)[d]	-37.0^b	-26.0^b	-15.5^c
$\Delta H^{\ddagger}$ (kcal mol^{-1})	7.8 ± 0.5	12.6 ± 1.5	16.5 ± 1.2

[a] Enzyme concentration = 3.0×10^{-5} M. Substrate concentration = 6.0×10^{-4} M. [b] At −41.0 °C, pH* 6.1, 60% dimethyl sulfoxide. [c] At −25 °C, pH* 6.2, 60% dimethyl sulfoxide. [d] Estimated error ±20%.

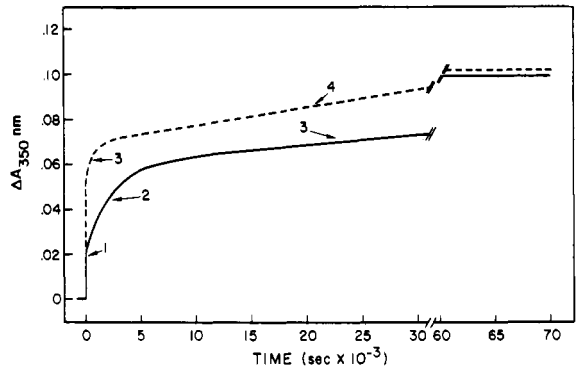


FIGURE 1: Time course of the reaction of N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide at 350 nm at −41.0 °C (—), pH* 6.8; and −5.0 °C (---), pH* 6.13; $E_0 = 4 \times 10^{-5}$ M, $S_0 = 3 \times 10^{-5}$ M, 60% dimethyl sulfoxide. The numerals 1–4 refer to the reactions discussed in the text.

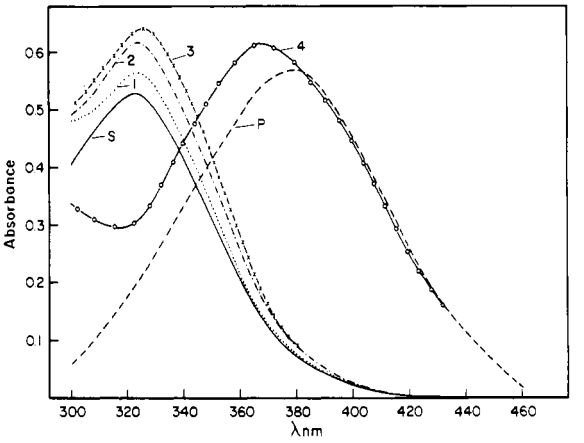


FIGURE 2: Spectra at the completion of reactions 1–4 and those of the substrate (S) (—) and product, *p*-nitroaniline (P) (---), for comparison; reaction 1 (···), reaction 2 (-·-), reaction 3 (X—X), and reaction 4 (O—O). Since the spectra are those of all the species present at the completion of the indicated reaction, they are not necessarily the spectra of individual intermediates. Thus spectrum 1 is that of a mixture of S + ES_1, spectra 2 and 3 are predominantly those of ES_1 and ES_1', respectively, while spectrum 4 is that of the tetrahedral intermediate TI (see text and Figure 6). Experimental conditions were −40 °C, 60% dimethyl sulfoxide, pH* 6.1, $E_0 = 3 \times 10^{-5}$ M and $S_0 = 3 \times 10^{-5}$ M, except for that of the spectrum of the product of reaction 4 and *p*-nitroaniline. These were obtained at −3 °C, with $E \gg S$, $E_0 = 7.5 \times 10^{-5}$ M, $S_0 = 2.7 \times 10^{-5}$ M in 60% dimethyl sulfoxide, pH* 9.3.

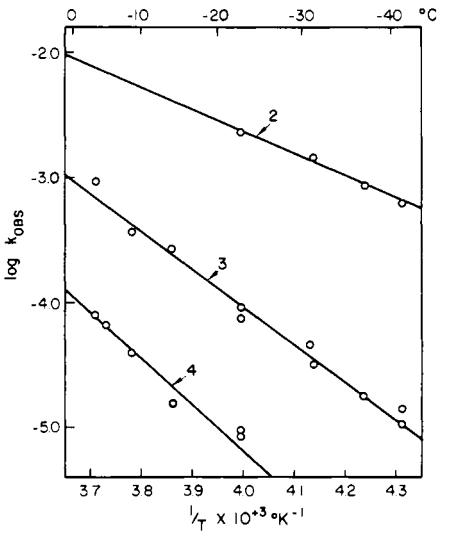


FIGURE 3: Arrhenius plots for reactions 2–4. The reaction conditions were identical for all the reactions, pH* 6.1, 60% dimethyl sulfoxide, $E_0 = 3 \times 10^{-5}$ M, $S_0 = 3 \times 10^{-5}$ M.

solvent system. The observed first-order rate constants were directly proportional to the concentration of the limiting reagent.

Low-Temperature Kinetics. Even at 0 °C in the cryosolvent, it was found that turnover was very slow under non-substrate-saturating conditions (i.e., $S_0 \ll K_m$) due to the large value of K_m in 60% dimethyl sulfoxide (Fink & Angelides, 1976). When papain and N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide were mixed at temperatures in the −30 to −50 °C range at pH* 6.1, an initial jump in the absorbance, which we have designated as reaction 1, was observed (Figure 1). This reaction was too fast for its kinetics to be resolved under the experimental arrangement. Reaction 1 was followed by two slower reactions which we have termed reactions 2 and 3, respectively (Figure 1). Although, under the experimental conditions used in Figure 1, reactions 2 and 3 are distinguished on the basis of the biphasic nature of the reaction curve, other conditions exist (vide infra) where their temporal resolution is much more complete.

Figure 2 shows the absorbance spectrum of the substrate prior to addition of enzyme, plus the resulting spectra at the completion of reactions 1–3. At temperatures above −25 °C an additional reaction, reaction 4, became detectable (Figure 1). The spectrum of the product of reaction 4 is also shown in Figure 2.

Good first-order plots were obtained for reactions 2–4 and Arrhenius plots for these reactions are shown in Figure 3. The rates for reactions 2 and 3 were pH* dependent over the range of pH*s examined (3.0–9.3). The limiting values of k_{obsd}, the pK^*s, and the activation parameters calculated from Arrhenius plots for reactions 2 and 3 are given in Table I.

The magnitude of the change in absorbance of reaction 2 decreased with increasing pH*, whereas the amplitude for reaction 3 was apparently unaffected except at the very high pH* range (i.e., pH* ≥8.6). At pH* 9.3 and −30 °C, a single exponential was observed corresponding to reaction 3, i.e., reaction 2 was not apparent at this pH*. The sum of amplitudes for reactions 1 and 2 was constant through the pH* range investigated.

The rate of reaction 4 shows bell-shaped pH* dependence (Table I). In addition the amplitude of the spectral change for reaction 4 increased with increasing pH* and was at a

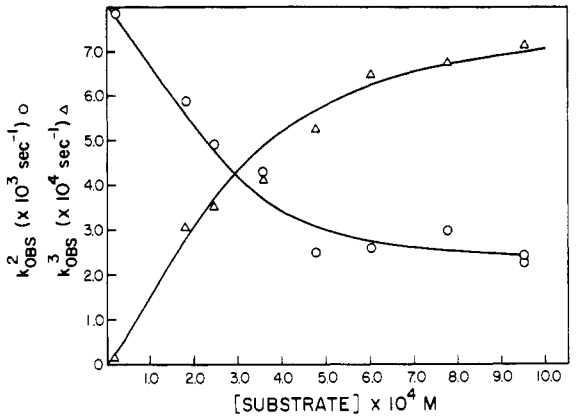


FIGURE 4: The observed rates of reaction 2 (○) and 3 (△) as a function of substrate concentration, pH* 6.26, −30 °C, 60% dimethyl sulfoxide. $E_0 = 1 \times 10^{-5}$ M. Note that the scale on the ordinate for each of the reactions differs by one order of magnitude. Solid curves are theoretical for values of $k_2 = 2.5 \times 10^{-3}$, $K_1 = 1.65 \times 10^{-4}$; and $k_3 = 9.3 \times 10^{-4}$, $K_1K_2 = 5 \times 10^{-4}$ (see Table II).

maximum at the most alkaline pH* examined, pH* 9.3. Detailed results of reaction 4 are given in the accompanying paper (Angelides & Fink, 1979). However, a few pertinent results are included here in order to facilitate comparison of reaction 4 with reactions 1–3.

Reaction 5, which was detectable at temperature above −5 °C, corresponded to turnover, as indicated by the release of *p*-nitroaniline. The spectrum of the product, *p*-nitroaniline, is also given in Figure 2. Under conditions of $S_0 \gg E_0$, the rates of reaction 2–4 varied as a function of the substrate concentration, and the magnitude of the spectral change (i.e., the concentration of the intermediate) varied as a function of the enzyme concentration. This is the type of behavior predicted for reactions preceding the rate-limiting turnover step under non-turnover conditions (Fink, 1976b). The rate of reaction 5, however, varied as a function of the enzyme concentration as would be expected for turnover. These observations are in accord with the overall rate-limiting step being the formation of the acyl-enzyme (Angelides & Fink, 1979).

Under the conditions of $S_0 \gg E_0$, the microscopic rate constants for reactions 2 and 3 were obtained by varying the substrate concentration over the range 2.4×10^{-5} to 1.3×10^{-3} M at pH* 6.3, −30 °C, in 60% dimethyl sulfoxide (Fink, 1976b). Interestingly, the rate of reaction 2 *decreased* as the substrate concentration increased, while the rates of both reactions 3 and 4 increased with increasing substrate concentrations. As a result, at the highest concentrations of substrate used (1.3×10^{-3} M), it was not feasible to accurately resolve the contributions of reactions 2 and 3 due to their similar rates and unequal amplitudes. However, as shown in Figure 4, reactions 2 and 3 tend toward saturation and a computer fit of reciprocal plots allowed the desired microscopic rate constants to be determined (Fink, 1976b). Microscopic rate constants obtained from the data at pH* 6.3, −30 °C, and 60% dimethyl sulfoxide are given in Table II. For reaction 2, the substrate concentration at half-saturation is related to K_1, and for reaction 3 the substrate concentration at half-saturation is related to $K_1(1 + K_2)$ (see Scheme III) (Fink, 1976b). By cross-checking the value of K_1K_2 obtained from each of these plots, we find that they are in good agreement with each other. The value of K_1K_2 is also given in Table II.

With the microscopic rate constants in hand, we are now able to carry out a detailed thermodynamic analysis and construct a free energy vs. reaction coordinate diagram for the reaction of papain with N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide at −30 °C and pH* 6.3. This is shown in Figure 5 (see Discussion for interpretation).

Table II: Microscopic Rate Constants for Reactions 2 and 3

reaction 2[a]	$k_{-2} = 9.3 \pm 0.2 \times 10^{-3}$ s^{-1}
	$k_2 = 2.5 \pm 0.3 \times 10^{-3}$ s^{-1}
	$K_2 = 3.7 \pm 0.6$
reaction 3[a]	$k_{-3} = \leq 1 \times 10^{-5}$ s^{-1}
	$k_3 = 9.1 \pm 0.5 \times 10^{-4}$ s^{-1}
	$K_3 \leq 1.1 \pm 0.5 \times 10^{-2}$
	$K_1 = 1.25 \pm 0.5 \times 10^{-4}$ M
	K_1K_2[b] $= 2.5 \pm 1.52 \times 10^{-4}$ M
	K_1K_2[c] $= 4.0 \pm 2.0 \times 10^{-4}$ M

[a] At −30 °C, pH* 6.3, and 60% dimethyl sulfoxide. [b] From k^3_{obsd} vs. S_0. [c] From k^2_{obsd} vs. S_0.

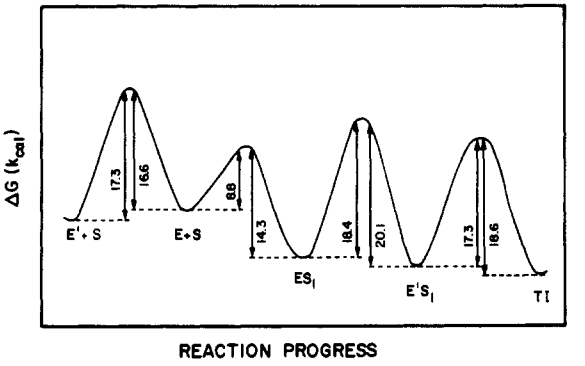


FIGURE 5: Free energy vs. reaction progress for the reaction of papain with N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide at −30 °C, 60% dimethyl sulfoxide. The profile is for Scheme III with the data of Tables I and II.

In order to characterize the nature of the intermediates observed and to determine whether the thiol of Cys-25 was involved as a catalytic group in any of the observed reactions, papain-S-S-CH_3 was used in place of the native enzyme. At pH* 6.1, −40 °C, reactions 1 and 3 were present and were similar, although of smaller amplitude, to those observed with nonmodified papain. Neither reactions 2, 4, or 5 were observed with papain-S-S-CH_3. Since reactions 2–4 are not observed when either mercuripapain or papain inactivated with Tos-LysCH_2Cl is used, we are certain that the observed spectral changes reflect substrate bound in the active site of the enzyme.

Fluorescence Lifetime Experiments. The fluorescence decay kinetics for papain was studied in aqueous solution at two different pHs at 25 °C. A good fit of the fluorescence decay, $F(t)$, is obtained with the biexponential equation

$$F(t) = a_1e^{-t/\tau_1} + a_2e^{-t/\tau_2}$$

where a_1 and a_2 are constants which will arbitrarily be normalized to $a_1 + a_2 = 1$, and τ_1 and τ_2 are fluorescence lifetimes. In addition the data can be described by an average lifetime, τ_{av}, defined as $\tau_{av} = a_1\tau_1 + a_2\tau_2$ (Angelides & Hammes, 1979). This average lifetime is the normalized area under the decay curve and, therefore, represents a relative quantum yield. At pH 6.1 the values found were $a_1 = 0.21$, $\tau_1 = 2.2$ ns, $a_2 = 0.79$, $\tau_2 = 5.5$ ns, $\tau_{av} = 4.7$ ns. At pH 9.0, $a_1 = 0.41$, $\tau_1 = 3.4$ ns, $a_2 = 0.59$, $\tau_2 = 6.7$ ns, and $\tau_{av} = 5.3$ ns. The amplitude ratios a_1/a_2 are different at the two pHs and are 0.27 at pH 6.1 and 0.69 at pH 9.0.

Discussion

The results of this investigation provide evidence for several intermediate transformations prior to turnover when papain reacts with N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide at subzero temperatures. In order to utilize the results of these findings

to explain the mechanism of action of papain, it is essential that their relevance to the reaction under normal conditions (i.e., no organic cosolvent and ambient temperatures) be established. In particular, it is necessary to determine whether the observed spectral changes correspond to transformations of productive enzyme–substrate intermediates, whether the same intermediates might be formed under normal conditions but not detected because of their low concentrations and short lifetimes, and whether any information concerning the nature of the postulated intermediates can be gleaned by the data obtained in this study.

We have previously shown that 60% aqueous dimethyl sulfoxide and subzero temperatures have no adverse effects on either the structure or catalytic properties of the enzyme (Fink & Angelides, 1976). However, an increase in K_m is observed as the cosolvent concentration increases. This is due to increasingly less favorable partitioning of the substrate to the active site from the bulk solvent (probably caused by the change in hydrophobicity of the solvent). The result is that the initial binding of substrate to enzyme is decreased and the dissociation constant, K_1, increases (Fink, 1974). Thus, under non-substrate-saturating conditions, the rates of all the steps in the reaction will be decreased (Fink, 1976b). In the case of papain and 60% dimethyl sulfoxide, the increase in K_m is of the order of 10^3 for N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester at 0 °C (Fink & Angelides, 1976). The consistent agreement between the overall catalytic properties of papain-catalyzed reactions (acylation and deacylation rates, pH dependence, bursts of *p*-nitrophenol from below 0 °C in cryosolvent) and those obtained under normal conditions indicates that the overall reaction pathway is the same in 60% dimethyl sulfoxide and subzero temperatures as it is in the absence of organic cosolvent at 25 °C (Fink & Angelides, 1976).

We are confident that the observed absorbance changes (Figures 1 and 2) reflect interactions between the substrate and the enzyme's active site since they are seen only with active enzyme present and are absent when mercuripapain or papain inactivated by Tos-LysCH_2Cl is used. In these enzyme derivatives, productive substrate binding is not to be expected since the active site is hindered or blocked. Since turnover eventually occurs if the temperature is raised sufficiently after mixing enzyme and substrate at very low temperatures, the observed intermediates formed by reactions 1–4 must be either on the productive catalytic pathway or in readily reversible equilibrium with an intermediate on the productive pathway. From an individual consideration of each intermediate reaction (see following discussion), we are able to eliminate the possibility that an irreversible dead-end complex or kinetically significant nonproductive complex may exist. We will now consider the processes which may be responsible for the observed reactions.

Reaction 1. We ascribe the initial rapid process after addition of enzyme to substrate in the −65 to −35 °C range (reaction 1 in Figure 1) to substrate binding resulting in the formation of the initial noncovalent Michaelis complex, ES_1. This assignment is supported by the observed rates since under the experimental conditions $k_{obsd} \geq 0.2$ s^{-1} at −65 °C and is in accord with expectations for the diffusion-controlled substrate binding reaction. Further support in this assignment of reaction 1 to substrate binding emanates from the observed absence of the reaction when papain inactivated with Tos-LysCH_2Cl was used since substrate would not be able to bind to this derivative. The spectrum is inconsistent with the substrate being in a distorted form since in this case it would be expected that the λ_{max} of the intermediate would be significantly shifted from the λ_{max} of the substrate.

Scheme I

$$\text{(a)}\quad E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} I \rightarrow$$

$$k_2 \downarrow$$

$$ES_{DE}$$

$$\text{(b)}\quad ES_{NP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \rightarrow$$

Scheme II

$$E' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1$$

Reaction 2. This reaction has several interesting features. It is pH* dependent with pK*s of 4.4 and ≥8.0 (at −25 °C). The magnitude of the increase in absorbance decreased with increasing pH* until it became absent at pH* ≥9.0. This reaction was completely absent with papain-S-S-CH_3. Under conditions of $S_0 \gg E_0$, the rate of reaction 2 was inversely proportional to the change in the substrate concentration (Figure 4). Two possibilities will be considered to describe the events responsible for the observed process(es) of reaction 2.

(1) The binding of the substrate results in the formation of an irreversible dead-end complex, e.g., ES_{DE} (Scheme Ia), or a nonproductive enzyme–substrate complex, e.g., ES_{NP} (Scheme Ib). These explanations can be eliminated by several observations. Kinetically the observed rate constant for the formation of ES_{NP} would be $k_{obsd} = k_{-2} + k_2S_0$ under conditions of $S_0 \gg E_0$. Hence an increase in the observed rate of reaction 2 would be expected with an increase in the substrate concentration. The fact that an inverse relationship between the observed rate constant (k^2_{obsd}) and the substrate concentration (Figure 4) is found is good evidence to exclude this pathway. An irreversible dead-end complex can be eliminated by a similar kinetic argument. When the reaction mixture is warmed, intermediate 2 converts to products as monitored by the appearance of *p*-nitroaniline at 380 nm. This implies that intermediate 2 lies in readily reversible equilibrium with the productive catalytic pathway. Furthermore, the Arrhenius plots for reactions 2 and 3 indicate that the product of reaction 2 would be a kinetically significant intermediate at ambient temperatures.

(2) The spectral changes associated with reaction 2 reflect a reorganization of the active-site structure. The reaction could involve a conformational change that follows substrate binding or a conformational change that precedes the binding step (Scheme II). The appropriate kinetic expressions for these two mechanisms are $k^2_{obsd} = k_{-2} + k_2/(1 + K_1/S_0)$ and $k^2_{obsd} = k_2 + k_{-2}/(1 + S_0/K_1)$, respectively, for $k_1 \gg k_2$.

It is fairly easy to distinguish the two mechanisms. Comparison shows that the observed rate constants from the isomerization steps may be either increased or decreased by increasing the substrate concentration, depending upon whether the isomerization occurs after or before the binding step, respectively. The mechanism of Scheme II requires that the value of k^2_{obsd} should decrease from $(k_2 + k_{-2})$ to k_2 as the initial substrate concentration is increased. This concentration dependence fits the data of Figure 4 and, hence, the mechanism in Scheme II is most consistent with the observations. This situation is formally analogous to the equilibrium between the active and inactive forms of α-chymotrypsin, in which a similar relationship has been derived (Fersht & Requena, 1971). The isomerization of E′ → E is detectable as reaction

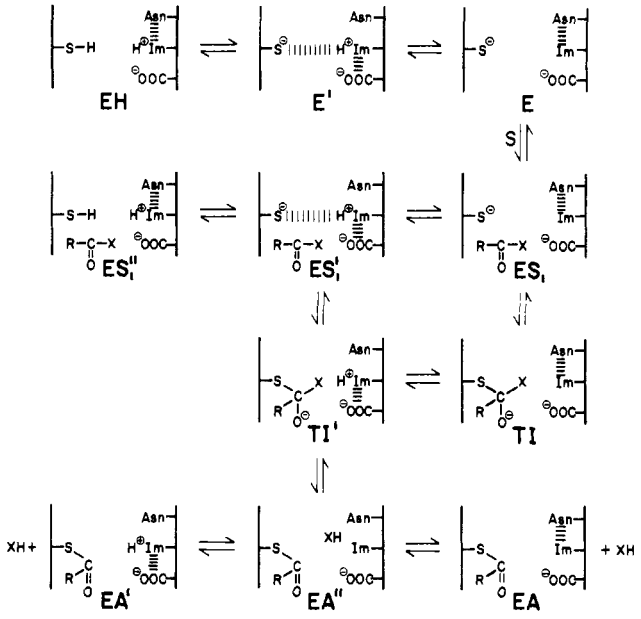


FIGURE 6: Proposed reaction pathway for papain catalysis. Reproduced from Angelides & Fink (1978).

2 because it is coupled with the faster substrate binding reaction, reaction 1. That is, the observed spectral changes for reaction 2 correspond to the uptake of substrate in the second step as

$$S + E' \xrightarrow{\text{slow}} S + E \xrightarrow{\text{fast}} ES_1$$

We therefore conclude that the catalytic mechanism of papain involves an isomerization of the enzyme before substrate binding. From the asymptotic values of k^2_{obsd} at high and low substrate concentrations (Table II), a value of k_{-2}/k_2 of 3.7 ± 0.6 is found, corresponding to approximately 80% E′ and 20% E at pH* 6.3 and −30 °C. This is in reasonable agreement with the results presented by Lewis et al. (1976) whose data are consistent with 90° E′ and 10% E based on potentiometric difference titrations of papain and papain-S-S-CH_3 in aqueous solution, 20 °C, and our fluorescence lifetime measurements (see below). The value of K_1 may be obtained from the data of Figure 4 and eq 2. Under the experimental conditions, −30 °C, pH* 6.3, the calculated value of K_1 is $1.2 \pm 0.5 \times 10^{-4}$ (Table II), and, thus, the equilibrium for $E + S \rightleftarrows ES_1$ is very much in favor of the ES_1 complex.

Since the enzyme will be present largely in the unreactive E′ state in the absence of ligand at pH* 6.3 and −30 °C, the addition of excess substrate causes the E′ to E equilibrium to be displaced toward E (at the rate controlled by k_2) by the formation of ES_1. Thus the rate of reaction 2 corresponds to the rate of isomerization of E′ to E, but the product of reaction 2 is ES_1 (Scheme III, Figure 6).

What can be said about the nature of the enzyme conformation change that precedes the binding of substrate? In a previous publication, we have presented the detailed mechanism of action of papain with a specific ester substrate (Angelides & Fink, 1978). In that report we were able to reconcile a substantial body of data concerning papain's structure and function by invoking a two-state model for the free enzyme. The postulated reaction pathway is reproduced in Figure 6.

A key factor in our proposed mechanism is that at high pH papain exists in a conformation (E) in which the active-site imidazole is hydrogen bonded to Asn-175 (the crystallographically determined structure) which we will designate the UP conformer. For reasons that will be be discussed below

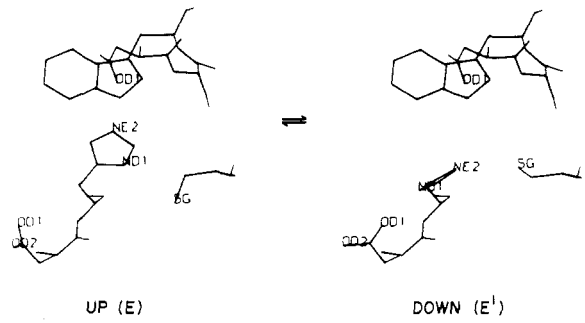


FIGURE 7: Photograph of the screen display of the Molecular Modelling computer graphics system representing the interconversion of catalytically active but "sterically" inactive papain (E′) to the sterically active, catalytically inactive form (E). Coordinates for the papain molecule used were those reported by Drenth et al. (1976). The side chains of Cys-25, Asp-158, His-159, Trp-177, and Asn-175 are shown. SG represents the S^γ of Cys-25; NE2 and ND1 are the imidazole ring nitrogens of His-159, and OD1 and OD2 represent the carboxyl oxygens of Asp-158. According to our proposed mechanism, the H bond between the imidazole and Asn-175 in E would in fact involve the Asn as the proton *donor* and imidazole as the *acceptor*.

we interpret the isomerization of E to E′ to involve a rotation of the imidazole of His-159 about its α–β axis by +79° and β–γ plane by +35° to a position which we have termed DOWN (E′). Repositioning of the Asp-158 carboxyl side chain by rotating C^α–C^β by 3° and C^β–C^γ by 68° also occurs.[2] In E′, the DOWN conformer, the imidazole is predominantly protonated ($pK \approx 8$) and is electrostatically "fixed" by its interactions with the carboxylate of Asp-158 and the thiolate of Cys-25. This is shown in Figure 7. There is substantial evidence that supports both the ion pair and conformational isomerization model (Polgar, 1973, 1974a,b, 1976a,b, 1977; Drenth et al., 1975, 1976; Lewis et al., 1976). Though the ion pair is the *catalytically* active form (Polgar, 1973, 1974a,b, 1976a,b, 1977; Shipton et al., 1975; Lewis et al., 1976; Drenth et al., 1975; Sluyterman & Wijdenes, 1976), access to the active site of papain is hindered when the imidazole is in the DOWN position. As a result the substrate is unable to bind to this form of the enzyme, at least at a comparable rate. When the imidazole of His-159 is in a position in which it is hydrogen-bonded to the carbonyl side chain of Asn-175 (UP), the substrate would be able to bind in the active site area. However, as has been previously described for the events in papain catalysis of a specific ester substrate (Angelides & Fink, 1978), this form of the enzyme is not the catalytically competent form, although it is capable of binding the substrate.

Our interpretation that reaction 2 involves this specific type of enzyme conformational change is based upon the data for the kinetics of the interconversion, the pH dependency, the absence of the reaction with papain-S-S-CH_3, and preliminary fluorescence lifetime studies of papain and papain-S-S-CH_3.

The following features of the transformation between E′ and E are of note. At high pH (>8.5), the predominant form of the enzyme is the UP conformation, E, in which the thiol is present as S^- and the imidazole as the free base. In this conformer the pK of the thiol is ~8 and of the imidazole ~4. On decreasing the pH, the thiolate becomes protonated, yielding the species EH (UP conformation). This form rapidly isomerizes to the corresponding DOWN conformer which undergoes a proton transfer (from thiol to imidazole) and yields the stable conformer E′, in which the imidazolium is

[2] These values were obtained from the Molecular Modelling System at the University of California, San Francisco, by using the X-ray crystallographic coordinates reported by Drenth et al. (1976).

electrostatically interacting with the carboxylate of Asp-158 and the thiolate. This is the stable and predominate species in the pH region 4–8, and has pK ~4 for the thiol and ~8 for the imidazole. The driving force for the isomerization can be either a pH change or binding of substrate. In the absence of substrate, the thiolate anion is required to stabilize the DOWN conformation (see results with papain-S-S-CH_3). At low pH (pK ~4), protonation of the thiolate weakens the salt-bridge interactions maintaining the DOWN conformation, and the initially formed E'H species isomerizes to the UP conformer EH''. Thus the top row of Figure 6 shows only the predominant species under acidic, neutral and basic conditions (Angelides & Fink, 1978). The reported cooperative ionizations of two groups with pKs ~4 (Bendall & Lowe, 1976) is consistent with the thiolate–imidazolium–carboxylate interactions (i.e., the pK of the carboxylate of Asp-158 will be sensitive to the ionization state of the adjacent imidazole, which in turn is dependent on the ionization state of the thiol).

As the pH* is increased from the acidic to the neutral region, the observed rate of reaction 2 increases corresponding to the increased concentration of E'. Further, the amplitude of reaction 1 increases on raising the pH* (see below) as more of the enzyme becomes in the E conformation. As is predicted for this mechanism (Figure 6), the total absorbance change for reactions 1 and 2 is constant, within experimental error, over the pH* range 4.5–8.6. At pH >8, however, the observed rate of interconversion (reaction 2) slows down as the histidine gradually becomes deprotonated, the thiol remaining the anion, regardless of whether its pK is 4 or 8 at this stage (as indicated before the thiol pK would change from 4 to 8 upon deprotonation of the histidine). Consequently the majority of free papain is in the UP form, the catalytically inactive but "sterically" active conformer. In fact, reaction 2 should disappear at high pH*s since the predominant species would be the free-base form of the histidine in which it is UP. This is exactly what is observed experimentally (see Results).

Furthermore the relative magnitudes of the absorbance change for reactions 1 and 2 vary as a function of the pH*. As the pH* increases, the ratio of the observed spectral changes are as follows:

$$\frac{\Delta A_{\text{reac 1}}}{\Delta A_{\text{reac 2}}} \geq \frac{\Delta A_{\text{reac 1}}}{\Delta A_{\text{reac 2}}} > \frac{\Delta A_{\text{reac 1}}}{\Delta A_{\text{reac 2}}}$$

pH*: 8.5 3.0 5–7

Accordingly the magnitude of reaction 1 is equivalent to the concentration of E, whereas the magnitude of reaction 2 is equivalent to the concentration of E' (see (Figure 1). The ratio of the amplitudes of the reaction is approximately 3 in the neutral pH* region and gives an estimate of the ratio of the species. This estimate is in accord with the value of the equilibrium constant of 4 obtained from the kinetic analysis of reaction 2. These observations are entirely consistent with the proposed mechanism.

On the basis of preliminary fluorescence lifetime experiments with papain at a few pH values, we have been able to obtain estimates for the relative proportions of the two conformers. It is well established that the majority of the fluorescence emission comes from Trp-177, located at the active site (Lowe & Whitworth, 1974). Upon lowering the pH from 9 to 6, Shinitsky & Goldman (1967) demonstrated that the tryptophan emission of papain is partially quenched (with pK = 8.5) and attributed this to quenching of the tryptophan by formation of a charge-transfer complex with an adjacent histidine.

If we assume that the lifetimes reflect the decay of Trp-177 and that the decrease from 0.69 to 0.27 for the ratio of a_1/a_2 on changing the pH from 9.0 to 6.1 is due to protonation of the adjacent histidine, then we can arrive at approximate assignments for the state of the free enzyme as a function of pH. Based on the effect of pH on the lifetimes and ratios of a_1/a_2, we interpret a_1 to reflect the E conformation since minimum quenching is observed with this form at the high pH, as would be anticipated from the model. The proportion of a_2 decreases as the pH increases which is consistent with it corresponding to E' in which the imidazole is DOWN and protonated. Accordingly at neutral pH the majority of the free enzyme is in the DOWN, ion-pair, form, whereas at high pH it is predominantly in the thiol–imidazole, UP, form.

These results are, of course, only semiquantitative because the true decay function is certainly more complex and energy transfer among the five tryptophan residues may also exist. Experiments are currently underway to selectively modify three of the five residues and to repeat the experiments described above.

Reaction 3. Reaction 3 is similar to reaction 2 in several respects. The spectra for the products of reactions 2 and 3 are similar in that no *p*-nitroaniline is indicated and they resemble the spectrum of the substrate. Reaction 3 is present with the *S*-methylthio derivatives of papain and, hence, cannot involve the formation of a covalent attachment to this residue.

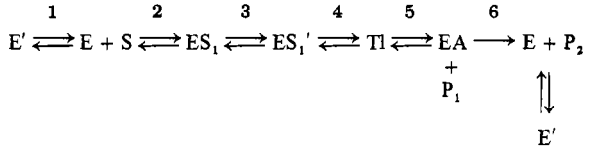
Again the question arises whether reaction 3 lies on the productive catalytic pathway. Based largely upon the kinetic analysis, and since the rate of reaction 3 has always been faster than the rate of turnover, the rate is consistent with the formation of a productive complex or dead-end complex in readily reversible equilibrium with the productive pathway.

Based on our proposed mechanism, reaction 3 corresponds to a repositioning of the active-site residues which activates the enzymes to the catalytically competent ion-pair state, i.e., the reverse of reaction 2. This is a similar interpretation of the events described for reaction 2 with the methyl ester substrate of N^{α}-carbobenzoxy-L-lysine, i.e., ES_1 to ES_1' (Angelides & Fink, 1978). We believe that reaction 2 with the methyl ester and reaction 3 with the *p*-nitroanilide represent the same process. Circular dichroism studies with the methyl ester substrate and *p*-nitroanilide substrate at low temperatures indicate that no *substantial* conformational change occurs in the enzyme (K. Angelides and A. L. Fink, unpublished results).

Both reaction 2 with the methyl ester substrate and reaction 3 with the *p*-nitroanilide substrate exhibit a pK* of ~4. We interpret this pK* to represent the cooperative ionization of the conformation-controlling Asp-158 and the thiolate of Cys-25 which maintain the imidazole in the DOWN conformation (e.g., see Figures 6 and 7). The importance of the carboxylate in the catalytic mechanism of papain has been discussed by Zannis & Kirsch (1978). The enzyme–substrate complex formed in reaction 3, ES_1', is now in the catalytically competent form in which the ion pair predominates and nucleophilic attack on the substrate carbonyl will subsequently take place. Furthermore, we suggest that the process(es) of reaction 3 result in proper orientation of the substrate and catalytic groups of the enzyme for this key chemical step to occur.[3]

[3] We believe that direct conversion of ES_1 to the tetrahedral intermediate TI is not a significant process for two reasons. First, the presence of the substrate induces the enzyme isomerization to the DOWN conformer ES_1' (at neutral pH, ES_1' is favored over ES_1 by a factor of 10^2, Table II); secondly, the substrate is not optimally oriented with respect to the thiolate in ES_1, but is in a more favorable orientation in ES_1' (Angelides & Fink, 1979).

Scheme III



Reaction 4. The pH* dependence of reaction 4 and its absence with papain-S-S-CH_3 indicate the critical involvement of Cys-25 as a catalytic agent. This reaction has been extensively studied and is the subject of a more detailed report (Angelides & Fink, 1979). Briefly all the available data suggest that reaction 4 corresponds to the formation of a tetrahedral intermediate, whose breakdown is rate limiting at neutral and high pH.

Reaction 5, Turnover. Reaction 5, the slowest of all the observed reactions, involves the formation of *p*-nitroaniline and corresponds to the turnover reaction. Although it is suspected that the rate of this reaction corresponds to the formation of acyl-enzyme, this cannot be unambiguously defined since a nondetected process which is rate limiting in the overall catalysis (e.g., a conformational change) may occur between reaction 4 and the acylation reaction.

The value of K_m for the proposed reaction pathway (Scheme III) under steady-state, turnover conditions is given by

$$K_m = \frac{K_1K_3K_4(1 + K_2)}{1 + K_4 + K_3K_4}$$

Unfortunately the self-consistency of the values of the dissociation and equilibrium constants determined at subzero temperatures (Table II) cannot be directly checked since we have not measured their temperature dependence. However, if we make the appropriate corrections for pH* and cosolvent effects, and assume that the values of $\Delta H°$ range from 2 to 5 kcal mol^{-1}, we estimate a value of $K_m = 0.3$ μM for pH 6, 25 °C, aqueous solution. The measured value for these conditions is 10 μM (Angelides & Fink, 1978). In the absence of experimental data concerning the temperature dependence of the equilibrium constants, we can only say that the values of K_m and K_1 to K_4 are not inconsistent.

Reaction Pathway. The spectral changes observed in the reaction of papain and N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide are best interpreted in terms of Scheme III. Reaction 6 may involve several intermediates, possibly analogous to ES, ES$_1$, ES$_1$′, and TI in which *p*-nitroaniline is replaced by water. Significant mechanistic points include: the isomerization of the predominant unliganded enzyme species E′ to the E conformer; the substrate-induced isomerization ES → ES$_1$′, i.e., the accumulation of a discrete tetrahedral intermediate at high pH, followed by its rate-limiting collapse to an acyl-enzyme. Additional discussion of the reaction pathway and mechanism is given in Angelides & Fink (1978, 1979).

The energy diagram shown in Figure 5 reveals a number of interesting features. For example, the two conformers differ by 1 kcal mol^{-1} in free energy. For the "internal states" (Albery & Knowles, 1976), the transition state involving tetrahedral intermediate formation is most stable, consistent with transition-state theory in which maximal affinity of the enzyme for this transition state would be expected. The similar free energies of the transition states and intermediates are in accord with expectations based on considerations of enzyme efficiency (Albery & Knowles, 1976; Fersht, 1974). Since the data in Figure 5 apply to the reaction at −30 °C, there are likely to be differences (probably relatively small) for the reaction under normal conditions.

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Mechanism of Thiol Protease Catalysis: Detection and Stabilization of a Tetrahedral Intermediate in Papain Catalysis†

Kimon J. Angelides‡ and Anthony L. Fink*

ABSTRACT: Evidence supporting the existence of a tetrahedral intermediate in the acylation reaction of papain with the specific substrate N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide is presented. This intermediate can be directly observed both at subzero temperatures by using cryoenzymological techniques as well as in aqueous solution at 25 °C by using rapid reaction techniques. The intermediate can be accumulated in essentially 1:1 stoichiometry with active enzyme at high pH* (≥9.3) and stabilized for indefinitely long periods at subzero temperatures (pH* is the apparent pH in the cryosolvent). Both the rate of formation and concentration of the intermediate are pH dependent ($pK_1 \simeq 4.3$; $pK_2 \simeq 8.6$). Formation of the intermediate is rate limiting at low pH, whereas breakdown is rate determining at high pH. The spectral characteristics of the tetrahedral intermediate are similar to those of *p*-nitroaniline except λ_{max} is blue-shifted by about 16 nm. If mercuripapain or the *S*-methylthio derivative of papain is used, no reaction is observed at ambient or subzero temperatures. Extrapolation of the rate of formation of the tetrahedral intermediate at subzero temperatures to 25 °C and 0% cosolvent yields a calculated value of $k_{obsd} = 65 \pm 10\ s^{-1}$, in excellent agreement with the observed value of $70 \pm 4\ s^{-1}$ from stopped-flow studies. The role of stereoelectronic factors in the formation of the tetrahedral intermediate is considered in the context of the proposed catalytic mechanism.

The delineation of the mechanism of action of thiol proteases requires a knowledge of all the intermediates on the reaction pathway. In particular a key question in the hydrolysis of peptides or esters by papain is whether or not a tetrahedral intermediate accumulates during the catalytic process. Tetrahedral intermediates were first implicated in nonenzymatic acyl-transfer reactions (Bender, 1951; Johnson, 1967). In enzyme-catalyzed reactions, however, there has been some question as to the ubiquity of a discrete intermediate on the reaction pathway (Fersht & Jencks, 1970; Fastrez & Fersht, 1973). Rather than a species with a finite lifetime, it has been suggested that the enzymatic acyl-transfer reactions may proceed via a single transition state, the distinction between an intermediate and transition state becoming obscured as the activation energy for the breakdown of the intermediate tends to zero.

Although many studies have been directed at providing evidence for the accumulation of tetrahedral intermediates in serine protease catalysis, there is little direct evidence for their existence as discrete intermediates. However, Hunkapillar et al. (1976) have recently reported the first evidence for the direct observation of such a species. They observed a transient spectral change at 410 nm in the elastase-catalyzed hydrolysis of Ac-Ala-Pro-Ala-*p*-nitroanilide which was attributed to the formation of a tetrahedral adduct. Most importantly their kinetic arguments were directly supported by the spectral observations and were entirely consistent with tetrahedral intermediate formation.

With the thiol proteases, papain and ficin, the search for tetrahedral intermediates has been less extensive and less successful. Perhaps one of the most suggestive items bearing on the question of the existence of such an intermediate is the X-ray crystallographic structure of a papain–inhibitor complex, N^{α}-carbobenzoxy-L-phenylalanyl-L-alanylmethylenepapain (Drenth et al., 1976). In this structure a notable feature is the presence of an "oxyanion hole" whereby the oxide moiety of the tetrahedral carbon atom is potentially stabilized by a hydrogen-bonding network formed between the NH peptide backbone of Cys-25 and the side chain ϵ-NH_2 of Gln-19, analogous to that found in the serine proteases (Henderson et al., 1971; Robertus et al., 1972; Birktoft et al., 1976).

Other evidence to support the existence of a tetrahedral intermediate in papain catalysis has come from structure–reactivity relationships. Using substituted phenyl esters and anilides of hippuric acid, Lowe & Yuthavong (1971) obtained Hammet ρ values which suggested that the formation of a tetrahedral intermediate was rate determining for the aryl esters and that breakdown was rate limiting for the anilides. O'Leary et al. (1974) determined the $^{14}N/^{15}N$ kinetic isotope effects for the papain-catalyzed hydrolysis of N^{α}-benzoyl-arginine amide. The isotope effects were found to be considerably larger in the papain-catalyzed hydrolysis than in the α-chymotrypsin-catalyzed reactions studied. From the observed large isotope effects, it was concluded that breakdown of a tetrahedral intermediate was the rate-limiting step for the overall catalysis at neutral pH.

In a recent study of the inhibition of papain by *N*-benzoylaminoacetaldehydes, nuclear magnetic resonance

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