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

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ABSTRACT: Evidence supporting the existence of a tetrahedral intermediate in the acylation reaction of papain with the specific substrate $N^{\alpha}$-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 $\lambda_{max}$ 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, $N^{\alpha}$-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 $\epsilon$-$NH_2$ 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 $\rho$ 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 $N^{\alpha}$-benzoyl-arginine amide. The isotope effects were found to be considerably larger in the papain-catalyzed hydrolysis than in the $\alpha$-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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(NMR) evidence was presented for a tetrahedral hemithioacetal intermediate formed from the aldehyde and active-site thiol group. This strongly supports the proposed involvement of a tetrahedral intermediate in papain-catalyzed substrate hydrolysis (Bendall et al., 1977). Other investigations with aldehyde substrate analogues also suggest that tetrahedral adducts are stabilized by papain (Westerik & Wolfenden, 1972; Lewis & Wolfenden, 1977). Nevertheless, despite numerous investigations, no tetrahedral intermediate has been directly observed in papain catalysis; hence, its presence as a discrete intermediate on the catalytic pathway can only be considered as speculative.

The present report is the result of a detailed kinetic, thermodynamic, and structural investigation of the papain-catalyzed hydrolysis of $N^{\alpha}$-carbobenzoxy-L-lysine *p*-nitroanilide at subzero temperatures, in which the chromophoric *p*-nitroanilide moiety was used as a sensitive probe for events occurring at the bond cleavage site. At subzero temperatures, pH* 6.0, and 60% dimethyl sulfoxide, the reaction between papain and $N^{\alpha}$-carbobenzoxy-L-lysine *p*-nitroanilide is represented by a series of four reactions prior to turnover. This report discusses only the slowest of these reactions, reaction 4, the formation of the tetrahedral intermediate. Details of the other three reactions are reported in the preceding paper (Angelides & Fink, 1979). The cryoenzymological results have been compared with a corresponding investigation in aqueous media at ambient temperatures by using rapid reaction techniques in order to demonstrate their relevance to the catalytic mechanism under "normal" conditions.

## Materials and Methods

Papain (two times crystallized) was a Worthington Biochemical Corp. product and was further purified by affinity chromatography by the method of Blumberg et al. (1970). The concentration of active enzyme was determined by active site titration by using $N^{\alpha}$-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester, or kinetic assay with $N^{\alpha}$-carbobenzoxy-L-lysine *p*-nitrophenyl ester (Bender et al., 1966; Bender & Brubacher, 1966).

Papain-S-S-$CH_3$,[1] in which cysteine-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-Lys$CH_2$Cl was prepared by a modification of the procedure described by Whitaker & Perez-Villasẽnor (1968).

$N^{\alpha}$-Carbobenzoxy-L-lysine *p*-nitrophenyl ester, $N^{\alpha}$-carbenzoxy-L-lysine *p*-nitroanilide, and L-tryptophanamide were all obtained from Vega-Fox Biochemicals, lots no. F7713 (mp 136–138 °C), F7877 (mp 86–89 °C), and 12050, respectively.

Aqueous organic buffers used for this investigation were made up on a volume/volume basis as described previously (Fink, 1973). The apparent protonic activity at the low temperatures (pH*) was obtained from the data of Hui Bon Hoa & Douzou (1973). The general experimental protocols used in the subzero temperature experiments have been reported previously (e.g., Fink & Angelides, 1976; Angelides and Fink, 1978, 1979).

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 ±0.2 °C. The steady-state hydrolysis of $N^{\alpha}$-carbobenzoxy-L-lysine *p*-nitroanilide was followed at 400 and 440 nm.

Stopped-flow experiments were performed on a Durrum Model 110 stopped-flow spectrophotometer with a dead-time of 5 ms. The syringe chamber and cuvette were thermostated at +21.5 °C. One syringe contained enzyme in 0.05 M borate buffer (pH 9.2) and the other contained $N^{\alpha}$-carbobenzoxy-L-lysine *p*-nitroanilide in 5% dimethyl sulfoxide. Volumes of both components were 100 μL. Reaction progress was monitored at 340 and 400 nm.

Alkylation of noncovalently modified active-site thiol groups by [$^{14}$C]iodoacetate was done by a method that has been previously reported (Angelides & Fink, 1978).

Table I: Steady-State Kinetic Parameters of Ester and Anilide Hydrolysis by Papain for a Series of $N^{\alpha}$-Carbobenzoxy-L-lysine Derivatives[a]

| | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) with L-tryptophanamide[d] |
|---|---|---|---|
| ester | | | |
| methyl[c] | 36 (6.65)[b] | 2350 (6.65)[b] | |
| | 29 (8.3) | 2950 (8.3) | |
| benzyl[c] | 44 (6.14) | 26 (6.14) | |
| | 39 (8.3) | 55 (8.3) | |
| *p*-nitrophenyl[c] | 44 (6.19) | 1.7 (6.19) | 72 (6.1)[b] |
| | 39 (8.3) | 4.9 (8.3) | |
| anilide | | | |
| *p*-nitro- | 0.25 (6.1) | 10 (6.1) | |
| | 0.09 (9.16) | 35 (9.16) | 0.25 (6.1) |

[a] At 25 °C. [b] The pH is given in parentheses. [c] From Brubacher & Bender (1966a,b). [d] $1 \times 10^{-2}$ M; see text.

## Results

*Kinetics of Ester and Anilide Hydrolysis in Aqueous Solution.* The steady-state parameters obtained by conventional kinetic methods for the hydrolysis of a series of substrates sharing the common $N^{\alpha}$-carbobenzoxy-L-lysine acyl group are shown in Table I. Values of $k_{cat}$ for all three esters are identical within experimental error (Brubacher & Bender, 1966). The value of $k_{cat}$ at any pH for the *p*-nitroanilide substrate is approximately 200 times smaller than those of the esters. In the presence of the added nucleophile L-tryptophanamide (10 mM), the hydrolysis of the esters is accelerated, $k_{cat}$ being increased by 64%; however, there is no effect on the rate of hydrolysis of the anilide. In addition, no effect on $k_{cat}$ or $K_m$ was observed when *p*-nitrophenol or *p*-nitroanilide ($1 \times 10^{-4}$ to $2.4 \times 10^{-3}$ M) was present under steady-state conditions at pH 6.2 and 9.2.

*Low-Temperature Kinetics.* At 0 °C, pH* 6.1, in 60% dimethyl sulfoxide, under nonsaturating substrate conditions, turnover, as monitored by the appearance of *p*-nitroaniline at 380 nm ($\lambda_{max}$), was very slow ($t_{1/2} = 2.3 \times 10^4$ s for $1 \times 10^{-5}$ M enzyme) due to the cosolvent-induced increase in $K_m$ (Fink & Angelides, 1976) (see Discussion). When papain and $N^{\alpha}$-carbobenzoxy-L-lysine *p*-nitroanilide were mixed at temperatures in the 0 to −25 °C range, under conditions in which turnover was negligible, a first-order increase in absorbance in the 400-nm region was observed as a function of time. The effects of temperature, pH*, and substrate concentration on the kinetics of the reaction were investigated under conditions of $S_0 >> E_0$. Some of the results are shown in Table II. An energy of activation of 15 ± 2 kcal $mol^{-1}$ and apparent p$K^*$s of 4.3 ± 0.5 and 8.6 ± 0.5 were calculated. As indicated in the representative low-temperature results in Table

[1] Abbreviations used: papain-S-S-$CH_3$, papain in which the thiol of Cys-25 has been modified to form the *S*-methylthiol derivative; Tos-Lys$CH_2$Cl, $N^{\alpha}$-tosyl-L-lysine chloromethyl ketone.

Table II: Effect of pH* and Temperature on the Kinetics of Reaction 4[a]

| pH* | $k_{obsd}$ ($s^{-1}$)[a] | temp (°C)[b] | $k_{obsd}$ ($s^{-1} \times 10^5$) |
|---|---|---|---|
| 3.09 | $4.2 \times 10^{-5}$ | −3.5 | 7.9 |
| 3.44 | $1.7 \times 10^{-4}$ | −5.0 | 6.6 |
| 3.90 | $2.8 \times 10^{-4}$ | −8.2 | 4.1 |
| 4.44 | $4.0 \times 10^{-4}$ | −14.0 | 1.6 |
| 6.10 | $1.0 \times 10^{-3}$ | −22.8 | 1.0 |
| 9.16 | $2.5 \times 10^{-4}$ | | |
| 9.30 | $1.8 \times 10^{-4}$ | | |

[a] In 60% dimethyl sulfoxide, −3 °C, $E_0 = 1.0 \times 10^{-5}$ M, $S_0 = 1.2 \times 10^{-3}$ M. [b] pH* 6.1, $E_0 = 3.0 \times 10^{-6}$ M, $S_0 = 3.0 \times 10^{-5}$ M.

Table III: Representative Kinetic Values for Reaction 4

| [enzyme] $\times 10^5$ (M) | [substrate] $\times 10^3$ (M) | $k_{obsd}$ ($s^{-1}$)[a] | $k_{obsd}$ ($s^{-1}$)[c] (stopped-flow) | [TI]/[$E_{total}$] (%)[d] |
|---|---|---|---|---|
| 1.0 | 1.2 | $2.03 \times 10^{-4}$[e] | | 91 ± 3 |
| 1.0 | 0.03 | $1.9 \times 10^{-5}$ | | |
| 6.0 | 1.2 | $2.0 \times 10^{-4}$ | | 91 ± 3 |
| 4.3 | 1.0 | | 72.0 | 33 ± 5 |
| 7.5[b] | 0.03 | $2.4 \times 10^{-5}$ | | |
| 7.7 | 0.5 | | 41.0 | 33 ± 5 |
| 7.7 | 1.0 | | 68.0 | |

[a] 60% dimethyl sulfoxide, pH* 9.3, and −3 °C. [b] [E] > [S]. [c] 0.05 M borate, pH 9.16, and +21.5 °C. [d] With $\epsilon_{400}$ of *p*-nitroaniline. See also Table IV. [e] Average deviations in the rate constants are less than ±10%.

III, the reaction rate increased as a function of increasing substrate concentration, and the magnitude of the spectral change ($\pi$) increased as a function of increasing enzyme concentration. From plots of $k_{obsd}$ vs. $S_0$ ($S_0 >> E_0$), it was possible to calculate the values of $k_4$ and $k_{-4}$ (Fink, 1976). The values obtained were: $k_4 = (2.2 \pm 0.4) \times 10^{-4}$ $s^{-1}$ and $k_{-4} = (1.8 \pm 0.6) \times 10^{-5}$ $s^{-1}$ at pH* 9.3, −3.0 °C, 60% dimethyl sulfoxide.

Under conditions of excess enzyme and very low substrate concentration, the background absorption of the substrate was considerably reduced so that the reaction could be followed by repetitive spectral scans covering the range 500–310 nm. The isosbestic point between the substrate and intermediate was at 342 nm. Correcting for the contribution of the enzyme to the UV absorption, the $\lambda_{max}$ of the spectrum of the intermediate (365 nm) was blue-shifted some 16 nm from the $\lambda_{max}$ (381 nm) of the product, *p*-nitroaniline, and 43 nm red-shifted from the substrate $\lambda_{max}$ (322 nm). The spectrum is shown in Figure 2 of Angelides & Fink (1979). The spectrum of *p*-nitroaniline is not significantly perturbed in solvents of low or high polarity: for example, $\lambda_{max}$ = 384 nm in formamide, and in acetone $\lambda_{max}$ = 379 nm. The kinetic parameters obtained under conditions of excess enzyme are also included in Table III and are consistent with those obtained for $S_0 >> E_0$ (Fink, 1976). Under conditions of excess substrate, extrapolation of the observed rate of reaction 4 to 25 °C, and correction to 0% solvent, yields a value of 65 ± 10 $s^{-1}$. This is in excellent agreement with the value obtained by using the stopped-flow technique of 70 ± 4 $s^{-1}$. If mercuripapain, papain inactivated by Tos-Lys$CH_2Cl$ or papain-S-S-$CH_3$ were used, or if either enzyme or substrate were omitted, no reaction corresponding to reaction 4 was observed.

At the low temperatures, it was possible to titrate the enzyme to determine the concentration of the intermediate by the isotopic alkylation method that we have previously used

Table IV: Concentration of Accumulated Tetrahedral Intermediate as a Function of pH* at Subzero Temperatures[a]

| pH* | [TI][b] $\times 10^6$ (M) | [TI]/[$E_{total}$] (%)[b,c] | [TI]/[$E_{total}$] (%)[d] |
|---|---|---|---|
| 3.09 | 0.266 | 2.6 | 3 ± 3 |
| 3.4 | 1.42 | 15 | |
| 3.9 | 1.7 | 17 | |
| 4.44 | 3.9 | 39 | |
| 6.1 | 6.7 | 67 | |
| 9.16 | 8.8 | 88 | 92 ± 4 |
| 9.3 | 9.2 | 92 | 98 ± 3 |

[a] In 60% dimethyl sulfoxide, −3 °C. [b] Concentration of accumulated tetrahedral intermediate as measured spectrophotometrically using $\epsilon_{400}$ of *p*-nitroaniline. [c] Fraction of total active enzyme present as the tetrahedral intermediate. [d] Based on titration with [$^{14}$C]iodoacetate.

(Angelides & Fink, 1978). In this procedure, the concentration of free thiol is determined by alkylation with [$^{14}$C]iodoacetate. These experiments require that the reverse catalytic reaction (i.e., $k_{-4}$ in Scheme I) be slower than the rate of thiol alkylation in the free enzyme. To test this condition, and to determine whether the results from the isotopic alkylation studies were correct, we incubated the enzyme and substrate at 0 °C in 60% dimethyl sulfoxide at pH* 9.3 and, after reaction 4 had proceeded nearly to completion, a portion of [$^{14}$C]iodoacetate (tenfold excess over enzyme) was added to the reaction mixture. Repetitive aliquots were subsequently withdrawn from the reaction mixture at selected time intervals, quenched with trichloroacetic acid, washed with acetone and anhydrous petroleum ether, and then counted. When the concentration of alkylated thiol, as determined by its radioactive counts, was plotted as a function of time, little increase (25% over 10 h) was observed. This implies that the reverse reaction rate constant $k_{-4}$ must be less than $2 \times 10^{-5}$ $s^{-1}$, as was found in the kinetic measurements. We conclude that the titration technique is well suited for determining the concentration of the intermediate, and that the addition of iodoacetate does not perturb the existing equilibria between free enzyme and intermediate(s). The stoichiometry of the intermediate formed by reaction 4 determined by this method is found to be 98 ± 3% at pH* 9.3, 60% dimethyl sulfoxide, and −3 °C.

At subzero temperatures the amplitude of the absorbance change for the reaction (i.e., the concentration of the intermediate) increased with increasing pH* (Table IV). These observations are supported by alkylation titrations with [$^{14}$C]iodoacetate in which the concentration of the intermediate was determined as a function of pH* (Table IV). From comparison of the two methods, we calculate $\epsilon_{400}$ 9736 $M^{-1}$ $cm^{-1}$ for the intermediate at pH* 9.3, −3.0 °C, 60% dimethyl sulfoxide.

*Stopped-Flow Kinetics.* Reaction 4 can also be detected at ambient temperatures using rapid-reaction techniques. A rapid increase in absorbance at 400 nm followed by a slow zero-order reaction was observed (Figure 1). The rate of the rapid phase was proportional to the substrate concentration, and the magnitude of the spectral change was proportional to the enzyme concentration, for $S_0 >> E_0$. The zero-order portion of the reaction curve was directly proportional to the enzyme concentration and yields a value of $k_{cat} = 0.08$ $s^{-1}$ at pH 9.2, in excellent agreement with that obtained under steady-state conditions (0.09 $s^{-1}$). Representative values for the reaction rate are given in Table III.

The magnitude of the observed spectral change was greatest at the most alkaline pH examined, i.e., 9.2; at lower pH (i.e., <4) no burst phase was observed. The subsequent turnover

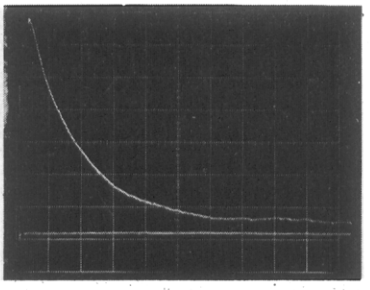

FIGURE 1: Stopped-flow oscilloscope trace of the reaction between papain and $N^{\alpha}$-carbobenzoxy-L-lysine *p*-nitroanilide. Conditions were $E_0 = 1.2 \times 10^{-5}$ M, $S_0 = 1.0 \times 10^{-3}$ M, pH 9.16, +21.5 °C. Ordinate: 50 mV/div (% transmittance). Abcissa: 10 ms/div.

**Scheme I**

$$E' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES' \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} TI \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} EA \overset{k_6}{\longrightarrow} E + P_2$$

$$+$$

$$P_1$$

reaction has the same rate as that under steady-state conditions. The addition of $5.0 \times 10^{-4}$ M *p*-nitroaniline inhibited neither the rate nor the amplitude of the fast phase.

## Discussion

The minimum reaction pathway that is consistent with the accumulated data for papain catalysis is shown in Scheme I (Angelides & Fink, 1978, 1979) where E′ and E represent conformational isomers of the free enzyme, $ES_1$ and $ES_1'$ are noncovalent isomeric complexes, TI is the tetrahedral intermediate, and EA the acyl-enzyme. The interpretation that reaction 4 represents the formation of a tetrahedral intermediate is based upon the following arguments which are considered in detail subsequently. (1) The intermediate formed by reaction 4 precedes the overall rate-limiting step, which, as is discussed below, is formation of the acyl-enzyme. Thus, based on kinetic reasoning, the intermediate cannot be the acyl-enzyme. (2) The spectral characteristics of the product of reaction 4 are entirely consistent with those expected for a tetrahedral intermediate involving *p*-nitroaniline. (3) The kinetic properties of the intermediate are completely in accord with those expected for the tetrahedral intermediate in this reaction.

*Comparison of Ester and Anilide Kinetics.* The $k_{cat}^{lim}$ for the papain-catalyzed hydrolysis of a series of $N^{\alpha}$-carbobenzoxy-L-lysine esters is approximately 40 $s^{-1}$ at 25 °C (Table I). For the enzymatic hydrolysis of $N^{\alpha}$-carbobenzoxy-L-lysine *p*-nitroanilide, $k_{cat}^{lim} = 0.25$ $s^{-1}$. The acyl-enzyme from all the substrates should be identical since they share common acyl groups. The similarity of the catalytic constants for the ester substrates with different leaving groups suggests the involvement of a common rate-determining step, most likely the breakdown of a common acyl-papain intermediate (Lowe & Williams, 1965; Brubacher & Bender, 1966; Kirsch & Igelstrom, 1966). In the presence of an added nucleophilic acceptor, acyl-papains may react with both water and the nucleophile (Henry & Kirsch, 1967). The addition of either amines or alcohols increases the rate of deacylation of both nonspecific (Brubacher & Bender, 1966a; Hinkle & Kirsch, 1971) and specific acyl-papains (Fink & Bender, 1969). Of these nucleophiles, L-tryptophanamide is the most selective and effective thus far examined in its reactions with acyl-papains (Hinkle & Kirsch, 1971; Fink & Bender, 1969; Brubacher & Bender, 1966).

Accordingly, the hydrolysis of the acyl-enzyme is rate determining for the esters since the rate of hydrolysis is accelerated by the addition of L-tryptophanamide. The facts that the $k_{cat}^{lim}$ for the hydrolysis of $N^{\alpha}$-carbobenzoxy-L-lysine *p*-nitroanilide is some 200 times smaller than those of the esters and that the rate is unaffected by the addition of L-tryptophanamide are good evidence that the breakdown of the acyl-enzyme cannot be rate determining for the hydrolysis of the *p*-nitroanilide. Thus, the observed value of $k_{cat}$ for the hydrolysis must reflect an earlier, slower step. That is, the rate-limiting reaction is either formation of the acyl-enzyme or a preceding step.

The experimental data are inconsistent with a rate-limiting conformational change following formation of a tight acyl-enzyme–*p*-nitroanilide complex. Briefly, if a simple desorption process is rate limiting in catalysis, leaving groups of similar size and structure (compare *p*-nitrophenol and *p*-nitroaniline in Table I) would be expected to give similar desorption rates. Furthermore, neither *p*-nitroaniline nor *p*-nitrophenol inhibits substrate hydrolysis (see Results). Even if the observed $k_{cat}$ for ester hydrolysis should represent rate-limiting dissociation of *p*-nitrophenol from the acyl-enzyme–*p*-nitrophenol complex (Holloway & Hardman, 1973), the value of $k_{cat}$ observed for the hydrolysis of the *p*-nitroanilide derivative must reflect a slower and earlier step along the catalytic pathway. Thus, reaction 4, the initial rapid increase in $A_{360}$ (Figures 1 and 2 of the preceding paper), cannot represent the release of *p*-nitroaniline concurrent with formation of the acyl-enzyme.

In 60% aqueous dimethyl sulfoxide, the turnover reaction is very slow at subzero temperatures. In our previous work, it was shown that the cryosolvent causes a 1000-fold increase in $K_m$ due to hydrophobic partitioning effects on substrate binding (Fink & Angelides, 1976). This effect, combined with a 30 °C drop in temperature and a change of 3 pH* units from the pH* optimum, serves to slow the turnover reaction by greater than four orders of magnitude under nonsubstrate saturating conditions. Consequently, the turnover reaction in the subzero temperature system is effectively negligible.

*Spectral Characteristics.* The most convincing evidence that characterizes the observed intermediate as being tetrahedral in nature is the absorbance spectrum. It is completely consistent with the predictions of Robinson (1970) and Hunkapillar et al. (1976) that the spectral characteristics of a tetrahedral intermediate species would closely resemble that of unprotonated *p*-nitroaniline while being quite distinct from that of the substrate anilide.

The possibility has been considered in which the observed absorbance changes represent active-site microenvironmental perturbations and/or substrate distortion. These conclusions are rendered unlikely and can be eliminated by the following data. Since the spectra of the intermediates preceding reaction 4 all resemble the substrate (Angelides & Fink, 1979), we believe it to be improbable that the active site environment *alone* could cause the observed 43-nm red shift. The similar $\lambda_{max}$ of *p*-nitroaniline in high and low polarity solvents indicates that microenvironmental effects involving polarity cannot be responsible for the observed spectral shift. Furthermore, if the 43-nm red shift of the intermediate is attributed to substrate distortion in the active site, the magnitude of the spectral shift implies a very substantial distortion from ground-state trigonal structure. Such a distorted species would be expected to be of high energy and, thus, more likely to be a transition state rather than an intermediate whose breakdown was rate limiting. Additionally, such distortion would presumably involve transformation of the trigonal C toward a tetrahedral form.

We have examined a number of other protease systems for the presence of detectable tetrahedral intermediates from *p*-nitroanilide substrates, using similar procedures to those of this investigation. In several instances, we have observed such species (A. L. Fink, K. J. Angelides, P. Compton, P. Meehan, and J. Zehnder, in preparation); in each case the spectrum was similar to that of the product of reaction 4, with a $\lambda_{max}$ in the vicinity of 350–360 nm.

*pH Dependence.* One of the most perplexing problems concerning the catalytic mechanism of papain centers around the state of ionization of the active-site thiol and imidazole groups. However, regardless of whether they are ionized (ion-pair mechanism) or not, a sigmoidal pH–rate profile for formation of the tetrahedral intermediate would be expected, the rate increasing with increased pH. Consequently, the observed decrease in acylation rate at pH values above 6 (e.g., Whitaker & Bender, 1965) could be interpreted as a change in rate-limiting step in the overall catalysis from formation of the tetrahedral adduct at pH values below 6 to its breakdown at higher pH. These suggestions are supported by the investigations of Lowe & Yuthavong (1971) and O'Leary & co-workers (1974).

In the present case, the pH*–rate profile for reaction 4, isolated as an elementary step in the hydrolysis of the *p*-nitroanilide by papain, also exhibits bell-shaped pH dependence with p$K$*s of 4.3 and 8.6 at −3 °C and 60% dimethyl sulfoxide. However, the concentration of the intermediate increases to a maximum above pH* 9. According to our proposed mechanism (see Figure 6, Angelides & Fink, 1979), the p$K$* of the thiol of Cys-25 is approximately 4 in $ES_1'$. We attribute the observed p$K_1$* of reaction 4 to this ionization. At lower pH* values, both the thiol and the imidazole are protonated and tetrahedral intermediate formation does not occur. In the neutral pH* region, the thiol is ionized and attacks the substrate carbonyl leading to tetrahedral intermediate formation. At high pH*, the $ES_1$ species is favored over $ES_1'$; i.e., the concentration of $ES_1'$ decreases (p$K$* ~ 8) and it is this decreased $ES_1'$ concentration[2] which is responsible for the observed decrease in the rate of reaction 4. At high pH*, the tetrahedral intermediate in which the imidazole is in its free base form predominates (TI in Figure 6 of Angelides & Fink, 1979) and is unable to act as a general-base catalyst for the departure of the *p*-nitroaniline from the tetrahedral adduct. Thus, the tetrahedral intermediate accumulates in high concentration at high pH.

The pH*–rate data (Tables III and IV) indicate that the formation of the tetrahedral intermediate is the overall rate-limiting step in catalysis at pH values below 6, and breakdown of the intermediate to the acyl-enzyme with concomitant release of *p*-nitroaniline is rate determining above pH 6.

By extrapolation of the Arrhenius plots at 25 °C (Angelides & Fink, 1979) and correction for the cosolvent effect on $K_m$ (Fink & Angelides, 1976), we estimate the following values for the observed rate constants for the reaction of Scheme I at pH 9.1, 25 °C, $S_0 = 1.0 \times 10^{-3}$ and $E_0 = 7.8 \times 10^{-5}$ M: $k^2_{obsd} \sim 1 \times 10^2$ s$^{-1}$, $k^3_{obsd} = 120$ s$^{-1}$, $k^4_{obsd} = 70$ s$^{-1}$, $k^5_{obsd} = 0.05$ s$^{-1}$, and $k^6_{obsd} = 27$ s$^{-1}$.

We believe this is the first reported direct observation of a tetrahedral intermediate in papain catalysis, and that this investigation demonstrates the utility of the cryoenzymological technique in several ways. For example, after detection of the intermediate at subzero temperatures, it was relatively straightforward to estimate the expected kinetic and spectral properties of the intermediate under normal conditions, and to ascertain that it should and could be detected on the stopped-flow time scale. In addition, the excellent agreement between the observed rate under normal conditions and that predicted from the subzero temperature experiments indicates that the low temperature and high cosolvent concentration does not affect the catalytic mechanism. The potential for stabilizing accumulated intermediates with low temperatures for long time periods facilitates the acquisiton of structurally related information about the intermediate, as illustrated by the ease of obtaining the spectrum and concentration of the intermediate.

*Implications for the Catalytic Mechanism.* On the basis of the results with ester and anilide substrates at low temperatures, a detailed mechanism for papain catalysis has been proposed (Angelides & Fink, 1978, 1979). The salient features are as follows. The reaction proceeds through a series of states: (1) association–activation; (2) the initial covalent bond making–breaking step in which a covalent tetrahedral intermediate is formed; (3) departure of the leaving group X and collapse of the tetrahedral intermediate to the acyl-enzyme; and (4) hydrolysis of the acyl-enzyme involving the reverse series of the reactions in which X is replaced by OH of water.

The process of association–activation (Schultz et al., 1977) involves the binding of substrate and subsequent changes in substrate and/or enzyme conformations and orientations. When a specific substrate binds to the enzyme, the binding forces between the enzyme and the substrate are utilized to force the enzyme into an energetically less favorable, but catalytically active, conformation. A poor substrate may bind to the active site but will not have the necessary structural features to force such a conformational change of the enzyme to the active form.

In the case of papain, the association–activation process occurs in discrete steps since the unliganded enzyme can exist in two conformers (Angelides & Fink, 1978, 1979). In particular, although neutral pH favors one conformer (E′), the substrate binds preferentially to the other (E), since access to the active site is sterically hindered in the E′ conformer. Thus, the formation of the first Michaelis complex, $ES_1$, will occur in two phases in the proposed mechanism. First, substrate will *associate* with the E (UP) conformation of the enzyme in a rapid bimolecular step. Secondly, the remaining enzyme in the E′ conformation will undergo isomerization to the E state to permit the binding of further ligand. Similar schemes have been reported for alkaline phosphatase (Reid & Wilson, 1971; Halford, 1972) and hexokinase (Cleland, 1975; Jentoft et al., 1977). Thus, the *activation* step involves a repositioning of the imidazole of His-159 in which the enzyme returns to the energetically preferred DOWN conformation. Some repositioning of the $S^\gamma$ of Cys-25 and the carboxylate of Asp-158 also occurs.

From the computer graphics system, a structure with bound substrate that is compatible with our model was found in which the His-159 $C^\alpha$–$C^\beta$ bond is rotated by 79°, $C^\beta$–$C^\gamma$ by 35°; for

[2] The amplitude of reaction 3, the formation of $ES_1'$, begins to decrease above pH* 7, being 20% of its maximum value at pH* 9.3 (Angelides & Fink, 1979). From examination of the proposed mechanism (see Figure 6 in the preceding paper (Angelides & Fink, 1979), one might have expected the reaction at high pH to go via the pathway E → $ES_1$ → TI. However, as shown in the preceding paper, the observed pathway is E → $ES_1$ → $ES_1'$ → TI′. This implies that the reaction $ES_1$ → TI is much slower than $ES_1$ → $ES_1'$ → TI′. As discussed subsequently, we believe that the orientation of the thiolate relative to the substrate carbonyl in $ES_1$ is not optimal for formation of TI, whereas in $ES_1'$ a better stereoelectronic relationship exists.

Asp-158 $C^{\alpha}$–$C^{\beta}$ by 3° and $C^{\beta}$–$C^{\gamma}$ by 68°; and for Cys-25 $C^{\alpha}$–$C^{\beta}$ by −101°. With this conformation, separations of 3.5 Å and 3.3 Å were found for $S^{\gamma}$ to $N^{\epsilon}2$ and $N^{\delta}1$ to OD1 of Asp-158, respectively. Concomitant with this conformational change, the p*K*s of the imidazole and thiol change so that, in the new complex, $ES_1'$, they exist predominantly as the activated ion pair (p*K* of the imidazole ~ 8 and the thiol p*K* ~ 4). The driving force for this activation step is a combination of factors stemming mostly from binding energy derived in the initial substrate association process as well as the electrostatic interaction between the imidazolium and the carboxylate of Asp-158 and thiolate of Cys-25.

At this stage in the catalysis, the carbonyl oxygen of the acyl group that is to be transferred to the Cys-25 thiolate is only weakly hydrogen bonded to the backbone NH of Gly-23 and Ser-24 backbone (Lowe & Yuthavong, 1971) and roughly points toward the $\epsilon$-$NH_2$ of Gln-19 (Drenth et al., 1976). Using the most recent electron density maps available (Drenth et al., 1976), and with the aid of the computer graphics system, we find that the distances between $N^{\delta}1$ of His-159 and the Asp-158 carboxyl oxygen (2.28 Å), and His-$N^{\epsilon}2$ and Cys-$S^{\gamma}$ (3.3 Å) are shorter in the $ES_1'$ complex than in $ES_1$ (approximately 6 Å in the former and 3.6 Å in the latter). The distance between the $N^{\epsilon}2$ hydrogen of His-159 and the $S^{\gamma}$ of Cys-25 appears to be too long to make a good hydrogen bond by 0.3 Å.

Judging by the coordinates of a highly specific tripeptide inhibitor of papain (Drenth et al., 1976) and with the position of the $S^{\gamma}$ of Cys-25 slightly changed from the initial $ES_1$ complex, the distance between the sulfur and the carbonyl carbon of the substrate at this stage is about 2 Å. Simultaneously the carbon–oxygen bond length of the substrate increases as the carbon tends toward being tetrahedral and the oxygen bearing a partial negative charge moves closer to the NH backbone groups of Cys-25 and $\epsilon$-$NH_2$ of Gln-19 forming stronger hydrogen bonds. It would be expected that the noncovalent product $ES_1'$ would readily form a tetrahedral intermediate by nucleophilic attack of the thiolate of Cys-25. An important factor that must also be considered during the course of the catalytic events is that the enzymatic rate enhancement is due in part to the requirements for the precise angular alignment of orbitals between the reactive center of the substrate and the functional groups of the enzyme.

*Stereoelectronic Requirements.* Substantial data has been accumulated that points to the existence of as yet unformulated stereoelectronic preferences, if not actual stereoelectronic imperatives in enzyme catalysis (Gorenstein, 1977; Knowles, 1976; Bizzozero & Zwiefel, 1975; Deslongchamps et al., 1975). Following the stereoelectronic requirements proposed by Deslongchamps et al. (1975; Deslongchamps & Taillefer, 1975) for proteolytic enzyme models, nucleophilic attack on the carbonyl carbon atom of the substrate would be facilitated by a binary antiperiplanar lone pair interaction between the oxyanion and leaving group nitrogen. Breakdown to acyl-enzyme and $P_1$ requires protonation of the nitrogen atom. In papain, the imidazole of His-159 is the protonating agent.

In the E (UP) conformation, shown by crystallographic studies, direct proton transfer from the $N^{\delta}1$ atom of His-159 would be improbable since the lone pair orbital of the leaving group nitrogen is not in the correct orientation. However, in the $ES_1'$ conformation (DOWN) of the enzyme the $N^{\epsilon}2$ proton of the imidazole is in the most favorable orientation with the lone-pair lobe of the leaving group nitrogen, thus satifying the stereoelectronic requirements for leaving group expulsion (Deslongchamps, et al., 1975) and proton transfer (Wang & Parker, 1967; Wang, 1968, 1970; Ingraham, 1972, 1973).

Rehybridization of the substrate $C_1$ to $sp^2$, and the relaxation of the imidazole of His-159, carboxylate of Asp-158, and Cys-$S^{\gamma}$ back to the positions they occupied in $ES_1$, would probably occur in the acyl-enzyme (Alber et al., 1976).

Though deacylation would most probably involve the reverse of the steps leading to acylation, an alternative possibility is that the imidazole is in the UP position in the acyl-enzyme and either the $N^{\delta}1$ *or* the carboxyl of Asp-158 acts as a general base to abstract a proton from water. Recent reports have suggested that the aspartate ultimately functions as the general base either through a water bridge or by direct proton abstraction from water to form hydroxide in the deacylation of nonspecific substrates (Zannis & Kirsch, 1978). Further understanding of the mechanistic details should result from X-ray crystallographic studies of the intermediates trapped at low temperatures.

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# Estrogen-Binding Proteins of Calf Uterus. Purification to Homogeneity of Receptor from Cytosol by Affinity Chromatography†

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ABSTRACT: The estrogen receptor has been purified to homogeneity from calf uterus cytosol by sequential affinity chromatography by using heparin–Sepharose 4B and 17-hemisuccinyl-17β-estradiol–ovalbumin–Sepharose 4B. The procedure yields about 1.2 mg of receptor protein from 1 kg of calf uteri, with a recovery of 53%. The receptor protein, as a complex with 17β-[$^3$H]estradiol, is purified more than 99%. A single band is seen on polyacrylamide gel electrophoresis under nondenaturing conditions. 17β-[$^3$H]Estradiol comigrates with the protein band. As computed from the specific activity of radioactive hormone, 64 450 g of purified receptor protein binds 1 mol of 17β-estradiol. 17β-[$^3$H]Estradiol bound to the proten is displaced by estrogenic steroids but not by progesterone, testosterone, or cortisone. As judged by chromatography on calibrated Sephadex G-200 columns, the purified receptor is identical with native receptor in crude cytosol: both show a Stokes radius of 6.4 nm. On sucrose gradient in low-salt buffer, the purified receptor sediments at 8 S. On electrophoresis in $NaDodSO_4$ gels, the purified receptor migrates as a single protein band with an apparent molecular weight of 70 000. The sedimentation coefficient measured on sucrose gradients in the presence of chaotropic salts [1 M NaBr or NaSCN (0.1 M)] is 4.2 S. We conclude that the estrogen receptor of cytosol consists of a single subunit weighing about 70 000 daltons and endowed with one estrogen binding site. Under native conditions in cytosol, several subunits associate to form a quaternary structure with a Stokes radius of 6.4 nm.

As soon as the estrogen receptor was identified as a main molecular link in the mechanism of estrogen action, attempts to achieve its purification began in several laboratories (Jungblut et al., 1965, 1967; De Sombre & Gorell, 1975) including ours (Puca et al., 1970, 1971a,b, 1972, 1975; Sica et al., 1973a,b; Molinari et al., 1977; Bresciani et al., 1978). The final goal was to make available purified receptor in tangible amounts, suitable to definitive physical, chemical, and biochemical characterization of this important regulatory molecule.

In a series of earlier publications we have described the results of purification work performed using both classical protein separation methods (Puca et al., 1970, 1971a,b, 1972, 1975) as well as affinity chromatography with solid-state adsorbents prepared linking 17β-estradiol 17-hemisuccinate to agarose via a polypeptide arm (Sica et al., 1973a,b). With classical protein separation methods we achieved partial purification of receptor. With affinity chromatography the complete purification was obtained but preparation of purified receptor on a large scale proved to be difficult, mainly because of considerable "bleeding" of hormone from the solid-phase adsorbent during incubation with uterine cytosol.

The above problem has now been solved by including in the purification procedure an initial step consisting of chromatography using heparin–Sepharose. Heparin binds the receptor protein but not most of the other cytosol components, including

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