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Resonance Raman Evidence for Substrate Reorganization in the Active Site of Papain†

P. R. Carey,* R. G. Carriere, K. R. Lynn, and H. Schneider

ABSTRACT: Resonance Raman spectra were obtained for the acylenzyme 4-dimethylamino-3-nitro(α-benzamido)cinnamoyl-papain prepared using the chromophoric substrate methyl 4-dimethylamino-3-nitro(α-benzamido)cinnamate. These spectra contained vibrational spectral data of the acyl residue while covalently attached to the active site and could be used to follow directly acylation and deacylation kinetics. Spectra were obtained at pH values ranging from those where the acyl-enzyme is relatively stable (pH 3.0, $\tau_{1/2} \simeq 800$ s) to those where it is relatively unstable (pH 9.2, $\tau_{1/2} \simeq 223$ s). Throughout this range acyl-enzyme spectra differed completely from that of the free substrate or the product (4-dimethylamino-3-nitro(α-benzamido)cinnamic acid), indicating that a structural change occurred on combination with the active site. The spectra are consistent with rearrangement of the α-benzamido group in the bound substrate, -NH—C(=O)Ph becoming —N=C(—OX)Ph, where the bonding to oxygen is unknown. Superimposed on these large differences, small changes in acyl-enzyme spectra also occurred as pH was raised to decrease the half-life. All of the above spectral perturbations are consistent with a structural change in the acyl-enzyme which precedes the rate-determining step in deacylation. Thus, deacylation proceeds from an acyl residue structure differing from that of the substrate in solution. Upon acid denaturation the spectrum characteristic of the intermediate reverts to one closely resembling the substrate, demonstrating that a functioning active site is necessary to produce the observed differences. Spectra in D_2O of native acyl-enzyme were identical with those in H_2O, indicating that the observed differences in rate constant were not due to solvent-induced structural changes. Activated papain purified by crystallization or by affinity chromatography formed the acyl-enzyme. However, the kinetics of formation and deacylation differed between these materials, as did the spectral properties. Small differences in active-site structure are considered to be responsible for this effect, and it is suggested that such spectral perturbations may be useful in directly relating small differences in structure of the substrate in the active site with corresponding differences in kinetics.

A knowledge of the events which occur in submolecular groupings of a substrate during catalysis would greatly extend the experimental basis for theories of enzyme mechanisms. A potentially powerful way of obtaining such data is by using chromophoric acylating groups which yield resonance Raman vibrational spectra when specifically combined with enzyme active sites (Carey and Schneider, 1974, 1976). The utility of this method (Carey et al., 1972) arises from its ability to monitor the vibrational spectrum of the substrate alone during enzymolysis and from the wealth of detail resonance Raman spectra contain about submolecular groupings in selected compounds. Since vibrational spectra are sensitive to the kind of chemical effects thought to be important during catalysis (e.g., bond strain and changes in charge distribution), they are ideal monitors for catalytic processes.

Papain-catalyzed hydrolysis of esters proceeds through the formation of a covalent acyl-enzyme intermediate linking the thiol group of the enzyme with the acyl residue of the substrate (Glazer and Smith, 1971). Several stable acyl-enzymes have been prepared (Lowe and Williams, 1965; Brubacher and Bender, 1966; Hinkle and Kirsch, 1970). The present paper describes the preparation and enzymatic and resonance Raman properties of 4-dimethylamino-3-nitro(α-benzamido)cinnamoyl-papain. The aim of the present study was to see if changes occurred in particular submolecular groupings of the acylating group during the course of the enzymatic reaction, and then to determine the nature of such changes.

Papain was chosen because of the known ability of sulfhydryl enzymes to produce large red shifts in the absorption spectra of acylating groups. The resonance Raman technique requires, at present, acylating groups with suitable absorption properties above 400 nm, and synthesis of such compounds is difficult. It was expected that the strong absorption band of the substrate at 350 nm (Figure 1) would be sufficiently red shifted on combining with papain so that a resonance Raman spectrum of the bound substrate could be obtained at low concentrations using 441.6-nm laser excitation. This expectation was born out experimentally. The particular substrate used (II, Figure 2) was chosen and synthesized because, in addition to its suitable chromophoric properties, the α-benzamido side chain introduces a degree of specificity into a synthetic substrate (Brocklehurst and Williamson, 1966; de Jersey, 1970).

The main thrust of the results was unexpected, and not apparent from examination of the absorption spectra alone. The

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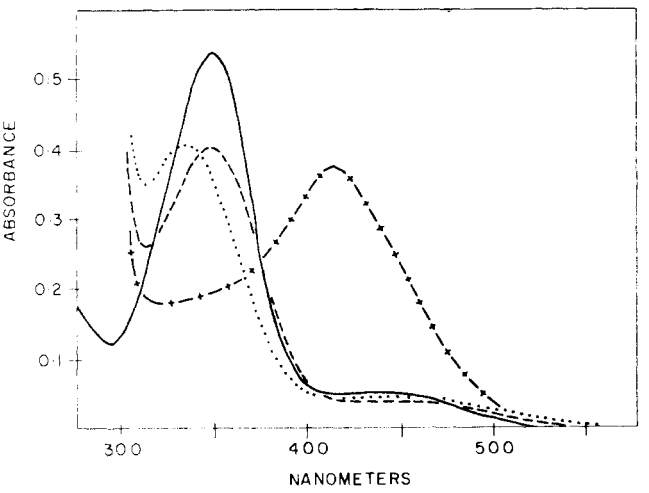

FIGURE 1: Absorption spectra of substrate, acyl-enzyme, and product. (——) Substrate (methyl 4-dimethylamino-3-nitro(α-benzamido)cinnamate), ~1.5×10^{-5} M in 20% CH_3CN, 80% H_2O, pH 6.0. (- - -) Product of enzyme hydrolysis, pH 3.0; ~1.2×10^{-5} M in water. (. . . . . .) Product of enzyme hydrolysis, pH 7.0; ~1.1×10^{-5} M in water. (—+—+) Acyl-enzyme; pH 3.0, ~1.4×10^{-5} M in bound αBA and 6.5×10^{-5} M in enzyme. 1-cm path length.

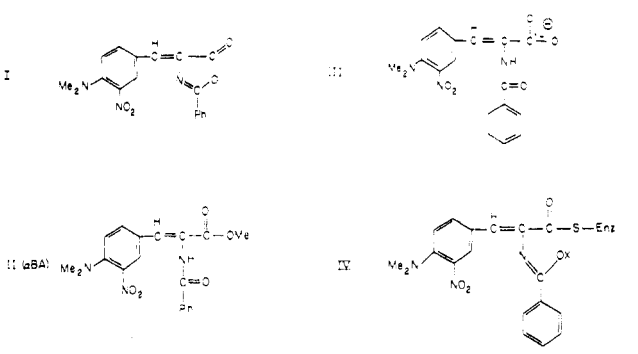

FIGURE 2: I, 4-(4-dimethylamino-3-nitro)benzylidene-2-phenyloxazolin-5-one; II (αBA), methyl 4-dimethylamino-3-nitro(α-benzamido)-cinnamate; III, 4-dimethylamino-3-nitro(α-benzamido)cinnamic acid; IV, proposed structure for the acyl-enzyme; the bonding to oxygen is not known.

resonance Raman spectra provided unequivocal evidence for a radical change in the geometry of the acyl residue upon binding to papain. The resonance Raman spectra also provided good evidence for the nature of this change. Additional unexpected findings were the differences in kinetics of formation and hydrolysis, as well as spectroscopic properties, of the acyl-enzyme formed from commercial, crystallized papain and from enzyme purified by affinity chromatography.

Experimental Section

Materials. 4-(4-Dimethylamino-3-nitro)benzylidene-2-phenyloxazolin-5-one (I, Figure 2) was prepared by stirring and heating at 100 °C for 2 h a mixture of 0.77 mol of 4-dimethylamino-3-nitrobenzaldehyde, 0.085 mol of hippuric acid, 0.23 mol of acetic anhydride, and 0.08 mol of freshly fused sodium acetate. The mixture was then allowed to cool slightly, dispersed in 20 ml of absolute ethanol, and filtered. The filtrate was resuspended in cold water, filtered, dried, and recrystallized from benzene (mp 181 °C). Anal. Calcd for $C_{18}H_{15}O_4N_3$: C, 64.09; H, 4.48; N, 12.46; mol wt, 337.34. Found: C, 63.88; H, 4.57, N, 12.55; mol wt (mass spectrometry), 337. The infrared, Raman, and resonance Raman spectra were consistent with the desired structure, and the latter two showed an intense band at 1559 cm^{-1} characteristic of azlactones (Shigorin and Syrikin, 1946). The NMR spectrum was also consistent with structure I.

Methyl 4-dimethylamino-3-nitro(α-benzamido)cinnamate (αBA; II, Figure 2) was prepared by alcoholysis of I. To 6.0 g of I (0.018 mol) suspended in 30 ml of dry benzene, 2.3 ml of 1 N sodium methoxide (0.023 mol) in anhydrous methanol was added slowly with stirring over a 5-min period. The product was precipitated by the addition of 3.0 ml of 1 N HCl, filtered, washed with water, dried, and recrystallized from absolute ethanol; mp 189–190 °C. Anal. Calcd for $C_{19}H_{19}O_5N_3$: C, 61.78; H, 5.19; N, 11.38; mol wt, 369.37. Found: C, 61.62; H, 5.38; N, 11.57; mol wt (mass spectrometry), 369. The Raman spectrum was characterized by absence of the 1560-cm^{-1} band due to the azlactone ring and the infrared spectrum was characterized by the appearance of the -N-H band at 3335 cm^{-1} and the carbonyl ester frequency at 1718 cm^{-1}. The NMR spectrum in Me_2SO showed the correct number of protons at expected positions. In addition, the N-H proton was rapidly and completely exchangeable in D_2O.

4-Dimethylamino-3-nitro(α-benzamido)cinnamic acid (III, Figure 2) was prepared by hydrolysis of the corresponding methyl ester (αBA; II, Figure 2). Three grams of the ester was suspended in 100 ml of ethyl alcohol and 66 ml of 0.5 N sodium hydroxide was added. The mixture was stirred at room temperature for 2 h, diluted with 66 ml of water, and extracted twice with diethyl ether. The aqueous layer was clarified by filtration and acidified, and the product was collected by filtration and recrystallized from ethanol (mp 265 °C). Anal. Calcd for $C_{18}H_{17}O_5N_3$: C, 60.84; H, 4.82; N, 11.83. Found: C, 60.64; H, 4.96; N, 11.85. The infrared and NMR spectra were consistent with structure III. Hydrolysis of αBA by aqueous alkali was found also to yield the cinnamic acid III as shown by identity of the absorption and resonance Raman spectra.

Crystallized papain (two times) was purchased from the Sigma Chemical Co., St. Louis, Mo., as a suspension in sodium acetate. The experiments reported were carried out with at least six different batches with identical results.

Papain purified by affinity chromatography was isolated as a mercury derivative. Crude Papaya latex (50 g, Sigma Type 1) was ground with 10 g of sand in 100 ml of "activation buffer" (Sluyterman and Wijdenes, 1970). After centrifugation, the supernatant was decanted and chromatographed (Sluyterman and Wijdenes, 1970; Lynn, 1973). The papain–mercury fraction was dialyzed against 10^{-2} M sodium acetate containing 10^{-4} M phenylmercuric acetate (Sigma) and stored at 4 °C.

The mercury-papain gave a single protein band on gel electrophoresis at pH 4.0 (Gabriel, 1971). The N-terminal end consisted of the sequence Ile-Pro-Gly (compare Glazer and Smith, 1971) when determined in a Beckman sequencer. The C-terminal residue was aspartic acid (Ambler 1967). Amino acid analysis further confirmed that the enzyme isolated was papain since the primary composition was identical with that reported (Glazer and Smith, 1971).

Preparation and isolation of the acyl-enzyme with papain (purified by crystallization) were carried out as follows: 0.6 ml of suspension was added to 4 ml of water containing 2 mg/ml of cysteine and 2 mg/ml of EDTA, resulting in solu-

[1] Abbreviations used are: αBA, methyl 4-dimethylamino-3-nitro(α-benzamido)cinnamate; EDTA, (ethylenedinitrito)tetraacetic acid; OD, optical density.

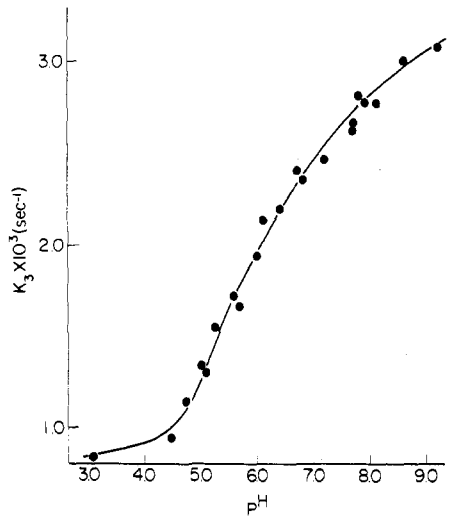

FIGURE 3: Effect of pH on deacylation rate of 4-dimethylamino-3-nitro(α-benzamido)cinnamoyl-papain. Phosphate buffer, μ = 0.20. Below pH 5.0 and above pH 8.0 in the presence of 0.06 M phosphate, μ = 0.20.

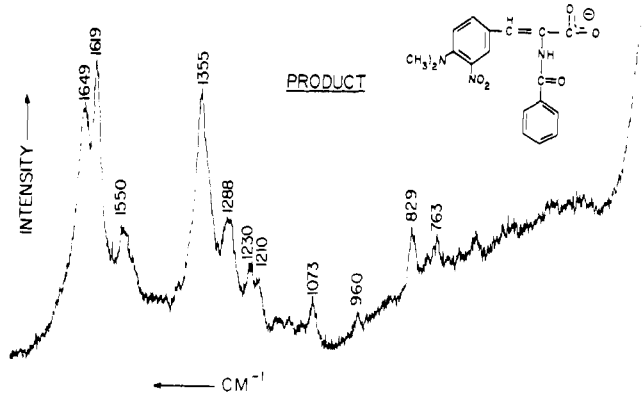

FIGURE 4: Resonance Raman spectrum of 4-dimethylamino-3-nitro(α-benzamido)cinnamic acid, pH 13.0, concentration ~3 × 10^{-4} M; 441.6-nm excitation; typical conditions, 50-mW power, 4000 counts full scale, 9-cm^{-1} spectral slit width.

tions containing approximately 3.2 mg/ml of protein. After adjusting the pH to 7.5, 0.2 ml of substrate stock solution (10^{-2} M αBA in dimethylformamide) was added and the solution incubated for 10 min at 25 °C. The solution was then adjusted to pH 3.0, passed through a Millipore filter (type HA) to remove precipitated substrate, and then put through a 10 × 1 cm G-15 Sephadex column, preequilibrated at pH 3.0 with HCl. The protein fraction eluted had an absorbance centered at 410 nm due to acylated enzyme molecules. After the protein passed through the column, at least 10 ml of eluent was collected before any trace of unbound substrate could be detected. Absorbance measurements on the products of deacylation showed that this protocol resulted in acylation of approximately 20% of the protein molecules. Prior passage of activated enzyme solutions through a Sephadex G-25 column, to remove low-molecular-weight material or through a CM-52 ion-exchange column did not change the extent of acylation. The low percentage of acylation is attributed, in part, to low solubility of the substrate in water, since aqueous solutions became cloudy within seconds after adding the substrate stock solution.

Methods. The apparatus used to obtain the Raman spectra has been described elsewhere (Kumar et al., 1974). Deacylation was sufficiently slow at most pH values studied, so that a flow system (Carey and Schneider, 1974, 1976) did not have to be employed to observe labile intermediates. Near neutral pH the deacylation rate is more rapid than at lower values. At these values a low-temperature cell adapted from an absorbance spectrometer was used to keep the temperature at 4 °C, thereby extending the half-lives by a factor of approximately five compared to 25 °C. Spectral conditions in the Raman experiments are given in the figure captions. All spectra were repeated at least three times. Ar, Ne, and Xe discharge lines were used as calibration standards and line positions are accurate to better than ±2 cm^{-1}; an accuracy of ±1 cm^{-1} was achieved in some regions.

Results

Characterization of the αBA-Papain Acyl-Enzyme and Hydrolysis Product using the Commercial Crystallized Enzyme. The absorption spectrum of the acyl-enzyme at pH 3.0 exhibits a large red shift relative to the substrate; λ_{max} moves from 350 to 412 nm (Figure 1). Such shifts characterize acyl-enzymes where the point of covalent attachment in the active site is a sulfhydryl group (Hinkle and Kirsch, 1970) and result from formation of thiol esters, RC(=O)S-enzyme. As expected if the active-site sulfhydryl were being acylated, addition of an equimolar amount of $HgCl_2$ to activated papain prevented formation of the complex and hydrolysis of substrate. In addition, no reaction was observed without prior activation of the papain.

Acid denaturation of the acyl-enzyme by exposure to pH 1.8–2.0 at room temperature produced a stable acyl-enzyme. This was indicated by the absorption spectrum of the denatured material remaining unchanged after 24-h dialysis at pH 3.0, whereas the absorbance above 300 nm in the native acyl-enzyme disappeared under identical dialysis conditions within 4 h. Denaturation caused λ_{max} to shift to 380 nm, indicating sensitivity of the acylating group to either structural or environmental changes in the active site.

Deacylation kinetics at various pH values were determined spectrophotometrically at 25 °C by monitoring acyl-enzyme concentration at 415 nm. Good first-order plots were obtained, and the half-lives may be calculated from Figure 3. The acyl-enzyme was relatively stable as shown by half-lives ranging from ~800 to ~200 s at pH values of 3.0–9.2. The pK for deacylation was estimated at ~6.0 (Figure 3). Study of the high pH region was difficult because of precipitation above pH 9.0. The pK value found is somewhat higher than that for several other substrates which, however, exhibit a fairly wide range of 3.3–4.7 (Glazer and Smith, 1971). Hippurate esters behave in an unusual way in that deacylation appears to be independent of pH (Sluytermann, 1964; Kirsch and Katchalski, 1965; Lucas and Williams, 1969).

The product of deacylation was identified as 4-dimethylamino-3-nitro(α-benzamido)cinnamic acid (III) by comparison with the synthetically prepared compound (III). The product of enzyme hydrolysis (Figure 1) and III both have a strong absorption centered at 348 nm (acidic form) and, although the spectrum of the enzyme product was contaminated by protein fragments, the overall absorption shape of the bands and the weaker peaks centered near 440 nm appeared to be the same. The basic species in both cases had a λ_{max} at 337 nm. Resonance Raman spectra of the product from enzyme hydrolysis were obtained after concentrating via dialysis at pH 7.0 followed by freeze-drying. These were of poor quality due to background luminescence. However, peaks could be observed at 1355 and 1618 cm^{-1}, the same positions as those of the intense peaks yielded by the cinnamic acid derivative (III) (Figure 4). The peak seen at 1647 cm^{-1} in III was obscured

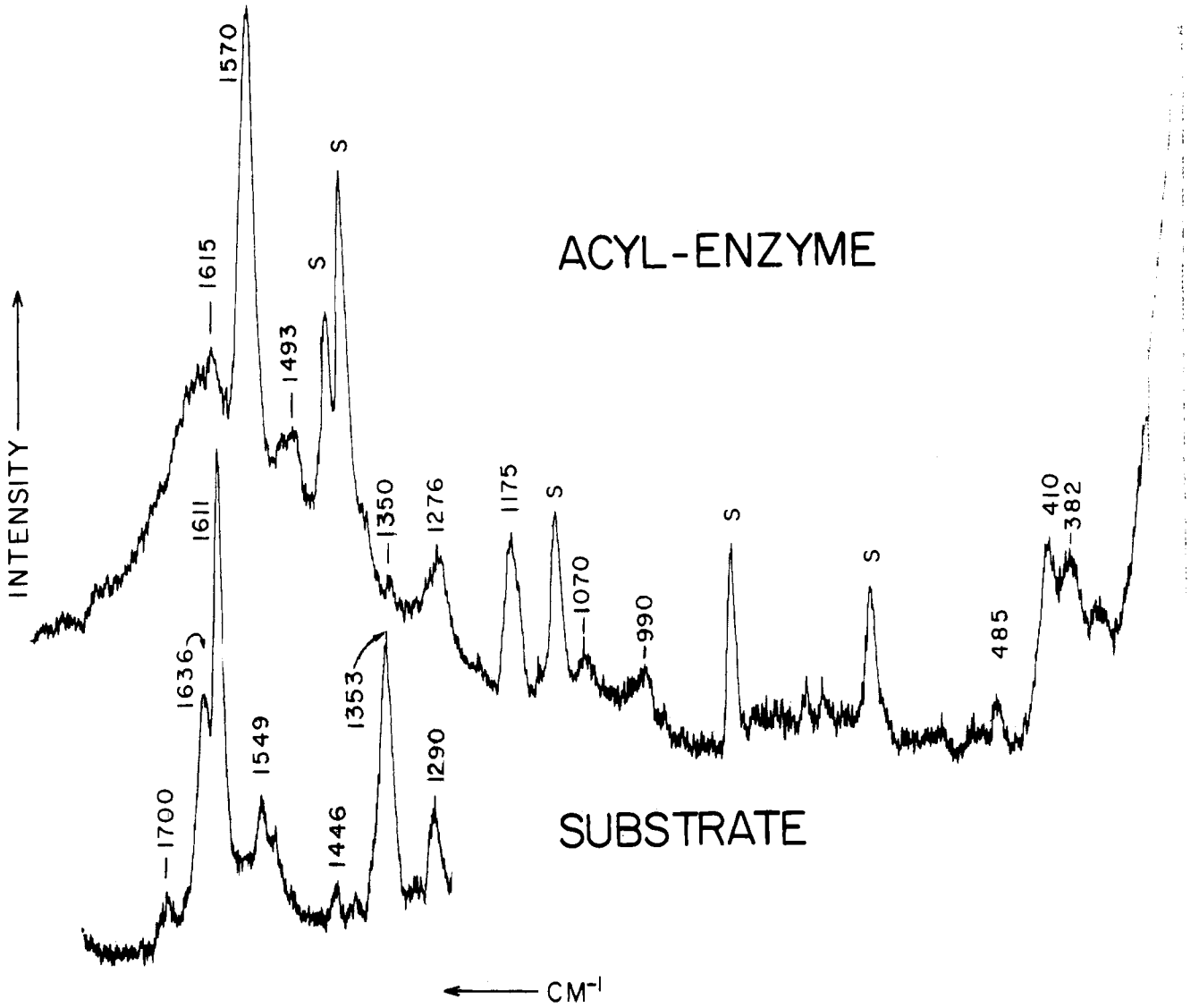

FIGURE 5: Resonance Raman spectra of 4-dimethylamino-3-nitro(α-benzamido)cinnamoyl-papain (commercial crystallized): intermediate (top) and the substrate (αBA) alone (bottom). Concentrations: acyl-enzyme; enzyme ~6.5×10^{-5} M; bound substrate ~1.4×10^{-5} M; substrate 10^{-4} M in 80% D_2O, 20% CD_3CN, 441.6-nm excitation, 50-mW power, ~9-cm^{-1} spectral slit width. The substrate spectrum below 1290 cm^{-1} contains only weak features (see Figure 6) which are obscured by solvent bands. S = solvent bands, resulting from dimethylformamide moving with the acyl-enzyme during chromatography.

by a water peak in the enzymatically produced product. As expected, the product spectrum closely resembles that of the substrate (Figures 5, 6) and was totally distinct from that of the acyl-enzyme (Figure 5).

Both enzyme product and III were found to have a pK of 4.0 ± 0.2 by spectrophotometric titration, a value consistent with that for cinnamic acid derivatives.

Resonance Raman Spectra of Substrate and Complex. The resonance Raman spectra of the substrate and complex are totally distinct (Figure 5). That for the substrate (Figures 5 and 6) is typical of an ester of cinnamic acid (Carey and Schneider, 1974, 1976). The intense feature at 1353 cm^{-1} can be assigned to the nitro group symmetric stretching frequency, while the intense bands at 1631 and 1611 cm^{-1} are associated with the -C=C- stretching vibration and a benzenoid ring mode, respectively, with the possibility that mixing may occur. The α-benzamido group in αBA does not contribute directly to the resonance Raman spectrum of the substrate (Figures 5 and 6). Conjugation does not extend through the -C—NH—C(=O)- residue and intense resonance Raman bands only come from parts of a molecule strongly conjugated with the π-electron system of the chromophore.

The resonance Raman spectrum of the acyl-enzyme differs mainly in the absence of the intense 1631, 1611, and 1353 cm^{-1} bands and by the presence of a new strong feature at 1570 cm^{-1}. With the substrate, bands were not found near 1570 cm^{-1} in the Raman (solid 647.1 nm excitation), resonance Raman (D_2O, CD_3CN solution 441.6 nm exitation), or the infrared (KBr pellet). The weak features in the acyl-enzyme spectrum close to those for the intense substrate peaks cited above (Figure 5) are due to the presence of product.

The profound spectral changes show that a marked change in structure of the acyl residue has occurred. Indications of the nature of this change are provided by the resemblance of the intermediate's resonance Raman spectrum to those of azlactones, and to those of polyenes, and by the increased Raman intensity of the intermediate compared to the substrate and product.

Spectra of the complex and the resonance and normal Raman spectra of azlactones are all characterized by a dom-

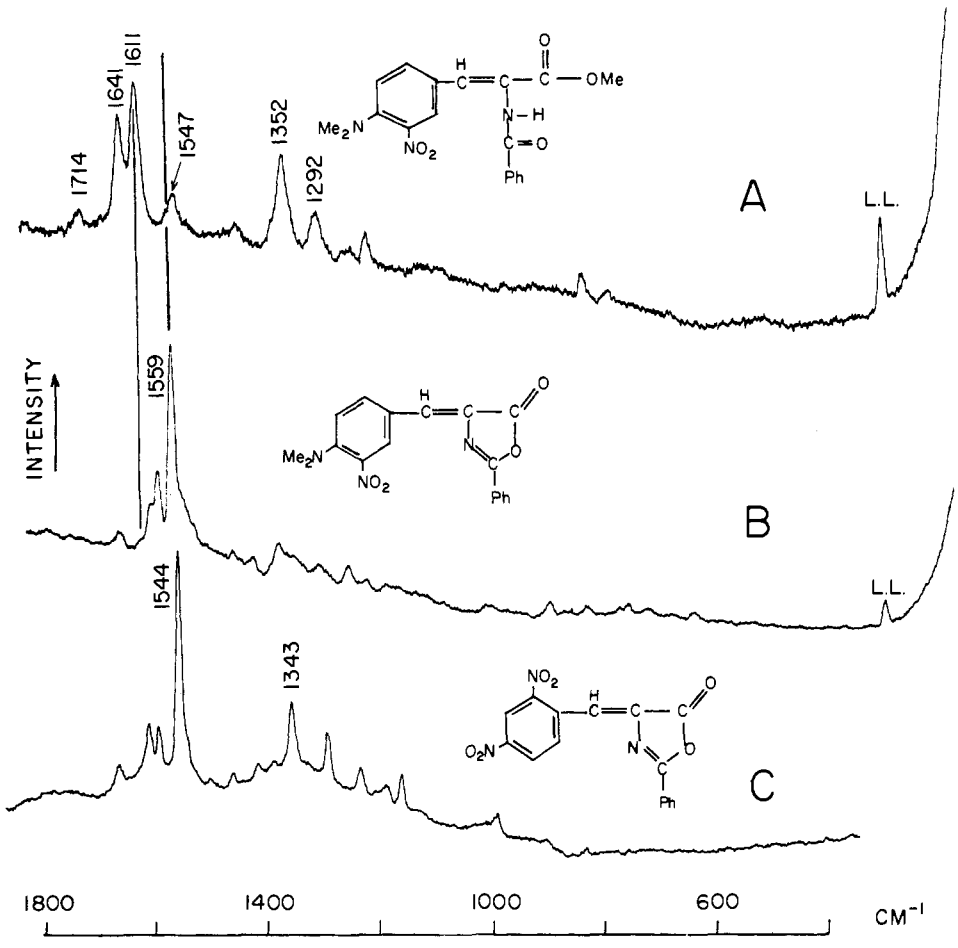

FIGURE 6: Resonance Raman or Raman spectra of the substrate and two azlactones. (A) Substrate, methyl 4-dimethylamino-3-nitro(α-benzamido)cinnamate; (B) 4-(4-dimethylamino-3-nitro)benzylidene-2-phenyloxazolin-5-one; (C) 4-(2,4-dinitro)benzylidene-2-phenyloxazolin-5-one. All spectra are of solid samples in a KBr matrix and were taken using a rotating sample holder. A and B, resonance Raman, 441.6-nm exitiation. C, normal Raman, 647.1-nm excitation.

inant feature in the 1560-cm^{-1} region. This feature is clearly evident in the spectra of the two azlactones shown in Figure 6 and in those of a variety of azlactones (Shigorin and Syrkin 1946). ^{15}N-substituted azlactones indicate that the 1560-cm^{-1} azlactone mode has a high degree of -C=N- stretching character (Carey et al., 1976). This, and the fact that the 1560-cm^{-1} azlactone feature is very intense in both resonance and normal Raman (Figure 6), indicates that an intense band in the 1560-cm^{-1} region is a good fingerprint for the PhC=C—N=CPh structure and one which is not subject to subtle vibronic effects upon changing excitation frequency. Thus, the intense intermediate band at ~1570 cm^{-1} is attributed to a stretching mode from a -C=C—N=C(—OX)- grouping formed from the α-benzamido side chain -C=C—NH—C(=O)-. The nature of the α-benzamido oxygen is not known. Among the possibilities are O^-, OH, or OR where R is a protein group. Since acid denaturation experiments (vide infra) indicate that the substrate is covalently bound to papain at cysteine 25, it is unlikely that a true azlactone is formed in the active site. Thus, the intermediate resembles an azlactone only in its conjugated path and a better model should be an acyclic azlactone of the form illustrated in Figure 2 (IV).

Resonance Raman spectra of polyenes are characterized by two intense spectral features, one near 1500 cm^{-1} and one between 1100 cm^{-1} and 1200 cm^{-1} (Rimai et al., 1973). The intermediate's bands near 1570 cm^{-1} and 1175 cm^{-1} may be assigned to analogous vibrations. In polyenes the 1500-cm^{-1} band is assigned to an "ethylenic" stretching mode (Rimai et al., 1973; Inagaki et al., 1975), and the analogous intermediate band at 1570 cm^{-1} can be associated with a stretching mode from the -C=C—N=C- group. Similarly the 1175-cm^{-1} feature in the intermediate can be ascribed to a mode possessing -C-C- (or C-N) stretching character as in polyenes (Rimai et al., 1973; Inagaki et al., 1975).

The high Raman intensity of the intermediate is demonstrated by the acid denaturation experiments (below) and the fact that it alone can be detected in a reaction mixture (see Discussion). The increase of Raman intensity in the intermediate is consistent with the intermediate's possessing a polarizable highly conjugated region.

The results of acid denaturation experiments suggest that the structure adopted by the acylating group depends on whether or not the enzyme is in a native or a denatured form. Acid denaturation causes a profound change in Raman intensity, as indicated by the disappearance of the 1570-cm^{-1} peak. It could not be detected when the denatured acyl-enzyme OD at 441.6 was greater than 0.2, conditions which yielded excellent spectra for the native material. However, weak features characteristic of a structure analogous to the substrate II could be distinguished. Thus, the spectral changes are consistent with denaturation destroying the extended, conjugated structure shown in Figure 2.

Relative to substrate, the shifts in the absorption spectrum of the acylating group upon binding and then upon acid denaturation of the acyl-enzyme are consistent with those observed for substrates which do not possess α-benzamido side

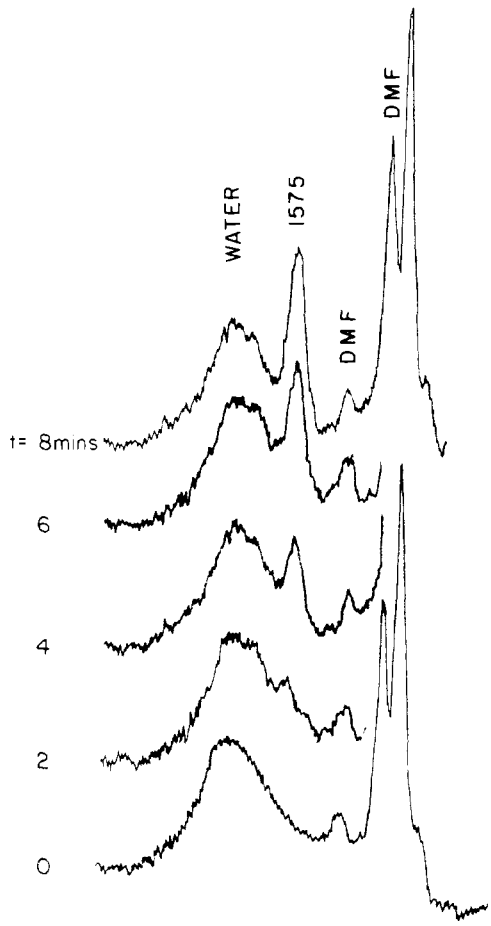

FIGURE 7: Resonance Raman spectra of intermediate detected in reaction mixture of αBA substrate and papain purified on an affinity column. Enzyme ~5 × 10^{-5} M; intermediate reaches a maximum concentration of ~5 × 10^{-6} M; DMF = dimethylformamide.

chains (Bernhard and Lau, 1972; Hinkle and Kirsch, 1970). Thus, the resonance Raman experiment detects changes in acyl group geometry which are not apparent from the absorption spectrum.

Comparison of Papain Purified by Crystallization and by Affinity Chromatography. Commercial, crystallized papain and material isolated and purified from Papaya latex, by Hg-agarose affinity chromatography, hydrolyzed the substrate. However, differences were evident in the reaction kinetics, as well as in resonance Raman spectroscopic properties of the acyl-enzyme. Isolated complexes could not be obtained with freshly prepared, affinity-purified papain. However, if the substrate and specifically purified enzyme were incubated for various periods of time (Figure 7) at pH 7.5 at 25 °C, accumulation took place of material yielding a resonance Raman band (1575 cm^{-1}) characteristic of the acyl-enzyme. Figure 7 demonstrates the buildup of such material. These spectra were obtained by removing samples at 2-min intervals and quenching the reaction by lowering the pH to 3.0. Resonance Raman spectra were then recorded following clarification through a Millipore filter, a process which removed precipitated unreacted substrate or product.

Differences in deacylation kinetics were observed by following the reduction in intensity of the 1575-cm^{-1} band. Half-lives estimated in this way were approximately one-half of those found with the crystallized material.

The spectroscopic differences are indicated by differences in position of the intense band in the resonance Raman spectra. In acyl-enzymes formed from crystallized, commercial papain the intense band is at 1570 cm^{-1}, but at 1575 cm^{-1} in the specifically purified material. This difference is outside the bounds of experimental error which in this spectral region is ±1.0 cm^{-1}. The difference is not due to the presence of unremoved substrate or product, since the differences still appear in control experiments with commercial papain. In addition, these differences are not due to small changes in solvent conditions. The resonance Raman spectra of the acyl-enzyme prepared with crystallized material was found to be unchanged in pH 3.0 HCl, pH 3.0 HCl + 0.1 M NaCl, and pH 3.0 HCl + 20% CH_3CN.

The different properties of the two types of papain are attributed to small differences in the structure of the active site resulting from autolysis in the crystallized, commercial material. The commercial material exhibited three bands on gel electrophoresis after prior passage through a Sephadex G-25 column to remove low-molecular-weight impurities, while the affinity-purified enzyme showed only one band. In addition, storage of the specifically purified material at pH 5.0 at 4 °C for 9 months allowed the preparation of acyl-enzymes using the Sephadex method. Repeated attempts to fractionate commercial, crystallized papain by ionic-gradient chromatography on CM-52 always resulted in a single symmetric peak which accounted for all of the protein. Thus, the chemical differences between the electrophoretically distinguishable components must be very small. It has been shown that papain can retain its activity after autolysis (Finkle and Smith, 1958) or limited proteolysis by another enzyme (Hill and Smith, 1960; Glazer and Smith, 1971).

When preparing acyl-enzyme with commercial, crystallized papain, yields never exceeded 20–25%, even though a wide variety of attempts were made at improvement. The low yield could be due in part to the low solubility of the substrate and to the poor leaving group nature of OCH_3. However, it is also possible that only one of the three electrophoretically distinguishable forms is acylated rapidly under the conditions employed. It is noteworthy that, in all spectra of the acylated commercial enzyme, no evidence was found of peak broadening in the 1570-cm^{-1} band. The presence of such broadening would be strong evidence for combination with more than one type of binding site (Carey et al., 1973).

pH Dependence of Resonance Raman Spectra of the Acyl-Enzyme. Half-lives of the crystallized papain αBA acyl-enzyme at 4 °C were about five times longer than those at 25 °C. Since it normally takes longer than 10 min to run a Raman spectrum, and half-lives in the pH range 6.0–9.0 varied from 5.5 to 3.5 minutes at 25 °C, pH dependency was studied at the lower temperature. Cooling produced better quality spectra than did a flow system (Carey and Schneider, 1974, 1976). The general procedure employed was to isolate the acyl-enzyme at pH 3.0 at 4 °C, adjust the pH to the desired value at this temperature, and then immediately run the spectrum. Small but definite changes in the acylating group structure occurred on making the acyl-enzyme more unstable by raising pH. These could result from alternations in structure of the acylating group or of the environment of the active site. They were detected by shifts in the position of the resonance Raman peak which occurs at 1570 cm^{-1} at pH 3.0. At pH 6.1 the peak moved to 1575, at pH 7.1 to 1574, at pH 9.5 to 1575, and at pH 9.8 to 1577 cm^{-1}. The differences among these values are at the limit of experimental reproducibility for this type of experiment (±1.5 cm^{-1}), but all are significantly different than the 1570-cm^{-1} peak observed at pH 3.0. No other changes were obvious apart from these shifts, although other

small changes may have occurred but were undetected due to the lower intensity of the remaining features (Figure 5). It is noteworthy that the shift to 1575 cm^{-1} seen in the 1570-cm^{-1} band on going to higher pH is similar to that seen when comparing the crystallized papain-αBA complex (1570 cm^{-1}) with the corresponding complex yielded by affinity purified papain (1575 cm^{-1}) at pH 3.0.

Resonance Raman Spectra of the Acyl-Enzyme in D_2O. Half-lives of the complex (crystallized papain) in D_2O were approximately three times greater than in H_2O. Because of interference from a luminsecent background in D_2O it was not possible to obtain resonance Raman spectra of the acyl-enzyme of comparable quality to those in H_2O (Figure 5). However, in D_2O at pD 3.0 the intense acyl-enzyme Raman band at 1570 $\pm$ 2 cm^{-1} was clearly seen. This indicates that the structure of the acyl-enzyme in D_2O is very similar to that in H_2O and, at pH 3.0, the observed differences in half-lives between D_2O and H_2O do not reflect a conformational change in the acyl-enzyme.

Complex of Azlactone with Papain. The evidence that the bound α-benzamido substrate resembles an azlactone suggests that the azlactone analogue, I, of methyl 4-dimethylamino-3-nitro(α-benzamido)cinnamate (II) might be a substrate. This material was in fact hydrolyzed by both crystallized and affinity-purified papain, and a colored protein fraction could be isolated using the Sephadex procedure. However, this material was not studied in detail because of evidence that noncovalent complexes were present. This evidence consisted of pronounced tailing of azlactone behind the protein band.

Discussion

Three important conclusions follow from the present work. (1) Resonance Raman spectra allow monitoring and identification of substrate rearrangements during an enzymatic reaction. (2) In the papain-catalyzed hydrolysis of an ester substrate large changes in acylating group structure can occur. (3) Small differences in the spectra of the intermediates formed from two sources of papain suggest a means of relating small differences in active-site structure to differences in kinetic constants.

The presently available evidence suggests that rearrangement in the active site of the substrate's α-benzamido side chain to yield -N=C(—OX)—Ph is responsible for the profound differences observed in the resonance Raman spectra. Further work on the nature of the intermediate is being actively pursued. Whatever the outcome, comparison of the resonance Raman and kinetic (Figure 3) data shows that the change in structure of the acylating group takes place prior to the rate-determining step in deacylation. Furthermore, the disappearance of the 1570-cm^{-1} band on denaturing the enzyme demonstrates that interactions characteristic of the functional active site, in addition to linkage to cysteine 25, are responsible for the substrate reorganization.

The differences between commercial, crystallized papain and specifically purified material with regard to kinetics of acylation and deacylation and spectral properties were unexpected. These results are of considerable potential utility. They identify a situation where changes in the structure of the active site produce measurable changes in kinetics and spectral properties of the acyl-enzymes. Thus, they may provide a means to study the effect of variation in active-site structure on the catalytic process, an approach which is of great interest. In particular, these results suggest a way in which differences in rate constants can be related to small differences in substrate geometry, probably involving distances of <1 Å, in the active site. By obtaining x-ray data on a series of analogues of the intermediate, in this case probably acyclic azlactones, it would be possible to determine the structure of these molecules in the crystalline state to better than 0.1 Å. Raman data on the same series of molecules in the same state could then establish a correlation between small structural and small Raman spectral differences. Thus, a combination of x-ray and Raman data on a series of intermediate analogues and resonance Raman studies on the substrate complexes with similar but distinct enzymes would permit correlation between small differences in geometry and reactivity.

The appearance of a characteristic spectral feature for an intermediate allows rate constants for the formation and breakdown of the intermediate to be obtained directly. Since the detection of intermediates in multistage reaction is often extremely difficult by conventional techniques, this suggests that the resonance Raman technique may become a useful tool for resolving some of the difficulties currently encountered in kinetic studies.

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