Charge Effects in the Active Site of Papain: Resonance Raman and Absorption Evidence for Electron Polarization Occurring in the Acyl Group of Some Acylpapains†

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ABSTRACT: Resonance Raman spectra are reported for three acylpapains, viz., furylacryloyl, 4-dimethylamino-3-nitrocinnamoyl, and isotopically substituted 4-dimethylamino-3-nitro-(α-benzamido)cinnamoylpapain. For each substrate, upon acylation, a red-shifted absorption spectrum is accompanied by the appearance of an intense double bond resonance Raman feature at an unusually low frequency. A series of model compounds, based on imidazole esters of cinnamic and furylacrylic acids, mimics the absorption and resonance Raman properties of the acylpapains investigated. The crucial property of these imidazole esters is that they have a very strong electron-attracting group (imidazole) at the carboxyl end and an electron-donating group (e.g., dimethylamino) at the other extremity of the molecular skeleton. Thus, these residues acting through chemical bonds set up a highly polarized π-electron system. It is proposed that a similar polarization of π electrons is produced in the acyl groups by papain's active site. Denatured acylpapains show that an intact active site is required to produce the observed spectral properties. Components of the active site which may be responsible for the proposed electron polarization are discussed. Resonance Raman spectra of 4-dimethylamino-3-nitro(α-benzamido)-cinnamoylpapain, separately labeled with ^{13}C in the ethylenic linkage and ^{15}N in the benzamido residue, were obtained to help clarify the role of the benzamido side chain in the active site. Isotope shifts of 7–11 cm^{-1} were observed.

Among the many factors cited for enzyme specificity and rate enhancement (Jencks, 1975), the effect of charge interactions between protein groups and substrate has long been regarded as important. For this reason the state of ionization of the amino acid side chains in active sites has been the subject of considerable study. However, in the case of papain the conclusions remain controversial (Lowe, 1976; Lewis et al., 1976). The differences in the serine-histidine-aspartate charge relay triad in the serine proteases (e.g., chymotrypsin) and the cysteine-histidine-asparagine triad in the cysteine proteases (e.g., papain) have been the cause of further interest. For both chymotrypsin and papain, ester hydrolysis proceeds through a covalently linked acyl-enzyme and this affords the opportunity for direct spectroscopic comparison of the effect of these active sites on bound acyl groups. The pioneering work on the absorption properties of chromophoric acyl-enzymes has been reviewed by Bernhard & Lau (1972).

Resonance Raman spectroscopy offers a useful approach in delineating the critical steps in the enzymolysis of chromophoric substrates in chemical terms. The technique yields vibrational spectral data on the substrate and such spectra can reflect the nature of chemical events of interest during enzymic catalysis, for example, bond strain and charge redistribution. Recently, resonance Raman spectra of an intermediate in papain catalysis were reported (Carey et al., 1976). The spectra were shown to be sensitive to active site functionality: the intermediate's spectrum differed markedly from those of product and substrate, and there were changes dependent on factors controlling rate of decomposition of the intermediate. Application of the resonance Raman technique depends on the design of suitable, chromophoric substrates (Carey & Schneider, 1974, 1976). Laser excitation into an absorption band of the substrate produces an intense (or resonance) Raman spectrum from the substrate and allows this spectrum to be selectively monitored in an enzyme–substrate complex at concentrations of 10^{-4} to 10^{-6} M in water. The normal Raman spectrum from the nonchromophoric part of the system is obscured by the spectral background.

The present study provides insight into the electron reorganization occurring in papain bound acyl groups. This reorganization is responsible for the radical changes observed in the resonance Raman spectra and does not depend, as was initially thought (Carey et al., 1976), on the rearrangement of a substrate side chain. These results are based upon the resonance Raman spectra of some new papain acyl-enzymes and on a series of appropriate model compounds. A key aspect is the use of ^{15}N and ^{13}C isotopically labeled substrates. Spectra of these and of the isotopically substituted acylpapains greatly aided band assignments and assisted in the design of further experiments.

Experimental Section

The 4-dimethylamino-3-nitro-(α-benzamido)cinnamoyl papains were prepared as described previously (Carey et al., 1976). The protocol for 4DMA-3NO_2-cinnamoyl-papain is: to 4 mL of H_2O containing 1 mg/mL each of cysteine and EDTA, add 0.6 mL of Sigma (2× crystallized) papain, adjust pH to 4.2, add 0.5 mL of 10^{-2} M 4DMA-3NO_2-cinnamoylimidazole in CH_3CN, incubate for 4.5 min, lower pH to 2.8, purify by column chromatography as before (Carey et al., 1976). Although small differences were not monitored, papain purified by affinity chromatography gave an intermediate with essentially the same spectral characteristics and active site occupancy (20–30%) as that prepared from Sigma papain. Furylacryloylpapain was prepared essentially according to the method of Hinkle & Kirsch (1970).

Raman measurements using laser excitation in the visible followed procedures detailed in previous publications. An

† From the Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A OR 6. *Received April 20, 1977; revised manuscript received October 7, 1977.* This paper is NRCC Publication No. 16530.

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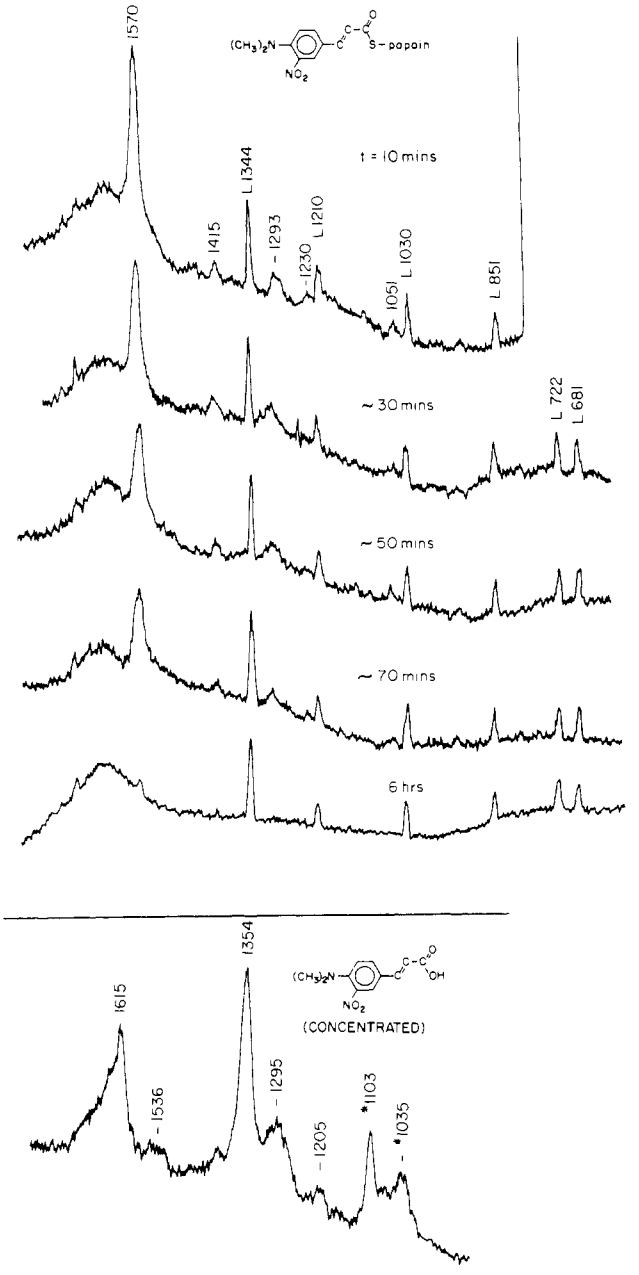


FIGURE 1: Resonance Raman spectra of 4-dimethylamino-3-nitrocinnamoylpapain as a function of time, 4 °C, pH 3.0; 10^{-4} M in enzyme, initially ~30% active site occupancy; 457.9 nm, 50 mW excitation, spectral slit ~8 cm^{-1}, L = laser plasma lines. The broad feature near 1650 cm^{-1} is a water peak. (Bottom) Preresonance Raman of 4-dimethylamino-3-nitrocinnamic acid, 10^{-4} M, pH 2.5, in 30% CD_3CN/H_2O. * = CD_3CN; 488.0 nm, 200 mW, 9 cm^{-1} spectral slit. Product spectrum does not appear under conditions used to record acyl-enzyme spectrum; see text. Substrate has a single intense band at 1600 cm^{-1} (Table I).

additional set of resonance Raman data on isotopically substituted 4DMA-3NO_2-(α-benzamido)cinnamoylpapain was recorded using a second Jarrell-Ash instrument in Dr. H. J. Bernstein's laboratory; the isotope shifts were in good agreement with those measured using our own equipment. Resonance Raman measurements with 350.7-nm excitation were made using a Spex 0.5-m double monochromater and a Coherent Radiation 3000 K laser. D.C. photodetection was employed. Sample cells consisted of a horizontal quartz capillary. The sample was flowed through the capillary, using a peristaltic or syringe pump, to minimize photodegradation.

Syntheses. Methyl-4-dimethylamino-3-nitro-(α-benzamido)cinnamate: The appropriate labeled glycine derivative was benzoylated with benzoyl chloride using Schotten-Baumann conditions to give the labeled hippuric acid in 60–70% yield. The following procedure for [^{15}N]hippuric acid is typical. Hippuric acid (0.18 g; 1 mmol), potassium bicarbonate (40 mg), and 4-dimethylamino-3-nitrobenzaldehyde were stirred at room temperature in acetic anhydride (5 mL) until everything has dissolved (45 min); then the reaction mixture was allowed to stand overnight. The resultant crystals were collected by suction, washed with water, and dried to yield 0.16–0.17 g (45–50%) of azlactone, mp 180–181 °C. The azlactone (100 mg) was refluxed in spectral grade methanol (20 mL) until no starting material was observed by TLC[1] (silica gel; C_6H_6). The methanol was removed to yield a solid ester (88 mg; 80%) mp 185–186 °C (189–190 °C; Carey et al., 1976). The ester was characterized by TLC, comparison with authentic material, and by its resonance Raman and enzymological properties.

4-Dimethylamino-3-nitrocinnamic Acid: This acid was synthesized by the method of Koo et al. (1963), from 4-dimethylamino-3-nitrobenzaldehyde (3.4 g; 17 mmol) in 94% yield, mp 180–181 °C (CH_3OH). Anal. Calcd for $C_{11}H_{12}N_2O_4$: C, 55.92; H, 5.12; N, 11.86. Found: C, 55.81; H, 5.15; N, 12.01. 4-Dimethylamino-3-nitrocinnamic acid thioethyl ester was synthesized from the corresponding acid by treating the mixed anhydride from ethylchloroformate with ethanethiol.

Imidazole Esters: 4-Dimethylaminocinnamoylimidazole. 4-Dimethylaminocinnamic acid (0.38 g; 2 mmol) in dioxane (15 mL) was treated with triethylamine (0.22 g; 2.2 mmol) and ethylchloroformate (0.22 g; 2.1 mmol) at room temperature. After 5 min the mixture was chilled, filtered, and treated with imidazole (0.14 g; 2.1 mmol). After 6 h at room temperature, the dioxane was removed at low temperature. The resultant bright yellow solid was crystallized from benzene to give 0.08 g (16%), mp 203–206 °C (dec). Anal. Calcd for $C_{14}H_{15}N_3O$: C, 69.64; H, 6.27; N, 17.42. Found: C, 69.35; H, 6.31; N, 16.95.

4-Dimethylamino-3-nitrocinnamoylimidazole. Carbonyldiimidazole (51 mg; 0.32 mmol) in tetrahydrofuran (4 mL) was treated with the acid (75 mg; 0.32 mmol) and the reaction flask swirled until everything dissolved. After 4 h cyclohexane was added until the mixture became cloudy. Refrigeration led to orange needles, 55 mg (60%), mp 190–192 °C. Anal. Calcd for $C_{14}H_{14}N_4O_3$: C, 58.73; H, 4.93; N, 19.57. Found: C, 58.55; H, 4.82; N, 19.71.

Results and Discussion

The Resonance Raman and Absorption Spectra of 4-Dimethylamino-3-nitrocinnamoylpapain and Furylacryloylpapain. The absorption and resonance Raman spectra of 4DMA-3NO_2-cinnamoylpapain are markedly similar to those reported for 4DMA-3NO_2-(α-benzamido)cinnamoylpapain (Carey et al., 1976). Both intermediates are characterized by λ_{max} near 410 nm and by an intense resonance Raman feature at 1570 cm^{-1}. Figure 1 shows the spectra of 4DMA-3NO_2-cinnamoylpapain at pH 3.0 and at 4 °C taken at ~20-min intervals and after 6 h. Figure 1 also compares the spectrum of concentrated product 4DMA-3NO_2-cinnamic acid and intermediate. As for 4DMA-3NO_2-(α-benzamido)-cinnamoylpapain, the resonance Raman spectrum is dominated by an intense band at 1570 cm^{-1}. However, the other characteristic band of the α-benzamido containing complex, at 1175 cm^{-1}, is absent in 4DMA-3NO_2-cinnamoylpapain.

[1] Abbreviations used: DMA, dimethylamino; TLC, thin-layer chromatography.

TABLE I: Comparison of Spectral Properties for Acyl-enzymes and Model Compounds.

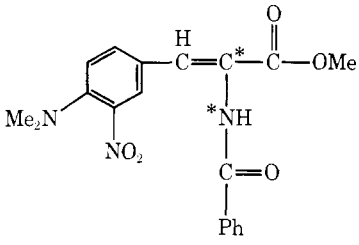
	λ_{max} (nm)	$\nu_{C=C}$ (cm^{-1})
4-Dimethylamino-3-nitrocinnamoylpapain	411	1570
Denatured 4-dimethylamino-3-nitro-cinnamoylpapain	370	1610[a]
4-Dimethylamino-3-nitro-(α-benzamido)-cinnamoylpapain	412	1570
Denatured 4-dimethylamino-3-nitro-(α-benzamido)cinnamoylpapain	370	1615[a]
4-Dimethylaminocinnamoyl-ImH$^+$	453	1568[b,c]
4-Dimethylaminocinnamoyl-Im	420	1583[b,e,f]
4-Dimethylaminocinnamaldehyde	398	1591[b,f]
4-Dimethylamino-3-nitrocinnamoyl-ImH$^+$	405	1591[b,c]
4-Dimethylamino-3-nitrocinnamoyl-Im	385	1600[b,f]
4-Dimethylamino-3-nitro-(α-benzamido)-cinnamic acid methyl ester	350[d]	1642[g]
4-Amino-3-nitrocinnamic acid methyl ester	343[d]	1643[g]
Furylacryloylpapain	360[h]	1613
Denatured furylacryloylpapain	337[h]	
Furylacrylic acid (pH 3.0)	304	1636
Furylacryloylimidazole	340	1625
5-Methylfurylacryloylimidazole	355	1613

[a] Partly obscured by water peak. [b] Intense in resonance Raman. [c] pH 3.0, 10% CD_3CN. [d] Strongest absorbance; there is a weaker absorbance at ~450 nm. [e] 1571 cm^{-1} in solid. [f] H_2O/CD_3CN solutions. [g] In CD_3CN. [h] Hinkle & Kirsch (1970).

For the latter, weak features are observed at 1414, 1290, 1230, and 1055 cm^{-1}. Figure 1 demonstrates that the resonance Raman spectrum can be used to monitor the kinetics of deacylation. The spectrum of product does not appear in the acylpapain spectra in Figure 1 since the resonance Raman intensity enhancement for the product is much less than that of the intermediate. This follows from the large blue shift in λ_{max} of product (343 nm, pH 3.0) compared with intermediate (410 nm).

The preparation and absorption spectral properties of furylacryloylpapain were reported by Hinkle & Kirsch (1970). The absorption data are given in Table I. Figure 2 compares the resonance Raman spectra of substrate, acyl-enzyme, and product. For the substrate, the imidazole ester of furylacrylic acid, $\nu_{C=C}$ occurs at 1625 cm^{-1} while $\nu_{C=C}$ is at 1613 cm^{-1} in the acyl-enzyme and at 1636 cm^{-1} in the product at pH 3.0. The change in the resonance Raman spectrum of the bound furylacryloyl moiety is not as dramatic as for the substituted cinnamoyl compounds. An explanation for this will be advanced under Model Compounds below.

^{15}N and ^{13}C-Substituted 4-Dimethylamino-3-nitro-(α-benzamido)cinnamic Acid Methyl Esters and Their Acylpapains. Resonance Raman and IR Spectra of the Isotopically Labeled Substrate. Figure 3 compares the resonance Raman spectra of the $^{14}N^{12}C$, $^{14}N^{13}C$, $^{15}N^{12}C$ analogues. The positions of the isotopic substitutions are indicated by * in the structural formula. Below 1200 cm^{-1} the spectra contain only very weak features (Carey et al., 1976) and this region is not



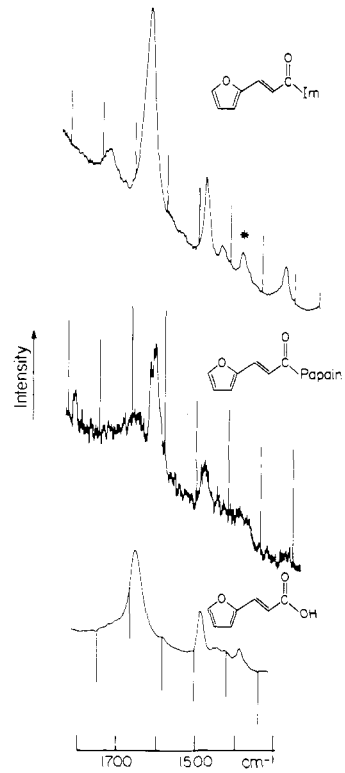


FIGURE 2: Resonance Raman spectra of furylacryloylimidazole (top), furylacryloylpapain, pH 3.0 (middle), and furylacrylic acid, pH 3.0 (bottom). Imidazole ester (6×10^{-4} M) in DMF (solvent indicated *). Papain (~10^{-4} M); active site occupancy ~20%; acid, 6×10^{-4} M; 350.7 nm, 150 mW, 8 cm^{-1} spectral slit.

shown. The spectra of the $^{15}N^{12}C$ and $^{14}N^{12}C$ derivatives are identical. However, they are clearly different from that of the $^{14}N^{13}C$ analogue. The latter lacks the band at 1642 cm^{-1} and compared with the other derivatives the 1613 cm^{-1}/1351 cm^{-1} intensity ratio is enhanced from ~1.3 to ~2.1. These observations suggest that the 1642-cm^{-1} band has a high degree of C=C stretching character and that it moves close to the 1613-cm^{-1} band upon ^{13}C substitution. In the ^{13}C derivative the 1613-cm^{-1} band would then represent a superposition of the ethylenic C=C stretch and the original 1613-cm^{-1} mode. This view is supported by better quality Raman data obtained in MeOH solution and from IR spectra of the solids in KBr. The Raman spectra taken in MeOH using 647.1-nm excitation show that, although the 1613-cm^{-1} band of the $^{14}N^{13}C$ analogue could not be resolved, the line width at half-height of the 1613-cm^{-1} band is 19 cm^{-1} compared with 16 cm^{-1} for the 1613-cm^{-1} band of the $^{14}N^{12}C$ analogue. Although the IR analysis is complicated by the presence of intense features from the α-benzamido moiety which do not appear in the resonance Raman spectra, the ^{13}C derivative has a shoulder at 1615 cm^{-1} in the IR which is absent in the other analogues. This shoulder may be assigned to the shifted C=C stretch. Since the shifted C=C stretch is not resolved its position can only be placed between the limits of 1613 cm^{-1} and approximately 1620 cm^{-1}. The corresponding shift of 29–22 cm^{-1} is somewhat less than the 30–32 cm^{-1} expected for a pure C=C vibration.

Resonance Raman Spectra of Isotopically Substituted 4-Dimethylamino-3-nitro-(α-benzamido)cinnamoylpapain. The resonance Raman spectrum of 4DMA-3NO_2-(α-benzamido)cinnamoylpapain is totally distinct from the spectra of the substrate and of the product (Carey et al., 1976). It is characterized by a very intense line at 1570 cm^{-1} and a line of medium intensity of 1175 cm^{-1}. Other weak broader lines

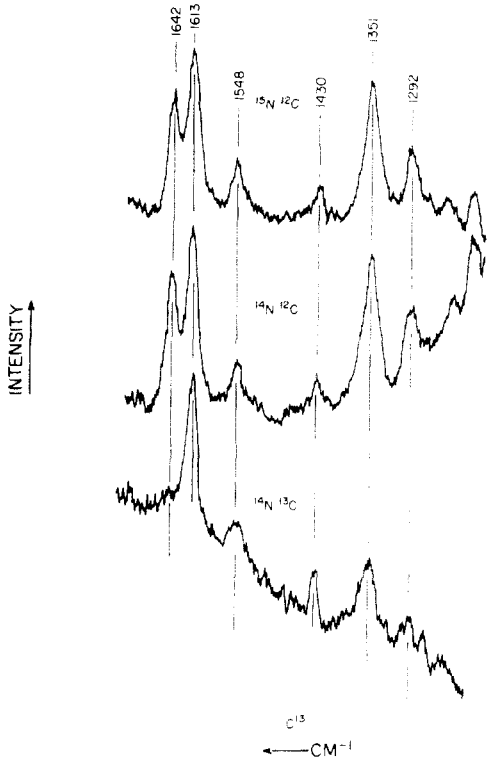


FIGURE 3: Resonance Raman spectra of isotopically substituted 4-dimethylamino-3-nitro-(α-benzamido)cinnamic acid methyl ester; ^{15}N in benzamido (top); unsubstituted (middle); ^{13}C in an α-ethylenic carbon (bottom). KBr pellet, rotating cell, 441.6 nm, 50 mW, 9 cm^{-1} spectral slit.

TABLE II: Line Positions (cm^{-1}) for Isotopically Substituted 4-Dimethylamino-3-nitro-(α-benzamido)cinnamoylpapain.

$^{14}N^{12}C$	1570	1175
$^{14}N^{13}C$	1562	1164
$^{15}N^{12}C$	1570	1167.5

appear in some but not all preparations of the acyl-enzyme and will not be discussed further. In the present work intermediates were made using ^{13}C- and ^{15}N-labeled substrate and the results, for the two intense lines, are given in Table II. Clearly discernible shifts occur with either ^{13}C or ^{15}N substitution. Thus the spectra selectively reflect a change of 1 dalton within a species of approximately 24 000 daltons. The reported isotope shifts are the average of 12 sets of data on four or five acyl-enzyme preparations for each intermediate. Two spectrometers (see Experimental Section) were used to record the spectra with good agreement. The reported shifts are thought to be accurate to within ±1 cm^{-1}. Both peaks moved upon ^{13}C substitution. However, upon ^{15}N substitution, a shift was detected only in the 1175-cm^{-1} feature. In our previous paper on this system the 1570-cm^{-1} band was assigned to a double-bond stretching mode from a conjugated system and the 1175-cm^{-1} band to a C–C (or C–N) single bond stretch. The isotopic substitution experiments fully support these assignments. The small isotope shift observed for the 1570-cm^{-1} feature upon ^{13}C substitution probably reflects conjugation and frequency mixing effects in the papain bound chromophore. The present results indicate that the α-benzamido N atom is not involved in the 1570-cm^{-1} mode.

Evidence for Benzamide Side Chain Rearrangment in 4-Dimethylamino-3-nitro-(α-benzamido)cinnamoylpapain. In our first paper on this acyl-enzyme (Carey et al., 1976) we noted the similarity between the resonance Raman spectrum

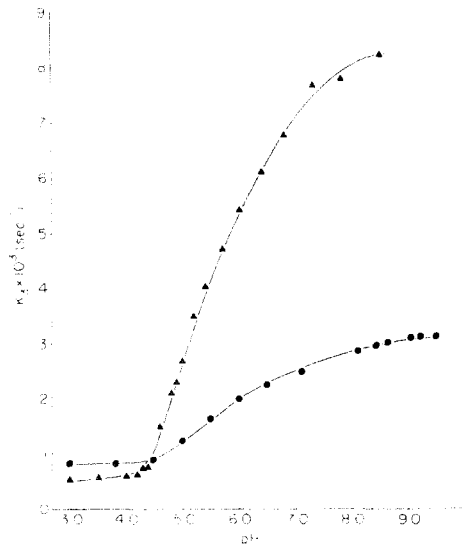


FIGURE 4: Effect of pH on deacylation rate of 4-dimethylamino-3-nitro-(α-benzamido)cinnamoylpapain (lower curve) and 4-dimethylamino-3-nitrocinnamoylpapain (upper curve). K_3 was measured by following the decrease in absorbance at 410 nm.

of the acylpapain and those of azlactones and polyenes. Furthermore, although the statement was not quantitated, we observed that the acyl intermediate appeared to have a high resonance Raman intensity. On the basis of this information it was suggested that the 1570-cm^{-1} band from the acyl-enzyme represented a double-bond stretching vibration from a conjugated π-electron system and that this delocalized electron system resulted from the α-benzamido side chain rearranging to –N=C(–OX)–Ph. The 1175-cm^{-1} band was said to possess C–C or C–N stretching character. The isotopic substitution results presented herein are consistent with the assignments. Moreover, comparison of the resonance Raman spectra and rates of deacylation of the cinnamoylpapains with and without the α-benzamido side chain (Figures 1 and 4 and Carey et al., 1976) provides evidence that this side chain causes changes in both deacylation and the resonance Raman spectrum. The 4DMA-3NO_2-cinnamoylpapain is the more labile intermediate and in addition lacks the 1175-cm^{-1} band of the α-benzamido analogue. Both intermediates have a pK of deacylation of ~6.0 (Figure 4). However, it is now evident that the properties of the α-benzamido side chain are not responsible for that acylpapain's intense 1570-cm^{-1} band since the 1570-cm^{-1} feature occurs in both complexes. It was emphasized that a true azlactone was not formed in the active site but that an acyclic analogue may be a model for possible α-benzamido rearrangment during enzymolysis. Unfortunately attempts at synthesis of such an analogue have thus far been unsuccessful. A resonance Raman study of true azlactones including isotopically substituted analogues has been completed (Kumar et al., 1978; Phelps et al., 1978).

In summary, although definite resonance Raman and kinetic differences exist between 4DMA-3NO_2-cinnamoylpapains with and without an α-benzamido side chain, the idea of α-benzamido side chain rearrangement remains tentative. However, it is now certain that a rearrangement of this type does not cause the intense 1570-cm^{-1} resonance Raman band, nor the large red shift in λ_{max} upon formation of the acyl-enzyme. A model for these effects is advanced below.

Model Compounds. The model compounds which mimic the absorption and resonance Raman spectra of the acylpapains are based on the cinnamoyl or furylacryloyl skeletons.

They have an electron-donating group attached to the phenyl or furyl ring and an electron-attracting group bound to the carbonyl. The concerted inductive effect polarizes the π electrons with the strongest "push-pull" pair setting up the greatest degree of polarization. It is immediately apparent from Table I that the combination of a strong push-pull pair, e.g., 4-dimethylamino and imidazole on the cinnamoyl skeleton, results in spectral properties closely resembling those of the cinnamoyl acyl-enzymes; λ_{max} is red shifted and there is a concomitant drop in $\nu_{C=C}$. We propose that these through bond inductive effects mimic the rearrangement of the electrons in the acyl group in papain's active site. However, it is thought that, while in the models the inductive effects act through chemical bonds, in the active site the electron rearrangement is caused by groups which are not covalently bound to the acyl group (see next section). The Raman spectrum of solid 4DMA-cinnamoylimidazole is dominated by an intense feature at 1571 cm^{-1} (Table I) and the same mode may be responsible for the strong absorbance seen at 1570 cm^{-1} in the IR (not shown). The high intensity of the IR mode could be accounted for by mixing with a benzene mode or by the polarized nature of the π system setting up a dipole across the C=C. Analogous Raman and IR spectra are observed for the other compounds listed in Table I. The intense 1571-cm^{-1} Raman band almost certainly has a degree of C=C stretching character. Although a luminescent background prevented resonance Raman spectra of the 4DMA compound from being obtained, both the preresonance and normal Raman spectra (not shown) of the 4DMA-$3NO_2$ analogue are dominated by $\nu_{C=C}$ to the extent that it is six times more intense than any other feature. The dominant 1571-cm^{-1} Raman feature and the position of λ_{max} in the absorption spectrum make 4DMA-cinnamoylimidazole an attractive model for the cinnamoylpapains.

As can be seen from Table I the net electron-donating ability of the 4DMA-$3NO_2$ groups is less than that of 4DMA (this probably results from the electron attracting nature of the nitro group and the distortion from planarity of the DMA caused by the ortho nitro). However, spectral comparisons suggest that 4DMA-cinnamoylimidazole is a better model for the cinnamoylpapains than 4DMA-$3NO_2$-cinnamoylimidazole. That is, the active site achieves a higher degree of electron polarization than that found in the imidazole ester of the substrate. A similar effect is seen for furylacryloyl.

Table I lists λ_{max} and $\nu_{C=C}$ for a variety of furylacryloyl complexes. As for the cinnamoyl derivatives discussed above the best model compound is an imidazole ester with an electron-donating group on the furyl ring. Thus methyl furylacryloylimidazole ester mimics the spectral properties of furylacryloylpapain. Again, further electron donation into the ring, now by a methyl group, brings the spectral properties of the furylacryloylimidazole ester closer to those of furylacryloylpapain. The spectral changes for furylacryloylpapain are not as dramatic as those for the cinnamoyl derivatives because the degree of electron polarization is less. This is probably the result of the cinnamoyl compounds possessing a strong electron-donating group in their skeleton which reinforces the polarizing forces due to papain's active site. The substituted cinnamoyl papains are "push-pull" compounds while furylacryloylpapain lacks a "push" from within the furylacryloyl skeleton.

Components of Papain's Active Site Which May Cause Electron Polarization in the Bound Acyl Group. Among the possible causes of the observed spectral changes are (a) binding to the S atom of cysteine-25; (b) binding to an imidazole side chain; (c) change in dielectric constant; (d) hydrogen bonding and charge effects.

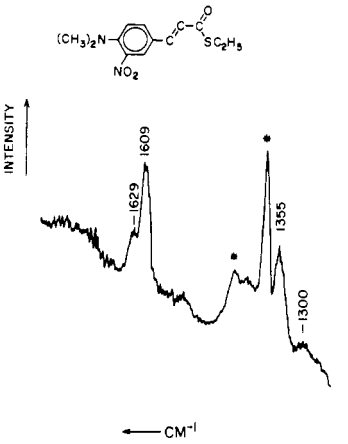


FIGURE 5: Preresonance Raman spectrum of 4-dimethylamino-3-nitrocinnamic acid thioethyl ester in CH_3CN. * indicates solvent.

(a) The chromophoric properties of the S atom of cysteine-25 (the normal site of acylation) can be eliminated as the source of the observed spectral effects. The preresonance Raman spectrum of the thioester 4DMA-$3NO_2$-cinnamic acid thioethyl ester is shown in Figure 5. The compound in CH_3CN has a λ_{max} of 363 nm. The Raman spectrum is essentially that of an oxygen ester of the acyl group and does not resemble those of the acylpapains.

(b) Evidence for the transient formation of an acylimidazole has been found during chymotrypsin esterolysis (Hubbard & Kirsch, 1972) and in the present case the spectral similarities between the acylpapains and the model acylimidazoles pose the possibility that acylation of papain may take place at an imidazole, e.g., the side chain of histidine-159. This, however, is ruled improbable on the basis of denaturation studies. By going to pH 1.8 4DMA-$3NO_2$-cinnamoylpapain denatures to give a species with λ_{max} at ~370 nm and a weak preresonance Raman spectrum closely resembling that of the product (Figure 1, bottom) and thioester (Figure 5). However, the absorption and resonance Raman spectra of 4DMA-$3NO_2$-cinnamoylimidazole at pH 1.8 (imidazole protonated) are characterized by a λ_{max} 405 nm and an intense resonance Raman feature at 1591 cm^{-1} with no trace of the bands seen at 1610 and 1355 cm^{-1} in the denatured acyl-enzyme. Above pH 4.0 (imidazole neutral), λ_{max} is at 385 nm and the dominant Raman feature is at 1600 cm^{-1} (Table I). Thus the spectra of protonated or neutral imidazole esters of 4DMA-$3NO_2$-cinnamoyl do not resemble the denatured acyl-enzyme and acylation of an imidazole side chain appears unlikely. Extensive dialysis shows that the acyl group remains covalently bound to the denatured enzyme.

(c) On binding to papain, acyl groups experience a change in the bulk dielectric constant. It appears, however, that such a change cannot account for the observed spectral properties. The resonance Raman spectra of 4DMA-$3NO_2$-cinnamoylimidazole were recorded in solvents of varying dielectric constant, 90% H_2O:10% CH_3CN, 100% CH_3CN, CCl_4, and $CHCl_3$. The spectra were the same in each solvent with $\nu_{C=C}$, in particular, occurring at 1600 $\pm$ 2 cm^{-1} in each case. Thus the chromophore appears insensitive to *bulk* dielectric changes. The difference of 12 cm^{-1} recorded in Table I for 4DMA-cinnamoylimidazole in solid and solution is probably a property of the solid phase which was made up of a small percentage of chromophore pressed into a KBr pellet.

(d) The importance of hydrogen bonds in active sites has been realized for some time and has recently received close scrutiny for the charge relay, hydrogen bonding system in serine proteases (Wang, 1970; Birktoft et al., 1976). In the

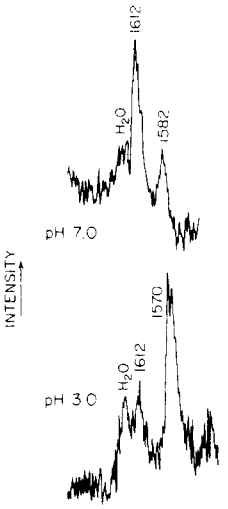


FIGURE 6: Resonance Raman spectra of a reaction mixture containing 4×10^{-4} M 4-dimethylamino-3-nitro-(α-benzamido)cinnamic acid methyl ester and 10^{-4} M activated papain. (Top) pH 7.0; (bottom) same reaction mixture with pH dropped to 3.0; 457.9 nm, 50 mW, 8 cm^{-1} spectral slit. H_2O feature contains a contribution from a 1636 cm^{-1} substrate band.

present case the formation of a strong hydrogen bond to the carbonyl of the papain bound acyl group could be responsible, at least in part, for increasing the electron-attracting power of the carbonyl. This would then lead to a polarization of the π electrons and the observed spectral effects.

Similarly a positively charged group close to the carbonyl could bring about the proposed electron polarization. The imidazolium ion of histidine-159, confirmed by x-ray crystallography (Drenth et al., 1976) to be an active site residue, would be a prime candidate at low pH. Involvement of an imidazolium residue is consistent with data for pH effects on deacylation rates. For the cinnamoyl intermediates the rate of disappearance of absorbance at 410 nm has a pK of ~6.0 for both intermediates (Figure 4). Although the pK behavior of histidine-159 has been the source of much speculation (Lowe, 1976), with recent evidence of a cooperative ionization of histidine-159 and aspartic acid-158 (Bendall & Lowe 1976), the value of 6.0 is suggestive of imidazole protonation.

The dramatic differences between the resonance Raman spectra of 4DMA-3NO_2-(α-benzamido)cinnamoyl as substrate, acylpapain, and product permit the observation of the intermediate in a reaction mixture containing all three entities (Carey et al., 1976). This was used in the present work to test the effect of pH and thus the protonation of active site side chains on the nature of the acyl-enzyme. A parallel experiment using absorption spectroscopy is impractical due to the overwhelming absorbance of the substrate and product. In a mixture at pH 7.0 consisting of 10^{-4} M activated papain and 4×10^{-4} M substrate a resonance Raman peak from the intermediate appeared at 1582 cm^{-1} and a substrate peak occurred at 1612 cm^{-1} (Figure 6). Under the conditions employed the concentration of substrate remained essentially constant, and the 1612-cm^{-1} band was used as an internal intensity standard. The intensity ratio 1582/1612 was 0.3. Upon dropping the pH to 3.0 the 1582 cm^{-1} peak moved to 1570 cm^{-1} and the 1570/1612 intensity ratio was 3.5 (Figure 6). Thus dropping the pH from 7.0 to 3.0 resulted in a tenfold increase in the intensity of the intermediate band and its movement to 1570 cm^{-1}. This is consistent with a protonation step in the active site bringing about an increased degree of electron polarization in the acyl group. Possibly the C=O of the acyl group is hydrogen bonded at both pH 3.0 and 7.0 and the protonation step further increases the electron-attracting power at the carbonyl group. This could occur via a H bond if, e.g., an imidazole side chain, already hydrogen bonded to the acyl carbonyl, became protonated. A negative charge placed near the ring of the acyl group could also account for the results near neutral pH.

Spectral invariance of the denatured acyl-enzymes or methyl esters in the pH range 1.9–10.0 makes direct protonation or deprotonation of the acyl groups unlikely.

Conclusion

The spectral analogies developed between model compounds containing polarized electron systems and the acylpapains suggest that, in the latter, substrate–active site interactions are instrumental in bringing about extensive electron rearrangement. Morover, the effect appears not to depend on the nature of the chromophore since three chemically distinct acyl groups show the same spectroscopic trends. Although the components of the site responsible for polarizing the acyl group electrons have not been identified, denaturation studies suggest that the interactions do not occur via the primary acyl-enzyme bond. It is therefore suggested that hydrogen bonding and, or the juxtaposition of charged groups are the prime factors involved. At first, it may seem surpising that the large spectral charges observed are brought about by essentially noncovalent forces. However, there is a body of evidence (Ebrey & Honig 1975; Honig et al., 1976; Warshel, 1976) suggesting that noncovalent charge effects in rhodopsin and bacteriorhodopsin can bring about large electron rearrangements in the retinal chromophore. Thus, it does seem likely that protein sites are capable of bringing about considerable electron rearrangements in bound groups and indeed Warshel & Levitt (1976) have recently stated that the abilities of proteins in this regard have been underestimated. The present studies suggest that resonance Raman data may provide crucial experimental support for these ideas.

On the basis of resonance Raman studies an interesting difference is emerging between the properties of acylpapains and acylchymotrypsins (Carey & Schneider, 1974, 1976) at pH 3.0. Although red shifts are observed in the absorption spectra of both sets of acyl-enzymes, only the resonance Raman spectra of the acylpapains are greatly changed from the spectra of the free chromophores. This means that the red shift in acyl-chymotrypsins is essentially an excited state effect, while in acylpapains extensive electron rearrangement occurs in the ground state. This difference may reside in the presence of a charged group in the active site of papain at low pH which is not found in chymotrypsin. Good supportive evidence can be found for the proposition that between pH 3.0 and 5.0 in chymotrypsin the imidazole side chain of histidine-57 is neutral (Fersht & Renard, 1974; Koeppe & Stroud, 1976), whereas in papain the imidazole of histidine 159 is protonated (Lowe, 1976). This difference probably resides in the characteristics of the serine-histidine-aspartate triad found in serine proteases and the cysteine-histidine-asparagine triad found in cysteine proteases.

Acknowledgments

We thank Dr. K. R. Lynn for a sample of papain purified by affinity chromotography and Dr. H. J. Bernstein for the use of his Jarrell-Ash Raman spectrometer.

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Triplet State of Tryptophan in Proteins: The Nature of the Optically Detected Magnetic Resonance Lines†

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ABSTRACT: Optical detection of magnetic resonance (ODMR) has been employed to examine the homogeneity of the tryptophan environment, both of the isolated residue in solvent, and of tryptophan in glucagon and lysozyme and azurin B (*Pseudomonas aeruginosa*). From the shifts in the zero-field splittings, we can safely conclude that tryptophan in lysozyme, azurin B, or glucagon does not have the same type of solvent interaction as the free residue. However, by "burning holes" in the ODMR lines, it is evident that the lines *in these cases* are inhomogeneously broadened. From the relative line widths and hole widths, it appears that ODMR can be used to examine the relative diversity of interactions for a luminescent amino acid in a protein. We have followed the ODMR line characteristics in a progression from free *N*-acetyl-L-tryptophanamide, to tryptophan in lysozyme, to "denatured" lysozyme, and present evidence that the line widths *narrow* as the tryptophan residues become less solvent accessible.

In the recent half decade, optical detection of magnetic resonance (ODMR)[1] (Sharnoff, 1967; Kwiram, 1967) has been introduced as a method for investigating the triplet state properties of tryptophan and tyrosine in proteins. Early studies in this field were carried out by Yamanashi & Kwiram (1970), and by Zuclich et al. (1972, 1973, 1974). The latter investigators reported that ODMR could be used to resolve tryptophan sites in proteins, particularly in lysozyme and horse liver alcohol dehydrogenase (HLAD).

HLAD is rare in that the two tryptophan sites have unique phosphorescence which allows them to be optically resolved (Purkey & Galley, 1970). von Schütz et al. (1974) noted a discontinuity in the zero-field-splitting parameters, $|D|$ and $|E|$, at the precise region of overlap of the two tryptophan phosphorescence peaks. In the case of lysozyme, there are six tryptophan residues but the optical spectrum shows no evidence for resolved sites. Yet, again discontinuities in $|D|$ and $|E|$ were found at a particular phosphorescence wavelength; specifically, a distinct ODMR doublet was observed. von Schütz et al. interpreted these results as evidence for at least two classes of tryptophan sites in both enzymes. However, they reported that the 100-MHz wide ODMR lines in lysozyme—in contrast to HLAD—were *homogeneously* broadened. This is rather surprising since one would expect magnetic resonance lines observed in condensed media to be *inhomogeneously* broadened as a result of a quasi-static distribution of interactions between the chromophores and their respective environments. (Unless, of course, there are unusual dynamic processes which facilitate spectral diffusion.) Moreover, if one assumes that the ODMR lines in lysozyme reflect the contribution of more than one distinct tryptophan site, then the

† From the Department of Chemistry, University of Washington, Seattle, Washington 98195. *Received August 29, 1977.* This research was supported by Grant GM 22603 awarded by the National Institutes of Health, Department of Health, Education and Welfare and in part by Grant GB 18016 awarded by the National Science Foundation and in part by an M. J. Murdock Charitable Trust grant of Research Corporation.

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[1] Abbreviations used: ODMR, optical detection of magnetic resonance; zfs, zero-field splitting, ELDOR, electron double resonance; CW, continuous wave; HLAD, horse liver alcohol dehydrogenase; Gdn·HCl, guanidine hydrochloride.

0006-2960/78/0417-1087$01.00/0