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Correlations between Reactivity and Structure of Some Chromophoric Acylchymotrypsins by Resonance Raman Spectroscopy[†]

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ABSTRACT: The resonance Raman (RR) spectra, excited by 350.7-nm irradiation, of 4-NH₂-3-NO₂-cinnamoyl- and 5-CH₃-thienylacryloylchymotrypsin are reported at various pH values. Changes in the RR spectra, taken with the corresponding changes in the absorption spectra with varying pH, show that at active pH a reduction in π -electron polarization throughout the chromophore occurs prior to deacylation. The reduction in polarization leads to a blue shift in λ_{\max} and an increase in frequency in $\nu_{\text{C}=\text{C}}$ and is thought to result from the generation of a negative or partial negative charge near the acryloyl carbonyl at active pH. The relation of the spectral changes to the activation of the carbonyl group is discussed. Intensity patterns in the RR spectra of the cinnamoyl intermediate obtained by using 350.7-nm excitation are markedly different from those reported earlier, obtained by using 441.6-nm excitation [Carey, P. R., & Schneider, H. (1976) *J. Mol. Biol.* 102, 679-693]. On the basis of comparison of

the alkaline rates of hydrolysis of the imidazole esters and the rates of deacylation of the acyl enzymes, three acyl enzymes, viz., 3-CH₃-thienylacryloyl-, α -CH₃-5-CH₃-thienylacryloyl-, and α -CH₃-5-CH₃-furylacryloylchymotrypsin, show unexpectedly low rates of deacylation. The carbonyl profiles or carbon-carbon double bond stretching profiles in the RR spectra of these acyl enzymes, taken with their anomalous $\langle \nu_{\text{C}=\text{C}} \rangle$ vs. $\langle \lambda_{\max} \rangle$ correlations, indicate that enzyme-substrate interactions perturb the geometry and π -electron distribution of the enzyme-bound chromophore compared to the free moiety in solution. On the basis of this evidence, it is proposed that steric hindrance in the active site, caused by the substrates' 3-CH₃ or α -CH₃ groups, leads to low rates of deacylation. Good quality RR spectra permitted a careful comparison of the RR spectra of 5-CH₃-thienylacryloylchymotrypsin in H₂O and D₂O. For this nonspecific acyl enzyme the spectrum was found to be totally insensitive to the change in solvent.

This paper is a direct sequel to the preceding paper (MacClement et al., 1981). The latter sets the basis for interpreting the resonance Raman spectra of several chromophoric acyl enzyme intermediates in terms of enzyme-substrate interactions at pH 3.0. At this pH the acyl enzymes are stable species. The present work deals more closely with the effects related to enzyme activity and the kinetics of deacylation. It explores

the consequences of taking the acyl enzymes to the pH range 7.0-8.5 wherein chymotrypsin has optimal activity [Fersht & Requena (1971) and references cited therein]. The resultant unstable acyl enzymes typically have half-lives in the 10-1000-s time span and must be studied in flow systems. The changes in the RR spectra as "active pH" is approached give clues to the effect on the substrate of the functioning catalytic apparatus and enable this effect to be distinguished from the consequences of merely binding at pH 3.0. In addition to the substrates dealt with in the foregoing paper, we have studied the effect of changing pH on the RR spectrum of the acyl

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enzyme 4-NH₂-3-NO₂-cinnamoylchymotrypsin. The RR spectrum of this moiety was formerly studied by using 441.6-nm wavelength excitation (Carey & Schneider, 1976), but the spectrum shows radical changes in its intensity patterns upon excitation with the 350.7-nm wavelength used in the present work. Importantly, the use of an excitation wavelength in the near-ultraviolet allows greatly improved data to be gathered on the unstable acyl enzyme. The RR spectra of three acyl enzymes, 3-CH₃-thienylacryloyl, α -CH₃-5-CH₃-thienylacryloyl, and α -CH₃-5-CH₃-furylacryloylchymotrypsin, are also reported and discussed in an attempt to explain their rates of deacylation which are unexpectedly slow compared to the analogues lacking the 3-CH₃ or α -CH₃ groups. An additional topic considered here concerns the effect of D₂O on the acyl enzymes. Rates of deacylation of the acyl enzymes under consideration are typically 2–3 times slower in D₂O compared to H₂O solutions. One possible cause of this effect is that the structure of the acyl enzyme in D₂O differs from that in H₂O (Jencks, 1969). RR spectra of 5-CH₃-thienylacryloylchymotrypsin in H₂O and D₂O are compared to look for possible perturbations to the acyl group structure.

It is important to keep in mind the main conclusions of the preceding paper (MacClement et al., 1981) when discussing the present work. One finding was that in solution the furylacryloyl and thienylacryloyl chromophores and their substituted analogues exist in a number of conformations about the acryloyl group. Moreover, upon binding to the active site of chymotrypsin, these acyl groups adopt two conformations having different environments about the carbonyl group. Measured spectroscopic parameters such as λ_{max} in the absorption and $\nu_{\text{C}=\text{C}}$ in the RR should therefore be considered as weighted mean values, e.g., $\langle \nu_{\text{C}=\text{C}} \rangle$, with contributions from more than one species. The model of π -electron polarization in the π -electron systems of the furyl- and thienylacryloyl chromophores was used to unify the RR and absorption data in the preceding paper. The term electron polarization refers to the setting up of a permanent dipole along the long axis of the chromophore by, e.g., active-site forces, solvent effects, or internal inductive effects within the chromophore. Changes in polarization lead to shifts in $\langle \nu_{\text{C}=\text{C}} \rangle$ in the RR spectra and corresponding perturbations to $\langle \lambda_{\text{max}} \rangle$ in the absorption spectrum. This gives rise to a correlation between $\langle \nu_{\text{C}=\text{C}} \rangle$ and $\langle \lambda_{\text{max}} \rangle$ such that a plot of $\langle \nu_{\text{C}=\text{C}} \rangle$ vs. $\langle \lambda_{\text{max}} \rangle$ generally gives rise to a straight line. One or more of several factors can vary the degree of electron polarization in a given chromophore, requiring care in assigning spectral shifts to a single cause. The models and concepts developed in MacClement et al. (1981) now find extensive use and support below.

Experimental Procedures

The preparation and properties of the imidazole substrates and acyl enzymes are given in the first paper in this series (MacClement et al., 1981). Samples in D₂O were made by freeze-drying batches of acyl enzyme at pH 3.0, then adding an equivalent amount of D₂O, and adjusting the pD to 3.0. As a control, the RR spectrum of a sample reconstituted with H₂O was compared to the spectrum of the sample taken prior to freeze-drying. The two spectra were identical.

The deacylation rates of the acyl enzymes were determined by using a Cary 118 spectrometer. The reference cell contained distilled water, the sample cell contained 2 mL of phosphate buffer ($\mu = 0.15$) at the desired pH, and up to 0.1 mL of acyl enzyme was placed on the disk shaped plunger handle of a micropipet. This was lowered into the sample cell (by using the plunger itself as a handle) and moved up and down to stir the mixture. This method allowed fairly rapid

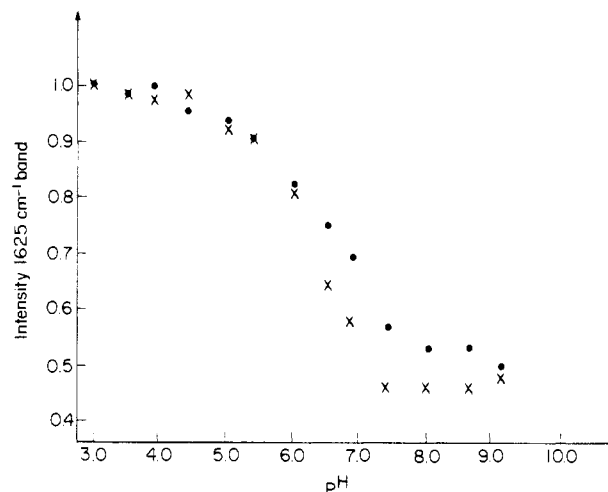


FIGURE 1: Variation of intensity of $\nu_{\text{C}=\text{C}}$, the ethylenic stretching mode in the RR spectrum of 4-NH₂-3-NO₂-cinnamoylchymotrypsin, with pH. (●) represents peak heights and (×) peak area. Since the latter is the total integrated intensity, it is the more accurate of the two. Intensity units are arbitrarily normalized to an intensity, at pH 3.0, of 1.0. Similar pK behavior was observed for the position of $\nu_{\text{C}=\text{C}}$ (see text).

mixing and very little dilution of the buffer. The deacylation rates were determined by setting the spectrometer wavelength at half the maximum absorption on the red side of the absorption peak and following the rate of decay. Good first-order exponential decays were observed in all cases. Since the pK_a of deacylation is not the same for all acyl enzymes, the maximum deacylation rate k_D (at high pH) was obtained by plotting $1/k_D(\text{obsd})$ against H^+ concentration, using

$$k_D(\text{obsd}) = k_D K_a / (K_a + [\text{H}^+])$$

as shown in Bernhard et al. (1966). Saponification rates of the imidazole esters were measured in 10 mM borate at pH 10.5. The change in absorbance at half the absorption maximum, on the red side, was again used to monitor the reaction. Saponification rates were not sensitive to borate concentration, indicating that borate does not catalyze the hydrolysis of these esters.

The conditions for recording Raman spectra are given in the accompanying papers (MacClement et al., 1981; Storer et al., 1981). For determination of the RR spectra of the acyl enzymes at active pH, stable acyl enzyme at pH 3.0 and buffer were mixed in a T-joint followed by a mixing chamber (Carey & Schneider, 1976). The solutions of acyl enzyme and buffer were each contained in a syringe and forced through the T-joint by a syringe pump. Subsequently, the mixture passed through a horizontal quartz capillary, comprising the Raman cell, 1 mm in diameter. The vertical laser beam was focused into the tube, and the scattered light was analyzed perpendicular to the directions of the tube and the laser beam. Intensities of Raman features measured in this apparatus were highly reproducible. For example, although for the pH titration of 4-NH₂-3-NO₂-cinnamoylchymotrypsin (below) a suitable internal intensity standard could not be found, a reliable titration curve (Figure 1) for the intensity of the 1625-cm⁻¹ band could still be generated. This was achieved by dividing a given preparation of acyl enzyme into three aliquots; the first sample was run in the mixing system against buffer at pH 3.0 and the second was run against buffer at pH >3.0. An aliquot of the original preparation was again run against buffer at pH 3.0 as a check for reproducibility.

Absorption spectra of the acyl enzymes at active pH were obtained from a flow system in which stable acyl enzyme was

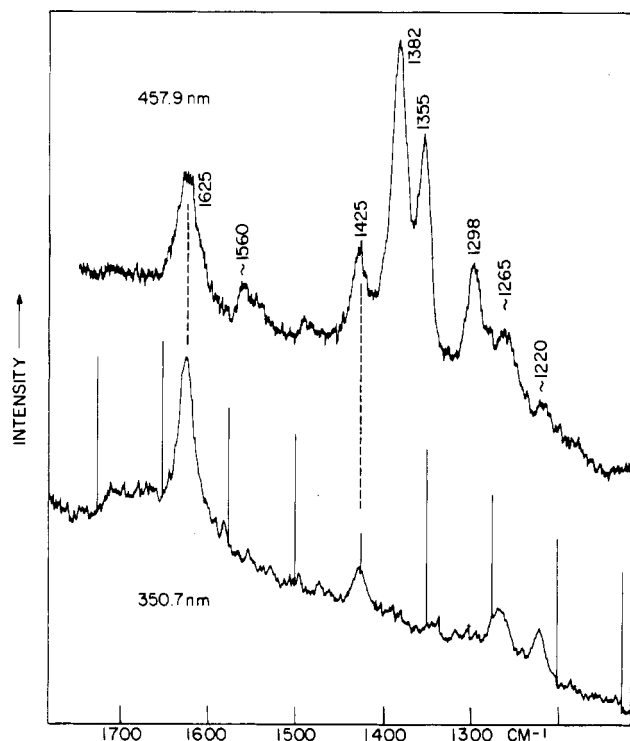


FIGURE 2: Comparison of RR spectra of the 4-NH₂-3-NO₂-cinnamoyl chromophore using 457.9- and 350.7-nm excitation. The top spectrum (457.9-nm excitation) is for the methyl ester (8×10^{-4} M in 40% CD₃CN and 60% H₂O); an identical spectrum is obtained for the acyl enzyme by using excitation in this region (Carey & Schneider, 1976). The bottom spectrum (350.7-nm excitation) is for acyl enzyme ($\sim 2 \times 10^{-4}$ M in enzyme, 30% acylated) in a flow system at pH 3.0. 50–100 mW of laser power was employed in both cases with the spectral slit width set at 8 cm⁻¹.

mixed with buffer. The two solutions were driven through an eight-jet mixing device (purchased from Dionex, CA) by a syringe pump and subsequently passed through a flow-through absorption cell of 1-cm path length.

Results and Discussion

Effect of Going to Active pH. Two intermediates, 4-NH₂-3-NO₂-cinnamoyl- and 5-CH₃-thienylacryloyl- (5-MeTA) chymotrypsin, have been studied in the pH range 7.0–8.5, wherein chymotrypsin has optimal activity. The RR and absorption spectra of the acyl enzymes at active pH were measured in flow cells (Experimental Procedures) after mixing stable acyl enzyme and buffer. Based on the known half-lives of the intermediates, it was possible to adjust the flow rate to ensure that <10% of the acyl enzyme had decomposed to product at the point of detection. The RR spectrum of the cinnamoyl derivative was examined by using near-UV excitation at 350.7 nm to compare with earlier results obtained by using 441.6-nm excitation (Carey & Schneider, 1976). Although the UV RR results generally confirm the previous work, interesting new effects were observed. The high-quality RR spectra of the 5-MeTA-chymotrypsin obtained at pH 3.0 prompted us to choose this compound for study at active pH.

4-NH₂-3-NO₂-cinnamoylchymotrypsin. The RR spectrum of this intermediate at pH 3.0, obtained by using 350.7-nm excitation, is compared in Figure 2 with the RR spectrum of the methyl ester of 4-NH₂-3-NO₂-cinnamic acid recorded by using 457.9-nm excitation. Since, by use of excitation in the visible region, the RR spectrum of the methyl ester is identical with that of the acyl enzyme (Carey & Schneider, 1976), Figure 2 essentially compares the RR spectra of the acyl enzyme obtained by using visible and near-UV excitation. The radical differences in the relative intensities of the RR peaks

in Figure 2 for the two excitation wavelengths result from the nature of the absorption spectrum of the acyl chromophore. The absorption spectrum of the intermediate is reported in Figure 1 of Carey & Schneider (1976); a 1.9×10^{-4} M solution of enzyme, with 30% of the enzyme acylated, has absorption maxima with OD's of 8 at 280 nm, 1.5 at 335 nm, and 0.35 at 430 nm. The absorption bands with λ_{max} 's at 335 and 430 nm are entirely due to the substrate. Different patterns of Raman intensity enhancement are thus obtained by exciting within these two absorption peaks. The spectrum in Figure 2, obtained by using 457.9-nm excitation, is, in the 1250–1450-cm⁻¹ region, characteristic of ortho amino-nitro substitution in a benzenoid ring (Kumar & Carey, 1975). The 430-nm absorption band of the chromophore is, on this basis, assigned to a mixed $\pi^*-\pi$, $\pi^*-\pi$ transition in which the -NH₂ and -NO₂ ring substituents make a substantial contribution. Since the RR spectrum recorded by using 350.7-nm excitation has little or no contribution from the ring modes, it seems likely that the 335-nm absorption is a $\pi^*-\pi$ transition coupled to the double bond feature at 1625 cm⁻¹. The 1625-cm⁻¹ band was originally (Carey & Schneider, 1976) assigned to the ethylenic double bond stretching mode, $\nu_{\text{C}=\text{C}}$, with the likelihood of some vibrational coupling with modes from the ring. The present findings strengthen this assignment.

The RR spectrum of 4-NH₂-3-NO₂-cinnamoylchymotrypsin excited by 350.7 nm (Figure 2) contains a weak feature in the carbonyl region. The carbonyl band appears at 1700 cm⁻¹ and is partially obscured by a broad feature near 1660 cm⁻¹. The latter is absent in spectra of the acyl enzyme in D₂O and is thus probably due to an O-H bending mode of water. Interestingly, there is no evidence for the sharp 1725-cm⁻¹ feature reported for the furyl- and thienylacryloylchymotrypsins in the foregoing paper (MacClement et al., 1981). Thus, on the basis of the interpretation given in the foregoing paper, the 4-NH₂-3-NO₂-cinnamoyl moiety appears to assume a single conformation in the active site with the C=O hydrogen bonded and the conformation *s-trans* about the carbon-carbon =C-C=O linkage. This is the same conformation, for the acryloyl group, as that reported for the indoleacryloyl group in the active site of chymotrypsin by Henderson (1970). Upon denaturation by the addition of sodium dodecyl sulfate (Na-DodSO₄), changes are observed in the RR spectrum of 4-NH₂-3-NO₂-cinnamoylchymotrypsin. For the acyl enzyme in D₂O denaturation causes a broadening of a feature at 1443 cm⁻¹ and a broadening of the peak due to $\nu_{\text{C}=\text{O}}$ at 1700 cm⁻¹. The double bond mode at 1625 cm⁻¹ appears to be unchanged by denaturation. The corresponding absorption data for the acyl chromophore are λ_{max} 's at 334 and 425 nm (native) and 326 and 423 nm (denatured).

The effect, on the RR spectrum, of taking 4-NH₂-3-NO₂-cinnamoylchymotrypsin to active pH was dealt with in an earlier paper (Carey & Schneider, 1976). The earlier work used 441.6-nm excitation and thus excited into the chromophore's 430-nm band, giving rise to a spectrum resembling that in the upper half of Figure 2. Spectral quality for the acyl enzyme was impaired, however, due to the presence of a background signal from a weak luminescence. Moreover, due to the limitations of our original flow system, reliable data could not then be obtained on the shorter lived intermediates generated above pH 7.0. Between pH 5.7 and 7.0 by use of 441.6-nm irradiation, the only change in the acyl enzyme's RR spectrum, compared to that at pH 3.0, was a substantial reduction in the intensity of the 1625-cm⁻¹ peak. In the present work, using 350.7-nm excitation, measurements were possible up to pH 9.1, and a much better signal to noise ratio was

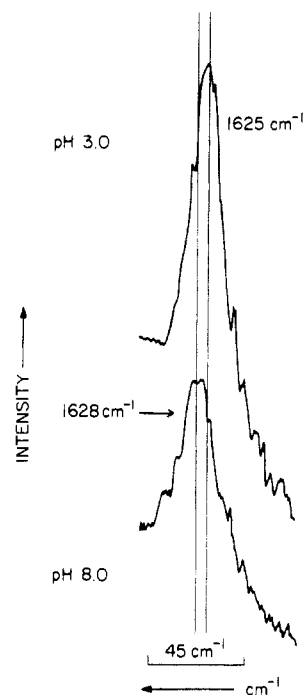


FIGURE 3: Comparison of $\nu_{\text{C=O}}$ in RR spectrum of 4-NH₂-3-NO₂-cinnamoylchymotrypsin at active (pH 8.0) and inactive (pH 3.0) pH. Conditions were as follows: flow system (see Experimental Procedures); 350.7-nm excitation; 100 mW; 8-cm⁻¹ spectral slit. Enzyme $\sim 2 \times 10^{-4}$ M, 30% acylated.

obtained for the 1625-cm⁻¹ peak. The improved data enabled us to detect a small frequency shift in $\nu_{\text{C=O}}$ in addition to the intensity change. This is illustrated in Figure 3 which shows that $\nu_{\text{C=O}}$ increases from 1625 to 1628 cm⁻¹, upon going from pH 3.0 to 8.0. The frequency change is an important finding because Raman and RR peak positions, in contrast to intensities, are a property solely of the electronic ground state. Since the catalytic processes we are concerned with here do not involve the electronic excited states, it is most useful to eliminate excited-state effects as a source of ambiguity. The improved data also allow us to present an apparent pK for the decrease in the intensity of the 1625-cm⁻¹ peak before deacylation occurs. As can be seen in Figure 1, the pK is near 6.3. Although the frequency changes are small, we were able to show that the position of $\nu_{\text{C=O}}$ also had a pK near pH 6.3. Each of the following values of $\nu_{\text{C=O}}$ was the mean of five measurements: $\nu_{\text{C=O}}$ = 1625.0 (pH 3.0), 1625.0 (pH 4.4), 1625.0 (pH 5.0), 1625.0 (pH 5.4), 1625.6 (pH 6.0), 1626.5 (pH 6.2), 1626.7 (pH 6.5), 1627.1 (pH 6.7), 1627.5 (pH 6.9), 1628.2 (pH 7.4), 1628.0 (pH 8.0), 1628.2 (pH 8.6), and 1628.2 cm⁻¹ (pH 9.1). Absorption data were also obtained for the first time for the active acyl enzyme in a flow cell (Experimental Procedures). Prior to deacylation, λ_{max} for the 335-nm peak at pH 3.0 moves to 330 nm at pH 7.0 and 321 nm at pH 8.0.

5-Methylthienylacryloylchymotrypsin. The effect on the RR spectrum of going to the active pH of 5-MeTA-chymotrypsin is less pronounced than that for the 4-NH₂-3-NO₂-cinnamoyl analogue. As can be seen in Figure 4 for 5-MeTA-chymotrypsin, upon going to pH 8.4, small shifts to higher cm⁻¹ are observed with ($\nu_{\text{C=O}}$) at 1611 cm⁻¹ moving to 1613.5 cm⁻¹ and the ring mode at 1464 cm⁻¹ moving to 1468 cm⁻¹. The shifts were observed for each of five separate acyl enzyme preparations. The integrated intensity of the peak near 1611 cm⁻¹ also falls as neutral pH is approached; if the integrated intensity of the 1611-cm⁻¹ peak at pH 3.0 equals 1.0, it is equal to 1.0 (pH 5.0), 1.0 (pH 5.5), 1.0 (pH 6.0), 0.96

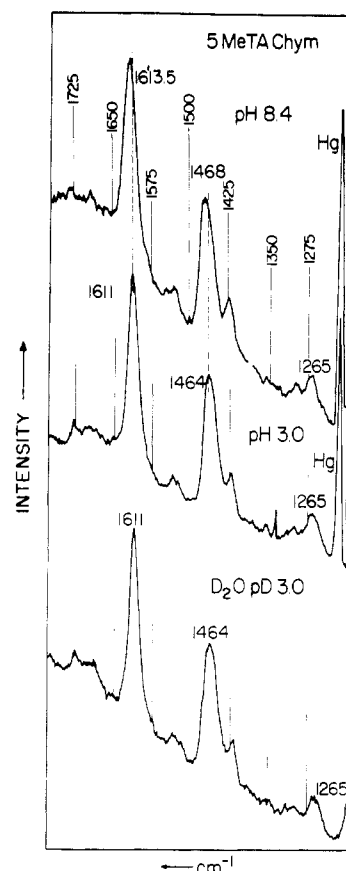


FIGURE 4: Comparison of RR spectra of 5-methylthienylacryloylchymotrypsin at pH 3.0 (enzyme inactive) and pH 8.0 (enzyme active) and in D₂O at pD 3.0. Conditions were as follows: flow system; 100 mW; 350.7-nm excitation; 8-cm⁻¹ spectral slit. OD's of solutions at 350 nm were typically 2; Hg = mercury discharge line from fluorescent room lighting.

(pH 6.5), 0.96 (pH 7.0), 0.90 (pH 7.5), 0.82 (pH 7.85), 0.84 (pH 8.45), and 0.79 (pH 8.55). A detailed examination of the "carbonyl region" between 1650 and 1730 cm⁻¹, in the RR spectrum of 5-MeTA-chymotrypsin, showed that no changes could be detected upon going from pH 3.0 to 8.0. The features in this region are weak, and, while gross changes are not occurring, subtle changes in band profile or shifts of <5 cm⁻¹ would not have been observed. Finally, in the absorption spectrum, the λ_{max} of 5-MeTA-chymotrypsin in the near-ultraviolet changes from 339 nm at pH 3.0 to 335 nm at pH 7.5 and to 332 nm at pH 8.1.

Possible Causes of Spectral Changes Seen on Going to Active pH. Any explanation of the observed changes in the acyl enzyme spectra must take the following points into account. (a) For both the 5-MeTA- and 4-NH₂-3-NO₂-cinnamoylchymotrypsins a change in pH from 3.0 to between 7.5 and 8.5 brings about a small shift to higher cm⁻¹ in $\nu_{\text{C=O}}$, together with a reduction in the integrated intensity of this feature (e.g., Figure 1). (b) The absorption maxima of both acyl enzymes are blue shifted 7–14 nm upon going to active pH. (c) While there is evidence for two conformers of the 5-MeTA acyl group in the active site (MacClement et al., 1981), the 4-NH₂-3-NO₂-cinnamoyl moiety appears to bind in a single conformation. (d) No major change was observed for the RR carbonyl profile of 5-MeTA-chymotrypsin upon going to active pH.

Using 441.6-nm excitation, we had previously reported an intensity change in the 1625-cm⁻¹ RR peak of 4-NH₂-3-NO₂-cinnamoylchymotrypsin upon going from pH 3.0 to the pH range 5.7–7.0. The possible explanations given for this

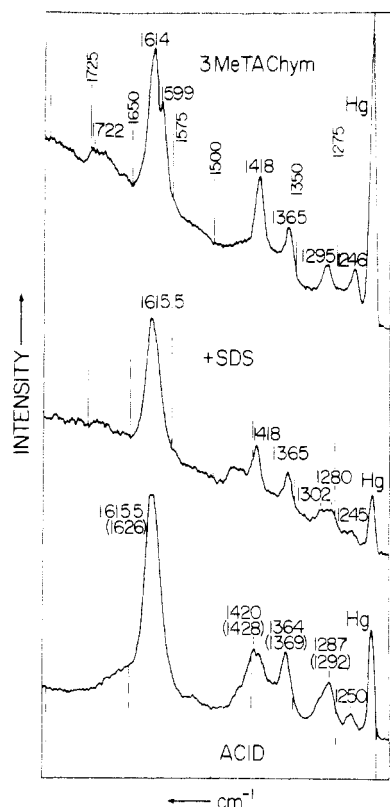


FIGURE 5: RR spectra of native 3-methylthienylacryloylchymotrypsin at pH 3.0 (top), that denatured by NaDodSO₄ (middle), and the acid form of the chromophore (bottom). The bottom spectrum is that of the -COOH form of the acid; the corresponding peak positions for the -COO⁻ species are given in parentheses. Spectral conditions were similar to those given in Figure 4. Hg = mercury line from room lighting.

5-MeFA- and 5-EtFA-chymotrypsin is greater than a/b for FA-chymotrypsin. The same trend is seen for the TA- and 5-MeTA-acyl groups. Several laboratories have reported that the rates of deacylation in a series of acylchymotrypsins reach a maximum for hydrophobic acyl groups of an optimum size (Baggott & Klapper, 1976; Dupaix et al., 1973; Hofstee, 1959; Marshall & Akgün, 1971). The reason for the increase in rate upon substituting with an alkyl group in the 5 position of the ring is not apparent from the RR data. Indeed, given the small rate enhancement factors involved and the complications of heterogeneous acyl group binding (MacClement et al., 1981), it is unlikely that an explanation will be readily forthcoming from the Raman results.

Three acyl groups, 3-CH₃-thienylacryloyl (3-MeTA), α -CH₃-5-CH₃-thienylacryloyl (α -Me-5-MeTA), and α -CH₃-5-CH₃-furylacryloyl (α -Me-5-MeFA), show, on the basis of their deacylation and saponification ratios (a/b , Table I), unexpectedly slow deacylation rates when compared to their analogues lacking the 3-CH₃ or α -CH₃ groups. The RR spectra of these acyl enzymes can now be examined for clues to this behavior.

3-CH₃-thienylacryloylchymotrypsin. The RR spectrum of this intermediate and the spectra of the NaDodSO₄-denatured form and the acid at pH 3.0 are shown in Figure 5. The notable features in the spectrum of the native acyl enzyme are similar to those discussed for several other intermediates in the preceding paper (MacClement et al., 1981), viz., (1) the distinctive carbonyl profile showing two weak peaks near 1720 and 1700 cm⁻¹, (2) the increase in relative intensity of bands in the 1250-cm⁻¹ region, and (3), especially noteworthy, the fact that for the first time the generally broad band attributed

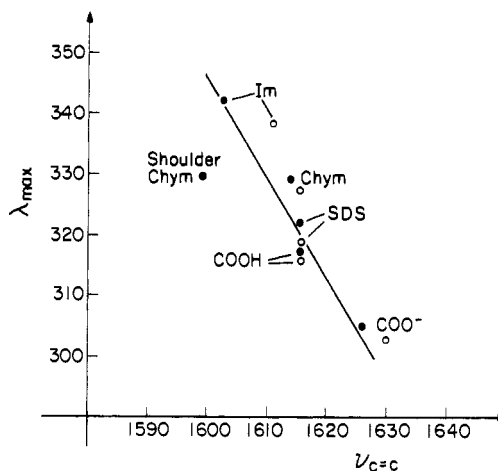


FIGURE 6: Plots of $\langle \lambda_{\max} \rangle$, the experimental absorption maxima, vs. $\nu_{C=C}$, the measured ethylenic double bond stretching frequencies, for the (●) 3-methylthienyl- and for the (○) α -methyl-5-methylthienylacryloyl chromophores.

to $\nu_{C=C}$ can be resolved into two components at 1614 and 1599 cm⁻¹. The two carbonyl bands and the two ethylenic features are further evidence for the proposal made, for other acyl groups, in the preceding paper that the acyl moiety is in two conformations in the active site. The differences in conformation arise from *s*-cis-*s*-trans isomers about one or both the -C=C- single bonds and lead to two environments for the C=O moiety. The splitting in $\nu_{C=C}$ is novel, and it is therefore apparent that the 3-Me group accentuates the differences in $\nu_{C=C}$ between the *s*-cis and *s*-trans isomers. Since the splitting is absent in the NaDodSO₄-denatured form, the imidazole ester and the -COOH and -COO⁻ species of the acid, it is likely that this splitting results from enzyme-substrate interactions slightly perturbing the geometries of one or both of the isomers compared to the geometries found in the free chromophore. The lack of perfect complementarity between the 3-MeTA acyl group and its binding site gives a clue as to the causes of the unexpectedly low deacylation rate. The contacts between the 3-MeTA group and the enzyme may be energetically favorable and mitigate against the conformational change needed for hydrolysis to occur (Henderson, 1970). It is also possible that the 3-Me moiety offers steric hindrance to the enzyme's catalytic apparatus.

A further interesting aspect of 3-MeTA-chymotrypsin is that it does not fit the $\nu_{C=C}$ vs. λ_{\max} correlation discussed in the preceding paper (MacClement et al., 1981). This is because the measured $\nu_{C=C}$'s at 1599 and 1614 cm⁻¹ (Figure 6) are no longer an average value for the isomers ($\nu_{C=C}$). However, the absorption spectrum for the 3-MeTA-chymotrypsin acyl chromophore is a broad peak containing unresolved contributions from both isomers, and thus the measured value of λ_{\max} is $\langle \lambda_{\max} \rangle$.

α -CH₃-5-CH₃-thienylacryloyl- and α -CH₃-5-CH₃-furylacryloylchymotrypsins. The ratio of deacylation to saponification rate constants a/b (Table I) is ~ 10 times lower for these acyl groups compared to the corresponding moieties lacking the α -methyl groups. The high rate of base-catalyzed hydrolysis, b , can be accounted for by steric crowding destabilizing the imidazole substrates. Similar crowding is probably also present in the acyl enzymes since the $\nu_{C=C}$ vs. λ_{\max} correlation is not obeyed. For example, in a plot of $\nu_{C=C}$ vs. λ_{\max} for the α -Me-5-MeTA chromophore neither the "imidazole" nor "native acylchymotrypsin" points (Figure 6) lie on the line defined by the -COOH, -COO⁻, and NaDodSO₄-denatured acyl enzyme points. This situation contrasts sharply with the

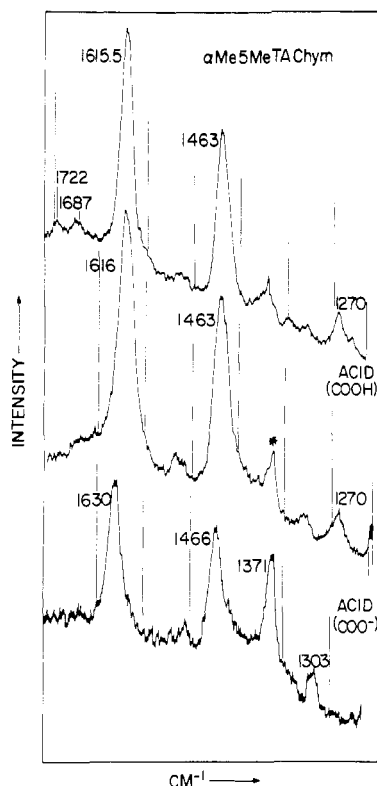


FIGURE 7: RR spectra of α -methyl-5-methylthienylacryloyl-chymotrypsin (native form, pH 3.0) (top) and the $-\text{COOH}$ (middle) and $-\text{COO}^-$ (bottom) states of the acid. Spectral conditions were similar to those given in Figure 4. A 5% CH_3CN and 95% H_2O solution was used to increase solubility of the $-\text{COOH}$ acid; the peak marked * may have some contribution from a CH_3CN mode.

acyl enzymes considered in the preceding paper (see Figure 9 of MacClement et al., 1981). As discussed in the preceding paper, a given acyl chromophore generally obeys the $\nu_{\text{C}=\text{C}}$ vs. λ_{max} correlations, with each chromophore having its own characteristic plot. Thus, the failure of the α -Me-5-MeTA-imidazole and -chymotrypsin to lie on the line indicates that the chromophore is altered in some way in these compounds. This may be caused in these complexes by marked deviations from planarity in the π system within the acryloyl group. Further evidence for distortion about the carbonyl group comes from the carbonyl profiles for both the α -Me-substituted acyl groups bound to chymotrypsin. The carbonyl profile for the furyl- and thienylacryloyl acyl enzymes discussed hitherto is distinctive and consists of two weak features near 1720 and 1700 cm^{-1} . The 1720- cm^{-1} feature is present in both the α -Me-substituted acyl enzymes (e.g., Figure 7), but the second carbonyl feature appears near 1682 cm^{-1} for α -Me-5-MeFA-chymotrypsin (not shown), while in α -Me-5-MeTA-chymotrypsin the 1700- cm^{-1} band is shifted to 1687 cm^{-1} (Figure 7). The steric crowding about the acyl carbonyls might be expected, as in the case of saponification of the imidazole compounds, to lead to rate acceleration for deacylation. However, for both α -Me-substituted compounds the ratios of deacylation are 2–3 times slower compared to those of their unsubstituted analogues (Table I). This implies that the α -Me groups are impeding the enzyme's catalytic apparatus, by directly blocking, e.g., the imidazole of histidine-57, or by locking the $\text{C}=\text{O}$ in an unfavourable conformation.

Both the α -Me-5-MeTA and the α -Me-5-MeFA chromophores show unusual Raman intensity enhancement of a feature near 1375 cm^{-1} in the $-\text{COO}^-$ forms of the acids (Figure 7). This may be due to a mode from the acryloyl moiety coming into resonance in the ionized acid. Since this

could provide a probe for charge buildup about the carbonyl in the acyl enzyme at active pH both, α -Me-substituted acyl enzymes were studied in the range pH 7–8. However, no intensity changes in the 1375- cm^{-1} region were observed at active pH. The changes at active pH were similar to those observed for the acyl enzymes discussed previously, viz., an increase of 4–7 cm^{-1} in $\nu_{\text{C}=\text{C}}$ in the RR spectrum along with a 7–9-nm blue shift in the chromophore's absorption spectra.

Effect of D_2O on Structure of Acyl Enzyme. A major obstacle to the interpretation of isotope effects of enzymic reactions in deuterium oxide arises from the possibility that the solvent change from H_2O to D_2O alters the conformation of the enzyme, by changing the energies of hydrogen bonds, hydrophobic interactions, and other forces. Since such conformational changes in the enzyme might be expected to perturb the acyl group, and therefore the acyl group's RR spectrum, we compared the RR spectra of 5-MeTA-chymotrypsin in H_2O and D_2O . This acyl enzyme was chosen for its good quality spectra. The acyl moiety has no exchangeable hydrogen atoms, and any change in the RR spectrum must be ascribed to a conformational change of the enzyme or to changes in enzyme-substrate interactions mediated by labile H atoms, e.g., in a hydrogen bond between the enzyme and the acyl group. 5-MeTA-chymotrypsin gave good first-order deacylation kinetics in D_2O and H_2O . The pK of deacylation in H_2O was 7.1, and the maximum rate of 1.39 min^{-1} at 20 $^\circ\text{C}$ was calculated according to Bernhard et al. (1966). The pK of deacylation in D_2O was at pD 7.4 with a maximal specific rate of hydrolysis of 0.46 min^{-1} . The reduction in the rate of hydrolysis in D_2O is in accord with that reported for other acyl enzymes (Bender & Hamilton, 1962; Zannis & Kirsch, 1978).

A comparison of several samples of 5-MeTA-chymotrypsin revealed that no differences could be detected between the RR spectra of the acyl enzyme in H_2O and D_2O . RR spectra in the two solvents are shown in Figure 4. In addition to these, approximately 30 separate spectra and partial spectra were obtained at slow scan rates and expanded scales enabling careful comparison of the profile of each RR peak. Again, a perfect match was found for the spectra in D_2O and H_2O . Thus, at the level of perturbations affecting the RR spectrum of the 5-MeTA group, no evidence was found for a conformational change. The possibility must be kept in mind, however, that since the binding of the 5-MeTA group is heterogeneous and probably nonspecific, imperfect steric complementarity between substrate and enzyme may permit small changes in enzyme conformation without affecting the conformation of the acyl group. A small shift might be expected for the 1700- cm^{-1} feature, assigned in the preceding paper to the acyl group's carbonyl in a hydrogen-bonded environment. However, the features in this region are too weak to be able to observe subtle changes in band profile. The samples in D_2O were made up from freeze-dried preparations of acyl enzyme (Experimental Procedures) and were left in D_2O for 2–5 h prior to recording RR spectra. It is also possible, therefore, that not all of the hydrogen atoms were exchanged in that time (Woodward & Hilton, 1979).

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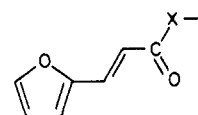
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Resonance Raman and Electronic Absorption Spectral Studies of Some β -(2-Furyl)acryloylglyceraldehyde-3-phosphate Dehydrogenases[†]

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ABSTRACT: Electronic absorption and resonance Raman spectra, together with rates of arsenolysis, are used to show that the acylated subunits of the rabbit furylacryloylglyceraldehyde-3-phosphate dehydrogenase (FA-rabbit enzyme), but not the FA-sturgeon enzyme, exist as a mixed population of at least two forms. One form of the FA-rabbit enzyme has a high rate of arsenolysis, and its near-UV absorption maximum (λ_{\max}) is red shifted on binding NAD^+ , while the other has a lower rate of arsenolysis, and its absorption spectrum appears to be unaffected by excess NAD^+ . The FA-sturgeon enzyme exists as a single population that has a high rate of arsenolysis, and its λ_{\max} is red shifted on binding NAD^+ . This red shift and the concomitant position of $\nu_{\text{C}=\text{C}}$, the band due to the ethylenic double bond stretching vibration in the resonance Raman spectrum, indicate that in the presence of NAD^+ the π electrons of the FA chromophore are polarized, i.e., a

permanent dipole has been set up along the long axis of the FA group. It is argued that this does not result in activation of the carbonyl group by setting up polarization (i.e., $\delta^+\text{C}-\text{O}^\delta$) within that group alone. A comparison of the $\nu_{\text{C}=\text{C}}$'s and λ_{\max} 's for a series of FA derivatives of the type



where X = H, N, or O, shows a clear correlation. However, when X = S as, e.g., in thiol esters, this correlation breaks down. This is explained in terms of a possible through-space $d\pi$ - $p\pi$ overlap between the ethylenic bond π orbitals and the empty sulfur 3d orbitals.

D-Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; GDH)¹ catalyzes the NAD^+ -dependent reversible oxidative phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate, via the formation of a covalent acyl enzyme intermediate in which the acyl group is linked to the sulfur of an active-site cysteine (Harris & Waters, 1976). The enzyme, composed of four chemically identical subunits, exhibits cooperativity in the equilibrium binding of NAD^+ and "half-of-the-sites reactivity" toward some active-site-specific acylating and alkylating agents (Seydoux et al., 1974b). To investigate the elementary steps of the enzyme reaction, Malhotra & Bernhard (1968, 1973) used the substrate analogue β -(2-furyl)acryloyl (FA) phosphate and obtained the chromophoric acyl enzyme furylacryloylglyceraldehyde-3-phosphate dehydrogenase with catalytic properties similar to those of the physiological intermediate. The absorption spectrum of the FA group was used to monitor the catalytic

steps in the enzyme reaction (Malhotra & Bernhard, 1968, 1973; Berni et al., 1977; Vars et al., 1979).

The FA group is also a suitable resonance Raman (RR) probe of the enzyme reaction allowing the vibrational spectrum of the substrate to be recorded during enzymolysis (Carey, 1978). In this paper we report kinetic data, together with absorption and resonance Raman spectroscopic studies, on the FA enzymes obtained by using GDH from rabbit and sturgeon muscle. The interpretation of the resonance Raman and some of the absorption data uses, as a framework, the ideas developed in the two preceding papers (MacClement et al., 1981; Phelps et al., 1981). The first area considered in the present work concerns the heterogeneity of furylacryloyl binding in

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¹ Abbreviations used: FA, β -(2-furyl)acryloyl; FA-P, FA-phosphate; FA-S, thiol ester or thiol acyl enzyme of FA; FA-rabbit enzyme and FA-sturgeon enzyme, respectively, rabbit and sturgeon muscle FA-glyceraldehyde-3-phosphate dehydrogenases; GDH, glyceraldehyde-3-phosphate dehydrogenase; FA-GDH, FA-glyceraldehyde-3-phosphate dehydrogenase; RR, resonance Raman; EDTA, ethylenediaminetetraacetic acid; NaDodSO₄, sodium dodecyl sulfate.