Biochemistry 1981, 20, 3438–3447

Koenig, S. H. (1978) *J. Magn. Reson. 31*, 1–10.

Koenig, S. H., & Brown, R. D., III (1980) in *ESR and NMR of Paramagnetic Species in Biological and Related Systems* (Bertini, I., & Drago, R., Eds.) pp 89–115, D. Reidel, Boston, MA.

Koenig, S. H., Brown, R. D., III, & Brewer, C. F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 475–479.

Koenig, S. H., Brewer, C. F., & Brown, R. D., III (1978) *Biochemistry 17*, 4251–4260.

Maret, W., Andersson, I., Dietrich, H., Schneider-Bernlöhr, H., Einarsson, R., & Zeppezauer, M. (1979) *Eur. J. Biochem. 98*, 501–512.

Evidence for Two Acyl Group Conformations in Some Furylacryloyl- and Thienylacryloylchymotrypsins: Resonance Raman Studies of Enzyme–Substrate Intermediates at pH 3.0[†]

B. A. E. MacClement, R. G. Carriere, D. J. Phelps, and P. R. Carey[*]

ABSTRACT: Resonance Raman (RR) spectra, obtained by ultraviolet laser excitation, are reported for 10 acylchymotrypsins at pH 3.0, in which the acyl groups are derivatives of furylacrylic and thienylacrylic acids. Spectra are also shown of the sodium dodecyl sulfate ($NaDodSO_4$) denatured acyl enzymes and the acid and ester analogues of the acyl groups. For most of the native acyl enzymes, the RR spectral profiles in the carbonyl stretching region suggest that the acyl groups bound to Ser-195 adopt two conformations, which are characterized by having either strong hydrogen bonds to the carbonyl oxygen or a nonbonding hydrophobic environment about the C=O group. It is also likely that in solution the ester and acid analogues of the acyl group adopt more than one conformation about the acryloyl linkages. Thus, the measured spectral parameters, such as the ethylenic double bond stretching frequency $\nu_{C=C}$ in the RR spectra, should be considered as a weighted mean, $\langle\nu_{C=C}\rangle$. For a series of compounds based on a given acyl group a correlation exists between $\langle\nu_{C=C}\rangle$ and the measured absorption maximum $\langle\lambda_{max}\rangle$. Possible explanations are given for the observed changes in $\langle\nu_{C=C}\rangle$ and $\langle\lambda_{max}\rangle$ when the acyl groups bind to the active site. A band appears near 1260 cm^{-1} in the RR spectra of some of the native acyl enzymes; it is not observed in the spectra of the $NaDodSO_4$-treated intermediates or in the spectra of any model compounds.

The study of organic reaction mechanisms is greatly aided by noting the change in reactivity with changing structure of the reagents. This approach has given considerable information on, for example, the electronic structure of transition states and the features that determine reactivity. However, similar approaches to the understanding of enzyme mechanism are much more limited (Jencks, 1969; Fersht, 1977). An important factor restricting their use is that structure–reactivity studies with enzymes tend to measure the effect of changes in the structure of the substrate on its interactions with the enzyme, rather than the effects on the electronic changes in the transition state. In this regard, it may be valuable to have a probe, at the level of atoms and chemical bonds, to independently characterize enzyme–substrate interactions during enzymolysis. The work presented here attempts to explore the possibility of using resonance Raman (RR) spectroscopy to delineate enzyme–substrate interactions within a series of related substrates and thereby isolate the effect of these interactions on kinetic parameters.

The resonance Raman spectrum of a molecule is obtained by using an excitation wavelength for the Raman effect which lies within an electronic absorption band of the molecule. Considerable intensity enhancement of the Raman spectrum is thereby obtained enabling vibrational spectra to be recorded from chromophores in the 10^{-4}–10^{-5} M range. By use of a chromophoric substrate whose absorption lies to the red of normal protein absorption bands, selective enhancement of the substrate vibrational modes, from an enzyme–substrate complex, can be achieved. As a result of the availability of laser wavelengths, the first RR studies on enzyme–substrate intermediates used chromophoric substrates, based on substituted cinnamic acid esters, having an absorption band in the 450-nm region (Carey & Schneider, 1974, 1976). Recent technological advances have made laser lines available in the 325–360-nm spectral range enabling a wider range of substrates, including specific enzyme–substrate intermediates (Storer et al., 1979), to be studied. The 10 substrates considered here are substituted derivatives of furylacrylic and thienylacrylic acids (Figure 1) absorbing in the near-ultraviolet and were chosen and synthesized on the basis of their differing electronic and steric properties. Chymotrypsin is used in this work as a "model" enzyme and was selected on the basis of its availability and known structural and mechanistic properties (Blow, 1976). One of the intermediates studied herein, namely, furylacryloylchymotrypsin has been the object of pioneering studies by absorption spectroscopy (Bernhard & Lau, 1972). Since the reaction of chymotrypsin with acyl imidazoles proceeds via the formation of an acyl enzyme intermediate the rate of breakdown of which is rate determining, it is possible, by manipulating pH and subsequent separation techniques, to obtain a pure stable acyl enzyme at pH 3.0, free from substrate or product. In each acyl enzyme the substrate's acyl group is covalently linked to the side chain of serine-195 in chymotrypsin's active site. This paper is concerned with interpreting the RR spectra of the stable acyl enzymes at pH 3.0 in terms of enzyme–substrate interactions. The following paper (Phelps et al., 1981) deals with those effects most related to kinetic aspects: the effects on the RR spectra of going to active

---

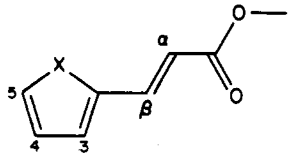
[†] From the Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A 0R6. N.R.C.C. No. 19393. *Received October 27, 1980.*

0006-2960/81/0420-3438$01.25/0

Table I: Physical Characteristics of Synthetic Materials

compound	% yield	mp (°C)	lit. mp (°C)	calcd (%)			found (%)		
				C	H	N	C	H	N
5-MeFA acid	81	165–157	152[a]	63.15	5.30		62.91	5.43	
5-EtFA acid	88	130–132		65.04	6.06		64.89	6.01	
α-Me-5-MeFA acid	74	130–131		65.04	6.06		65.11	6.20	
5-MeTA acid	81	168–169	165–166[b]						
3-MeTA acid	61	168–171	172–173[b]						
α-Me-5-MeTA acid	76	175 (dec)		59.31	5.53		59.18	5.66	
α-Me-3-MeTA acid	13	155 (dec)		59.31	5.53		59.59	5.63	
FAIm[e]	66	110–112	113–114[c]	64.41	4.34	14.89	63.87	4.47	14.99
5-NO_2FAIm	51	130 (dec)		51.55	3.03	18.02	51.14	3.07	17.84
5-MeFAIm	77	85 (dec)		65.33	4.98	13.86	64.09	5.01	14.32
5-EtFAIm	56	72–75		66.65	5.59	12.96	66.49	5.62	13.05
α-Me-5-MeFAIm	78	69–72		66.65	5.59	12.96	66.46	5.71	13.09
TAIm	42	134–135		58.80	3.90	13.72	58.69	3.92	13.58
5-NO_2TAIm	63	203–207 (dec)		48.19	2.83	16.86	48.16	2.71	17.08
5-MeTAIm	91	127		60.52	4.57	12.84	60.79	4.58	13.06
3-MeTAIm	73	128–130		60.52	4.57	12.84	60.68	4.69	12.82
α-Me-5-MeTAIm	73	93–95		62.47	5.21	12.06	61.59	5.38	12.33
5-NO_2FAOMe	100	147–149	148–149[d]						
5-MeFAOMe	90	36	37[a]						
5-EtFAOMe	90	24		66.65	6.71		66.12	6.68	
5-MeTAOMe	44	52–53		59.86	5.53		59.39	5.63	

[a] Janda & Radouch (1967). [b] King & Nord (1949). [c] Bernhard et al. (1965). [d] Kai & Ogawa (1963). [e] Im = imidazole.



SYMBOL	NAME	X	3	4	5	α	β
FA	furylacryloyl	O	H	H	H	H	H
5MeFA	5-methylfurylacryloyl	O	H	H	CH_3	H	H
5EtFA	5-ethylfurylacryloyl	O	H	H	C_2H_5	H	H
5NO_2FA	5-nitrofurylacryloyl	O	H	H	NO_2	H	H
αMe5MeFA	α-methyl-5-methylfurylacryloyl	O	H	H	CH_3	CH_3	H
TA	thienylacryloyl	S	H	H	H	H	H
5MeTA	5-methylthienylacryloyl	S	H	H	CH_3	H	H
3MeTA	3-methylthienylacryloyl	S	CH_3	H	H	H	H
5NO_2TA	5-nitrothienylacryloyl	S	H	H	NO_2	H	H
αMe5MeTA	α-methyl-5-methylthienylacryloyl	S	H	H	CH_3	CH_3	H

FIGURE 1: Acyl groups used in this work. The favored conformation in solution about the ring to ethylenic single bond is, as shown, *s*-trans. The favored conformation about the ethylenic to carbonyl C–C bond is not known.

pH and the spectra of those acyl enzymes possessing anomalous deacylation rates.

Experimental Procedures

Synthesis. Thienylacrylic (TA) acid was purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Furylacrylic (FA) acid (Aldrich) was crystallized from ethanol and water after decolorizing with activated charcoal. All of the aldehydes and the malonic acids employed in the acrylic acid syntheses were from Aldrich and were used without further purification.

The acids were synthesized in good to excellent yields (see Table I) by the method of Koo et al. (1963). The low yield of α-methyl-3-methylthienylacrylic acid is attributed to steric hindrance. The imidazole derivatives were synthesized in good yields by using carbonyldiimidazole (Aldrich). The esters were obtained by Fischer esterification. A typical example of each of the three synthetic reactions is described. Synthesis of the 5-NO_2FA and 5-NO_2TA acids was carried out according to literature methods (Gilman & Wright, 1930). Physical data on the compounds are listed in Table I.

5-Ethylfurylacrylic Acid. 5-Ethylfuraldehyde (2.48 g, 20 mmol), malonic acid (4.16 g, 40 mmol), and pyridine (8 mL) were placed in a 100-mL round-bottomed flask, and all solid was dissolved by heating and shaking. After the addition of piperidine (0.3 mL), the reaction mixture was heated at 80 °C for 0.5 h and then heated at reflux for 3 h. The reaction mixture was cooled and poured into 500 mL of water containing 100 mL of 1 N HCl. The resultant tan-colored solid [3.12 g (94%); mp 129–130 °C] was crystallized from ethanol and water to give 2.93 g (94% recovery; 88% yield; mp 130–132 °C) of analytically pure material.

5-Ethylfurylacryloylimidazole. 5-Ethylfurylacrylic acid (0.16 g, 1 mmol) and carbonyldiimidazole (0.16 g, 1 mmol) were dissolved with swirling in tetrahydrofuran (5 mL). After 22 h at room temperature, the solvent was removed via rotary evaporation at <40 °C. The resultant oil was treated with 25 mL of cold water. Refrigeration led to a yellowish solid [0.12 g (56%); mp 72–75 °C]. This proved to be analytically pure.

Methyl 5-Ethylfurylacrylate. 5-Ethylfurylacrylic acid (0.42 g, 25 mmol) was heated to reflux in methanol (100 mL) and sulfuric acid (10 drops). After 5.5 h, thin-layer chromatography (TLC) (silica gel; $CHCl_3$) showed no acrylic acid remained. The reaction mixture was cooled to room temperature and treated with 5% sodium bicarbonate solution to neutralize the sulfuric acid. The solvent was removed via rotary evaporation, and the oily product was refrigerated. The resultant solid was collected and washed with 5% bicarbonate and water. This gave 0.40 g (90%) of analytically pure solid with mp 24 °C.

Preparation of Acyl Enzymes. The flow system used for Raman measurements required relatively large volumes of material, and thus acyl enzymes were prepared in 25-mL batches. The procedure used was the same for all substrates. The imidazole substrate was dissolved in dimethylformamide to give a concentration of 7–10 mg/mL (~5 × 10^{-2} M). The pH of a solution containing 10 mg/mL (~4 × 10^{-4} M) of chymotrypsin (3× crystallized, Sigma Chemical Co.) was adjusted to 7.1 and sufficient fresh substrate stock solution added to give a 4-fold molar excess of substrate over enzyme. After the solution was stirred for 25–30 s, the pH was rapidly reduced to 2.9 by the addition of 1 N HCl. Substrate, product, and dimethylformamide were removed by dialyzing the sample twice against 4 L of 10^{-3} M HCl at 6 °C for 12 h or by passing it down a Sephadex G-15 column. Approximately 80% of the enzyme molecules were acylated, and the OD at 350 nm of the solutions used to record to record RR specftra was in the range 2–4. The above solutions of acyl enzymes were added dropwise to a third of their volume of $NaDodSO_4$ (24 mg/mL, pH 2.9), stirring continuously to obtain acyl enzymes denatured by sodium dodecyl sulfate ($NaDodSO_4$).

The pH of a sample of each acyl enzyme was raised to 7.5 for a period of at least 6 times the measured half-life to test for the possibility that some acyl groups had become irreversibly bound, e.g., by linking to amino acids other than Ser-195. The pH was then reduced to 3, and the samples were redialyzed or passed through a Sephadex G-15 solumn. By measurement of the ratio of absorption at 280 and 350 nm before and after this treatment, an estimate of the percentage of irreversible binding could be made. For most intermediates, other than NO_2FA- and NO_2TA-chymotrypsin, 10–15% of acyl groups were found to be irreversibly bound according to this criterion. This level of "impurity" is essentially undetectable in the RR spectra of the acyl intermediates. The NO_2FA and NO_2TA compounds, however, had up to 30% of irreversible binding, and this must be taken into account in spectral interpretation. The only substrates, apart from the 5-NO_2 substituted compounds, showing a higher degree of irreversible binding were those with α-methyl substituents. The latter gave rise to 15–20% irreversible binding.

Raman Measurements. The resonance Raman spectra were obtained by using a Spex 0.5m double monochromator equipped with an Model EM1 9789 QB photomultiplier connected via an RC time constant to a picoammeter (Gencom Model 1012). Excitation was provided by the 350.7-nm line of a Coherent Radiation 3000K krypton laser. The laser beam passed vertically in front of the spectrometer slit with the plane of polarization parallel to the front of the spectrometer. A Pellin-Broca prism and two diaphragms were used to separate the selected line from the two other main ultraviolet lines (337.5 and 356.4 nm) and other plasma radiation. Typical conditions were as follows: 100 mW of laser power at the sample; picoammeter 1 μA full scale; time constant of 1 s; a spectral slit width of 8 cm^{-1}; a scan speed of 1.5 cm^{-1}/s. Peak positions were carefully calibrated with Ar, Xe, Kr, Ne, or Hg discharge lines; they were reproducible to ± 1 cm^{-1} and are thought to be accurate to ± 2 cm^{-1}.

Possible photochemical effects of the laser beam on the acyl chromophores were removed by continuously flowing fresh sample through the focused beam. The flow cell consisted of a horizontal thin-walled quartz capillary cell, ~1.5-mm internal diameter, through which the solution was driven by means of a syringe pump (Sage Instruments, Model 355). The nitro-containing chromophores were particularly photolabile, and flow speeds of ~20 cm/s, giving a residence time of $\sim 10^{-3}$ s for each molecule in the vertical beam, had to be employed. For the other substrates slower flow rates were used. The most frequently encountered photochemical effect was the loss of Raman signal due to photodecomposition. Occasionally Raman lines were broadened at slow flow rates; the broadening was removed by increasing flow rate. In rare instances, extra, well-defined peaks appeared which, in all likelihood, were due to photoisomerization; these are discussed below.

Results and Discussion

Assignment of the Most Intense Resonance Raman Bands. The extensive analyses of the vibrational modes of furan and thiophene, existing in the literature (Reitz 1938; Thompson & Temple 1945; Katritzky & Lagowski, 1959; Rico et al., 1965, 1967), permit the assignment of the main features associated with these rings in the resonance Raman spectra of furylacryloyl (FA) and thienylacryloyl (TA) compounds. Further confirmation is made possible by the fact that the ring modes are relatively insensitive to changes in the acryloyl moiety, e.g., –COOH going to $-COO^-$ or –C(=O)–imidazole. Furan has an intense Raman mode at 1486 cm^{-1} associated with a totally symmetric vibration of the ring atoms (Thompson & Temple, 1945; Katritzky & Lagowski, 1959). In all likelihood, this is the mode giving rise to the intense RR band near 1475 cm^{-1} in FA and 5-NO_2FA compounds (Figures 2 and 3). The addition of a methyl or ethyl group in the 5 position appears to shift this intense mode to 1520 cm^{-1} and brings about an intensity increase in a band near 1575 cm^{-1} (Figures 4 and 5). The latter is of weak intensity in preresonance Raman spectra of FA derivatives obtained by using, e.g., 406-nm excitation (data not shown). The RR band at 1389 cm^{-1} in FA and 5-NO_2FA compounds (Figures 2 and 3) may also be assigned to a furan ring mode (Thompson & Temple, 1945; Katritzky & Lagowski, 1959; Rico et al., 1967). The band near 1350 cm^{-1} in the RR spectra of 5-NO_2 analogues (Figure 3) is associated with the symmetric stretching frequency of the NO_2 group (Carey et al., 1973).

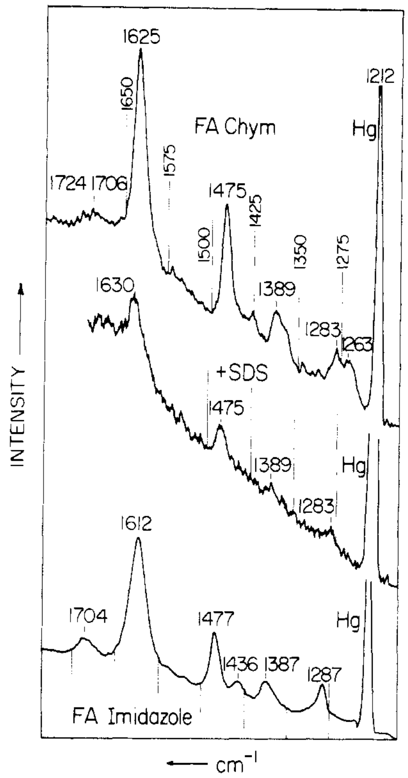


FIGURE 2: Resonance Raman spectra of furylacryloylchymotrypsin (FAchym) in the native form at pH 3.0 (top) and denatured by $NaDodSO_4$ (middle); resonance Raman spectrum of FA-imidazole in aqueous solution, pH ~7.0 (bottom).

In the RR spectra of TA (Figure 6) and possibly 3-MeTA compounds, the intense feature near 1420 cm^{-1} can be identified with the totally symmetric ring mode found, in thiophene, at 1404 cm^{-1} (Thompson & Temple, 1945; Rico et al., 1965). As for the FA analogues, substitution of a methyl group in the 5 position appears to shift the intense ring mode to higher cm^{-1}, giving rise to the intense feature near 1465 cm^{-1} in 5-MeTA (Figure 7) and α-Me-5-MeTA compounds. In the α-Me-5-MeFA and -TA molecules the band near 1380 cm^{-1}, showing remarkable intensity enhancement in the $-COO^-$

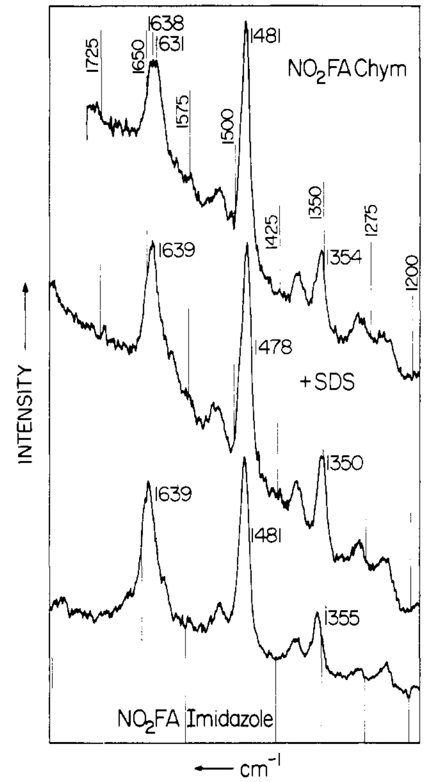


FIGURE 3: Resonance Raman spectra of 5-nitrofurylacryloylchymotrypsin (5-NO_2FAchym) in the native form at pH 3.0 (top) and denatured by $NaDodSO_4$ (middle); spectrum of 5-NO_2FA-imidazole in aqueous solution, pH ~7.0 (bottom).

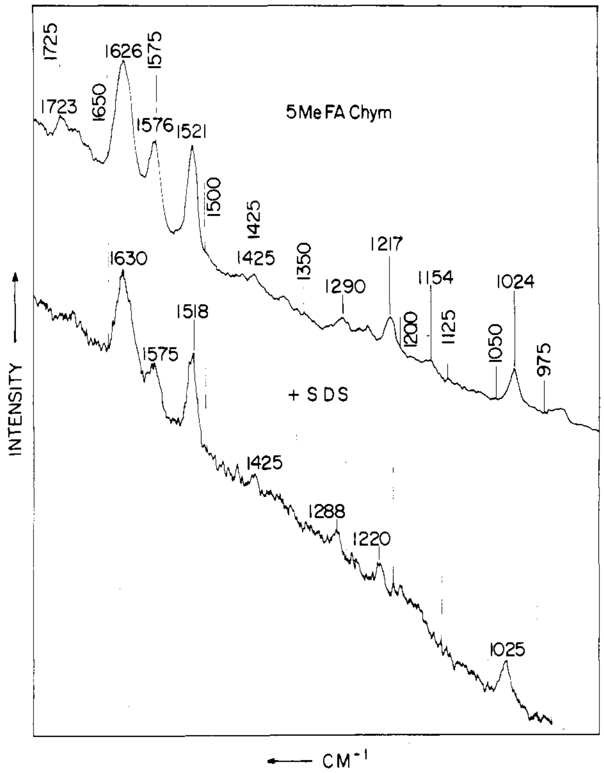


FIGURE 4: Resonance Raman spectra of 5-methylfurylacryloylchymotrypsin (5-MeFAchym) in the native form at pH 3.0 (top) and denatured by $NaDodSO_4$ (bottom).

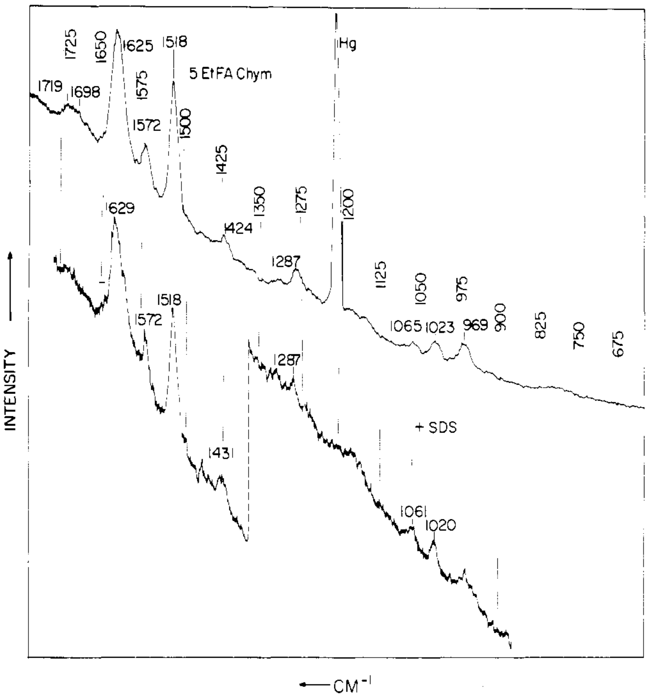


FIGURE 5: Resonance Raman spectra of 5-ethylfurylacryloylchymotrypsin (5-EtFAchym) in the native form at pH 3.0 (top) and denatured by $NaDodSO_4$ (bottom).

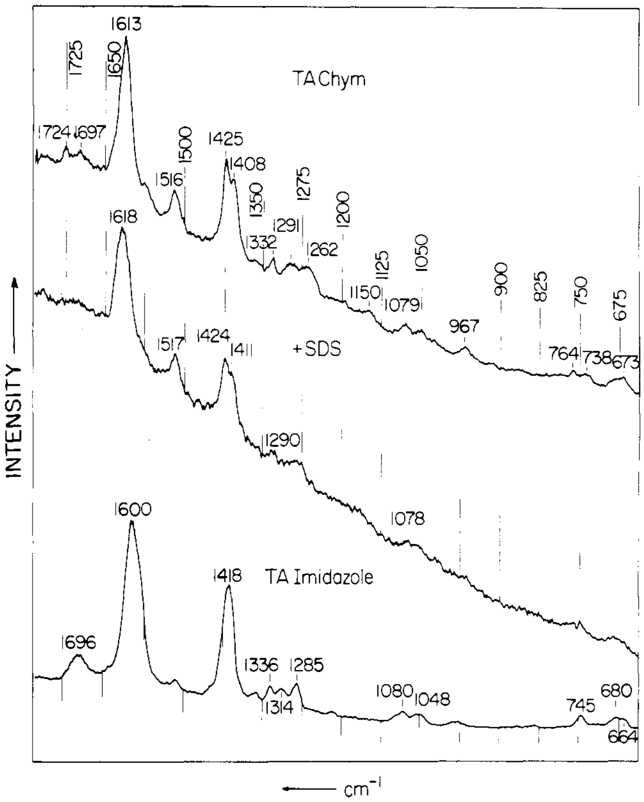


FIGURE 6: Resonance Raman spectra of thienylacryloylchymotrypsin (TAchym) in the native form at pH 3.0 (top) and denatured by $NaDodSO_4$ (middle). The bottom spectrum is that of thienylacryloylimidazole in water at pH ~7.0.

species (Phelps et al., 1981), probably has a substantial contribution from the acryloyl portion of the molecules.

Key Assignments Common to FA and TA Compounds. Since catalytic modification occurs to bonds within the acryloyl moiety, an understanding of the vibrational spectrum associated with this part of the molecule is important. Though the two fundamental vibrational bands of α,β-unsaturated esters and acids are probably coupled to a certain extent, it is customary to identify the higher wavenumber feature with the localized C=O stretching mode and the band at lower wavenumbers with the C=C stretching mode. This approach will be retained here. Thus, the intense RR feature occurring

Table II: Absorption Maxima $\langle\lambda_{max}\rangle$ and Position of the Ethylenic Mode $\langle\nu_{C=C}\rangle$ of Furylacryloyl and Thienylacryloyl Derivatives

	α-chym,[a] pH 3.0	α-chym + $NaDodSO_4$	-COOH	$-COO^-$	imidazole	methyl ester in CCl_4	methyl ester in H_2O
FA	324; 1625[b]	311; 1630	304; 1637	292; 1643	339; 1612		
TA	325; 1613	316; 1618	311; 1620	300; 1633	343; 1600		
5-EtFA	341; 1625	325; 1629	321; 1632	306; 1641	360; 1612	314; 1637	320; 1631
5-MeFA	337; 1626	323; 1630	319; 1631	305; 1642.5	360; 1613	314; 1637	321; 1632
5-MeTA	339; 1612.5	320; 1615	324; 1617	311; 1626	359; 1599		
3-MeTA	329; 1614[c]	322; 1615.5	318; 1615.5	306; 1626	343; 1603		
$5-NO_2FA$	352; 1638, 1631	356; 1639	355; 1639	365; 1641	360; 1639		
$5-NO_2TA$	368; *d*	360; *d*	363; 1632	372	364; 1615		
α-Me-5-MeFA	329; 1626	318.5; 1626	316; 1630.5	301; 1647	341; 1624		
α-Me-5-MeTA	327.5; 1615.5	319; 1616	316; 1616	303; 1630	338; 1611		

[a] α-chym = α-chymotrypsin. [b] The first value represents $\langle\lambda_{max}\rangle$ (in nanometers); the second value represents $\langle\nu_{C=C}\rangle$ (in cm^{-1}). [c] Shoulder at 1599 cm^{-1}. [d] Poorly resolved $\nu_{C=C}$ due to nonspecific binding.

in all the spectra between 1600 and 1640 cm^{-1} (Figures 2–7) is assigned to a double bond stretching mode having a substantial contribution from the ethylenic stretching vibration $\nu_{C=C}$. Because of its high intensity and sensitivity to conformational and electronic effects, $\nu_{C=C}$ will play an important part in the following discussion. The second assignment, common to all FA and TA compounds, is that of the carbonyl stretching mode $\nu_{C=O}$ to a feature near 1700 cm^{-1}. This feature is intense in the IR (data not shown) and weak in Raman and RR spectra (Figures 2 and 4–7).

The third common assignment involves the band which appears near 1285 cm^{-1} in the RR spectra of all FA and TA compounds. No Raman peaks appear between 1140 and 1360 cm^{-1} in the 647.1-nm excited Raman spectra of thiophene or furan (data not shown), and the 1285-cm^{-1} band from FA and TA molecules can thus be tentatively associated with a mode originating from the acryloyl group. However, in this spectral region it is difficult to assign group frequencies. Since the 1285-cm^{-1} band appears in the RR spectra of the imidazole esters of the acyl groups, as well as in the acids, esters, and acyl enzymes, it is unlikely that this band involves the C–O bond in the —C—(C=O)—O— group. It may have a substantial contribution from one or both of the ethylenic C–C single bond stretching motions, with CH in plane deformations of the vinyl protons being possible contributors.

Changes in Resonance Raman Spectra of Acyl Groups upon Binding to the Active Site. (*a*) *FA- and TA-chymotrypsins.* Figures 2 and 6 compare the resonance Raman spectra, taken by using 350.7-nm excitation, of FA- and TA-chymotrypsins in the native forms at pH 3.0, with the spectra of the denatured forms after treatment with $NaDodSO_4$ and the spectra of FA- and TA-imidazole esters. The intact active site of chymotrypsin brings about the following perturbations to the RR spectra. Firstly, the ethylenic double bond stretching frequency decreases by 5–10 cm^{-1}, e.g., $\nu_{C=C}$ in FA-chymotrypsin is 1625 cm^{-1} compared to 1630 cm^{-1} for the $NaDodSO_4$-treated acyl enzyme and 1636.5 cm^{-1} for the FAOMe ester in H_2O. The 1625-cm^{-1} peak in native FA-chymotrypsin (Figure 2) probably corresponds to the band reported at 1633 cm^{-1} by Schmidt et al. (1978) using 488.0-nm, i.e., preresonance, excitation. Secondly, in the RR spectra of both FA- and TA-chymotrypsin a new feature appears near 1260 cm^{-1}. This is not observed in the spectra of the $NaDodSO_4$-treated forms or in the acids or methyl or imidazole esters. Thirdly, in the RR spectrum of TA-chymotrypsin (Figure 6) a weak feature, consisting of a peak at 1724 cm^{-1} with a broader shoulder at 1698 cm^{-1}, appears in the carbonyl region. The same profile is seen in the RR spectra of FA-chymotrypsin in the 1700-cm^{-1} region. However, it is not well resolved in the spectrum shown in Figure 2. Again, like the 1260-cm^{-1} feature the "carbonyl profile", appears to be characteristic of the native acyl enzyme; it disappears on $NaDodSO_4$ treatment and is not seen in the RR spectra of the acids or the methyl or imidazole esters. The slight shifts (~2 cm^{-1}) in the peaks assigned to the ring modes of the furan and thiophene moieties in Figures 2 and 6 are reproducible and may result from a minor redistribution of electrons in the π electron system upon binding to the enzyme.

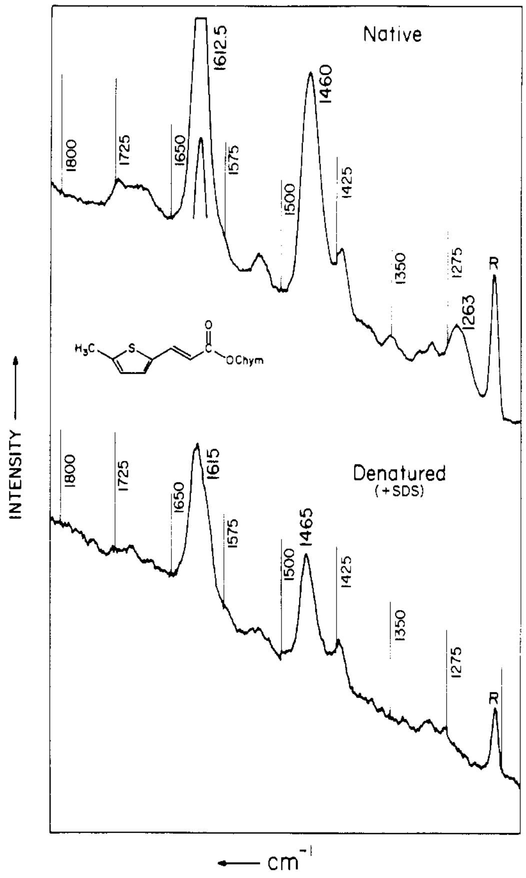


FIGURE 7: Resonance Raman spectra of 5-methylthienylacryloyl-chymotrypsin (5-MeTAchym) in the native form at pH 3.0 (top) and denatured by $NaDodSO_4$ (bottom).

(*b*) *5-MeFA-*, *5-EtFA-*, *and 5-MeTA-chymotrypsins.* Figures 4 and 5 compare the RR spectra, taken with 350.7-nm excitation, of 5-MeFA-chymotrypsin at pH 3.0 and its $NaDodSO_4$-treated form and 5-EtFA-chymotrypsin at pH 3.0 and its $NaDodSO_4$-treated form, respectively. Figure 8 shows the 350.7 nm excited RR spectra of the methyl esters of these two acyl groups in CCl_4 and in aqueous solution (98% H_2O and 2% CD_3CN v/v). Compared to the $-COO^-$ or -COOH forms of the acids (Table II) or the methyl esters in CCl_4 or water or the $NaDodSO_4$-treated acyl enzymes, the ethylenic

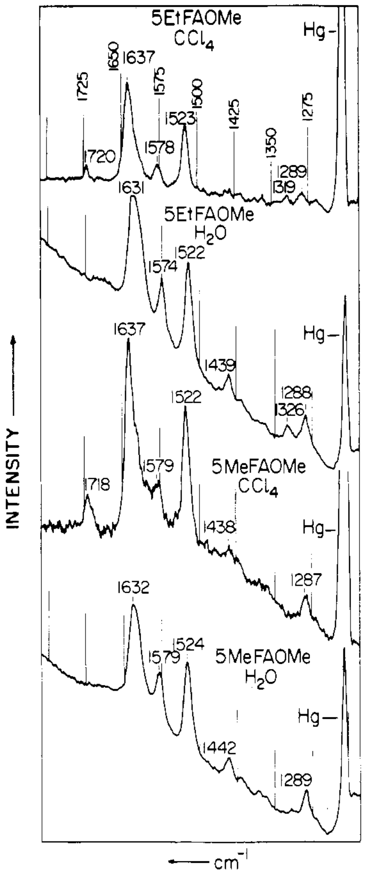


FIGURE 8: Resonance Raman spectra of the methyl esters of 5-methyl- and 5-ethylfurylacrylic (5-MeFA and 5-EtFA) acids in CCl$_4$ and H$_2$O (containing 2% CD$_3$CN v/v).

double bond stretching frequency, $\nu_{C=C}$, in the native acyl enzymes decreases by 5–15 cm^{-1}. The RR spectra of the native acyl enzymes also show a weak carbonyl feature consisting of a peak near 1720 cm^{-1} with a broader shoulder at 1700 cm^{-1}. Interestingly, a carbonyl band appears at 1720 cm^{-1} in the RR spectra of the esters in CCl$_4$ (Figure 8). There is no evidence for a new band near 1260 cm^{-1} in the RR spectra of 5-MeFA- or 5-EtFA-chymotrypsin.

The RR spectrum of native 5-MeTA-chymotrypsin (Figure 7) is typical of the acyl groups considered in this work: there is a small decrease in $\nu_{C=C}$ in the native acyl enzyme compared to the other derivatives of the acyl group, the 1725-cm^{-1} C=O band, with the broader shoulder at 1700 cm^{-1}, is evident, and the "new feature" appears at 1263 cm^{-1}. The bandwidth at half-height of $\nu_{C=C}$ is smaller in the native acyl enzyme (23 cm^{-1}) compared to the NaDodSO$_4$-treated form (30 cm^{-1}). This results from a fortuitous coincidence of the $\nu_{C=C}$'s from the two conformers in the active site (see below).

Since chymotrypsin forms dimers and higher aggregates as its concentration is raised (Pandit & Rao, 1975), a sample of 5-MeTA-chymotrypsin was used to search for any possible effects of concentration on the RR spectrum of the acyl group. A solution of 5-MeTA-chymotrypsin was made ~3 × 10^{-4} M in enzyme and had an OD of 5.8 at 340 nm; the RR spectrum of this solution was compared to those obtained by diluting by 2, 4, 8, 16, 32, 64, 128, and 256 times with aqueous HCl, pH 3.0. The spectrum of each diluted sample was identical with that of the original solution showing that concentration effects were not detected.

5-NO$_2$FA- and 5-NO$_2$TA-chymotrypsins. The major difficulty with these acyl enzymes concerns nonspecific acylation. Under the terms defined under Experimental Procedures up to 30% of the NO$_2$FA-acyl groups and 40% of the NO$_2$TA-groups are irreversibly bound. The electron-withdrawing character of the nitro group apparently makes the NO$_2$FA- and NO$_2$TA-imidazoles such good acylating agents that a substantial percentage of acylation occurs in a nonspecific manner, probably to a group on the enzyme other than serine-195. Spectroscopic interpretation thus becomes difficult since a heterogeneous population of acyl groups has to be taken into account. Only poor quality RR spectra of 5-NO$_2$TA-chymotrypsin were obtained, and, given the high percentage of nonspecific binding, this species will not be discussed further.

The photolability of the 5-NO$_2$FA moiety meant that flow rates of 20 cm/s through the focused laser beam had to be used to obviate problems of photochemical degradation. The resulting RR spectra are shown in Figure 3. The broad band assigned to $\nu_{C=C}$ was reproduced in each of ~10 preparations. The shape of the band suggests an unresolved doublet whose maxima occur near 1631 and 1638 cm^{-1}. Upon denaturation with NaDodSO$_4$ the broad band sharpens considerably and shows a single maximum at 1639 cm^{-1}. In the native acyl enzyme the 1638-cm^{-1} feature could be due to the 30% of nonspecifically bound acyl groups. Alternatively, the 5-NO$_2$FA bound to Ser-195 may be in more than one conformation. The small increase in the nitro stretching frequency near 1350 cm^{-1} in the native compared to the denatured acyl enzyme (Figure 3) is reproducible. The carbonyl stretching mode is not observed in the RR spectra of 5-NO$_2$FA compounds. This is due to the fact that the electronic transition giving rise to RR intensity enhancement is, because of the nitro group, more localized on the furan ring. This is apparent by comparing in Figures 2 and 3 the relative intensities of $\nu_{C=C}$ and the 1480-cm^{-1} ring mode in FA and the NO$_2$FA compounds. Consequently, $\nu_{C=O}$ is less intensity enhanced in the RR spectra of 5-NO$_2$FA compared to the other acyl groups and is not observed above the spectral background. Compounds based on the NO$_2$FA chromophore do not follow a $\nu_{C=C}$ vs. λ_{max} correlation (below). This chromophore differs from the others listed in Table II in that it has an electron-attracting group in the ring. The inductive effect of the NO$_2$ group will oppose the tendencies of the active site (below) which promote a partial positive charge on the ring. However, complications due to nonspecific acylation and, additionally, possible twisting of the NO$_2$ group in the active site (Carey et al., 1973) prevent further discussion of this effect.

Resonance Raman Evidence for Two Conformational States for Bound Acyl Groups. A feature occurring in the RR spectra of the chymotrypsin-bound acyl groups is the distinctive carbonyl band profile. This can be seen in most of the intermediates' RR spectra and consists of *two* components, namely, a sharp band near 1720 cm^{-1} and a broader feature centered near 1700 cm^{-1}. There is clear evidence for two components in the RR spectra of the bound acyl groups 5-MeFA (Figure 4), 5-EtFA (Figure 5), TA (Figure 6), and 5-MeTA (Figure 7). The carbonyl profile for FA (Figure 3) is less clear, but, on the basis of many separate spectra, we believe that there are two components near 1720 and 1700 cm^{-1}. The carbonyl modes of the 5-NO$_2$ analogues (e.g., Figure 3) are not strongly resonance enhanced, and no statements can be made on the conformers of the bound acyl groups 5-NO$_2$TA and 5-NO$_2$FA. For the majority of the acyl groups, the appearance of two bands is evidence that, while the acyl groups giving rise to the RR spectra are all bound to serine-195, two binding sites exist for the chromophores, each having a different protein environment about the C=O moiety. For accommodation of the acyl group in either of two binding sites, changes in conformation within the acryloyl moiety may be needed. Different conformers would have slight differences in their $\nu_{C=C}$'s (below). It is interesting to note that Baggott & Klapper (1976), to explain deacylation behavior of some [(5-*n*-alkyl)-2-furyl]chymotrypsins, hypothesized that the furyl ring may lie in more than one position. Additionally, NMR evidence has been presented for more than one environment for the fluorine atoms in *p*-(trifluoromethyl)benzenesulfonyl- and *p*-(trifluoromethyl)cinnamoylchymotrypsins (Maddox et al., 1975).

The carbonyl band near 1720 cm^{-1} in the RR spectra of the acyl enzymes has the same position and band shape as $\nu_{C=C}$ in, e.g., 5-EtFAOMe and 5-MeFAOMe in CCl_4 (Figure 8) and 5-MeTAOMe in cyclohexane (not shown). Thus, the 1720-cm^{-1} acyl enzyme peak is interpreted as being due to a C=O linkage in a hydrophobic, nonbonding environment, in which interactions with H_2O, charges, or dipoles are excluded. The second C=O peak in the acyl enzyme RR spectra is shifted by 20 cm^{-1} and broadened compared to the 1720-cm^{-1} feature. The shift to lower wavenumber and increased bandwidth suggests strong hydrogen bonding. Phenol, for example, by associating with ester carbonyls, reduces $\nu_{C=O}$ by 15–25 cm^{-1} (Bellamy, 1968). In X-ray studies of (indoleacryloyl)chymotrypsin, Henderson (1970) found evidence for two hydrogen bonds from two water molecules to the acryloyl carbonyl. One of these molecules was strongly hydrogen bonded to the carbonyl and to the N$^{\epsilon_2}$ of His-57, while the other water hydrogen bonded the carbonyl of Phe-41 and the acryloyl carbonyl. Two hydrogen bonds of the kind reported by Henderson (1970) would account for the 1700-cm^{-1} band seen in the present acyl enzyme RR spectra. The furyl and thienyl derivatives differ from the indoleacryloyl intermediate in that latter acyl group is bound in a single conformation. Unfortunately, intense fluorescence prevented us from obtaining RR data for (indoleacryloyl)chymotrypsin. In the present systems, it appears from the shape of the carbonyl profile (Figures 4–7) that the bound acyl groups are distributed approximately equally among the conformers having nonhydrogen- and hydrogen-bonded environments, with, perhaps taking the integrated intensities into account, the "hydrogen-bonded" conformer predominating. An additional effect possibly contributing to the differences in frequency between the 1720- and 1700-cm^{-1} carbonyl bands is that the 1720-cm^{-1} feature may come from molecules in the conformation *s*-cis about the =C–C=O single bond while the 1700-cm^{-1} band is due to molecules in the *s*-trans conformation. This is discussed further below. The carbonyl "profile" disappears upon denaturing the acyl enzymes because the carbonyls are then exposed to bulk water. This also hydrogen bonds to the carbonyl but in many slightly different and rapidly changing orientations. The randomness of the bulk water hydrogen bonding broadens the $\nu_{C=O}$ band so that it is not often resolvable above the spectral background. The weak features observable in some of the spectra of $NaDodSO_4$-denatured acyl enzymes are artifacts due to base line fluctuations or incomplete denaturation. The weak features could not be reproduced for a series of preparations.

Other possible assignments of the 1700- and 1720-cm^{-1} bands may be eliminated. Water has a weak Raman band at 1645 cm^{-1}, but this is too far removed from the 1700-cm^{-1} region to create confusion. Moreover, the characteristic 1700–1720-cm^{-1} profile remains in D_2O solution. Similarly, the non-resonance-enhanced amide I mode of the protein, occurring at 1668 cm^{-1} (Yu & Peticolas, 1974), cannot contribute to the spectrum above 1690 cm^{-1}. A further possibility is that the 1720–1700-cm^{-1} profile is due to a Fermi doublet. The constancy of the profile among the chemically different acyl groups on the native enzyme and its absence in the RR spectra of the denatured acyl enzyme and model compounds render this possibility unlikely.

The existence of multiple conformers in the active site and in solution (below) each with a slightly different $\nu_{C=C}$ makes it impossible to use the band profile of $\nu_{C=C}$ as a monitor of conformational heterogeneity. If a single conformer existed in the active site the $\nu_{C=C}$ profile would be expected to sharpen for the native acyl enzyme, and this is observed in the case of a furylacryloylglyceraldehyde-3-phosphate dehydrogenase (sturgeon enzyme) (Storer et al., 1981). However, in the present case either sharpening or broadening of the $\nu_{C=C}$ profile may be observed depending on the exact position of $\nu_{C=C}$'s for the different conformers in solution and in the active site.

Possible Conformations of Furyl- and Thienylacryloyl Esters in Solution. Rotational isomers about both the ethylenic single bonds and the ester C–O–C bonds must be considered. Concerning rotational isomerism about the C–C bond linking the ring with the ethylenic linkage, X-ray data show that the double bonds assume the *s*-trans configuration in solid *trans*-furylacrylic acid (Filippakis & Schmidt, 1967) and furylacrylamide (Leiserowitz & Rabinovich, 1969). Importantly, NMR data (Parr et al., 1976) on some vinyl furans and vinyl thiophenes demonstrate that the *s*-trans conformer also predominates about this bond in polar and nonpolar solvents. The preferred isomer about the =C–C=O single bond for α,β-unsaturated esters in solution has not been as well characterized. Both the *s*-cis (Filippakis & Schmidt, 1969; Leiserowitz & Rabinovich, 1969; Leiserowitz & Schmidt, 1965) and *s*-trans [unpublished work quoted in Leiserowitz & Schmidt (1965)] have been found in the crystal phase. Although, in the solid, the *s*-cis isomer appears to be favored, the small differences in enthalpy between the *s*-cis and *s*-trans forms and the importance of packing forces probably prevent this correlation from being extrapolated to conformations in solution. The quoted X-ray and NMR data being taken together, it is likely, therefore, that the furyl- and thienylacryloyl skeletons in solution are essentially planar, predominantly *s*-trans about the single bond linking the ring and the ethylenic double bond and either *s*-cis or *s*-trans about the =C–C=O single bond. Crucially, the enthalpy differences for the *s*-cis and *s*-trans forms about either single bond are likely <1 kcal/mol (Bowles et al., 1970a; Parr et al., 1976). Thus, the probability must be kept in mind that significant populations of all planar isomers are in equilibrium. Rotational isomers about the ester C–O–C bonds typically have enthalpy differences of <0.5 kcal/mol (Bowles et al., 1970b), again giving rise to a variety of isomers about these linkages in solution. Therefore spectral features, such as λ_{max} and $\nu_{C=C}$ will likely contain unresolved components, with each component due to one of several isomers and reflecting the spectral signature and population of that isomer. In recognition of the composite nature of features such as $\nu_{C=C}$ and λ_{max}, we will call the measured values $\langle\nu_{C=C}\rangle$ and $\langle\lambda_{max}\rangle$, the fences standing for the weighted mean. Rotational isomerism in the acryloyl moiety will primarily affect the acryloyl vibrational modes. This fact, and the distribution of isomers, accounts for the greater bandwidth at half-height for $\nu_{C=C}$ compared to the ring modes, seen for the esters (Figure 8) and $NaDodSO_4$-treated acyl enzymes (Figures 4, 5, and 7).

Observed Correlation between $\langle\nu_{C=C}\rangle$ and $\langle\lambda_{max}\rangle$ for a Series of Compounds Based on Each Acyl Group. For a conjugated π-electron system containing one or more ethylenic linkages, there is often a marked correlation between $\nu_{C=C}$,

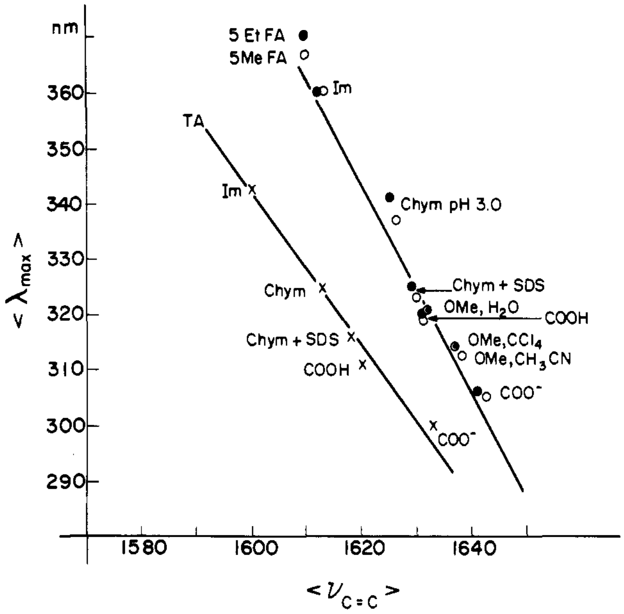
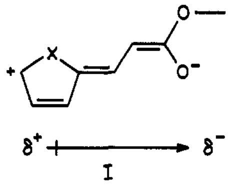


FIGURE 9: Representative plots of measured values of absorption maxima ⟨λ_{max}⟩ vs. the position of the ethylenic stretching mode ⟨$\nu_{C=C}$⟩. TA = thienylacryloyl, 5-Me- and 5-EtFA = 5-methyl- and 5-ethylfurylacryloyl, Im = imidazole ester; chym = chymotrypsin; OMe, H_2O = methyl ester in H_2O.

the vibrational frequency of the ethylenic mode, and λ_{max}, the position of the electronic absorption maximum (Heyde et al., 1971; Carey, 1978). Within a series of compounds based on the same chromophore, a plot of $\nu_{C=C}$ vs. λ_{max} reveals a straight line. This demonstrates that a red shift in λ_{max}, resulting from a narrowing of the electronic excited and ground-state energy gap, is accompanied by a reduction in $\nu_{C=C}$, indicating a decrease in C=C bond order and therefore increased electron conjugation in the electronic *ground state.* Since the energetics of catalysis are concerned with only the electronic ground state, it is an important advantage of the RR method that Raman and RR peak positions, being a property of the ground state alone, provide a direct probe of the ground state unencumbered by possible complications of excited state effects.

As Table II and the representative plots in Figure 9 show, within a series of compounds based on each acyl group, an essentially linear relationship exists between ⟨$\nu_{C=C}$⟩ and ⟨λ_{max}⟩. It is important to keep in mind at this point the conclusions from the previous sections—that a number of isomers exist for each acyl group free in solution and, moreover, the acyl groups are bound to the enzyme in two conformations. Given the heterogeneity of conformers in the free acyl groups and the heterogeneity of binding modes, there is still an overall red shift in ⟨λ_{max}⟩ and concomitant drop in ⟨$\nu_{C=C}$⟩ for the acyl groups bound to the native active sites at pH 3.0. The possible sources of such effects are as follows.

(*a*) *π-Electron Polarization.* In this mechanism electron conjugation is increased in the acyl group's π-electron system by protein forces setting up a dipole moment. In valence bond terms structures such as I assume an increased weighting in



I

the true structure.

Electron polarization may be brought about by the disposition of charged groups or dipoles associated with the protein or by strong hydrogen bonding to the carbonyl. This kind of effect is thought to be responsible for the large shifts in λ_{max}, from 480 to 620 nm, and the 25-cm^{-1} decrease in $\nu_{C=C}$ occurring when the carotenoid astaxanthin binds to some carotenoproteins (Salares et al., 1979). Certain acyl papains based on cinnamoyl or furylacryloyl groups also show large changes in λ_{max} and $\nu_{C=C}$ when the acyl groups bind to the active site (Carey et al., 1978), and again a π-electron polarization mechanism is thought to be operative.

(*b*) *Conformational Changes in Acryloyl Skeleton.* In this mechanism electron conjugation is increased in the π-electron system by enzyme–substrate interactions bringing about a *conformational change* in the acryloyl moiety, rather than, e.g., charge effects changing the π-electron distribution within a single conformer. Both the single bond ethylenic linkages are capable of *s*-cis–*s*-trans isomerization, and rotational isomerism about the C–O ester linkages is also possible. The most widely studied conjugated systems involving the effects of *s*-cis–*s*-trans isomerism on the vibrational spectra are α,β-unsaturated ketones (Erskine & Waight, 1960; Noack & Jones, 1961a,b; Bowles et al., 1969). For ketones the values of *trans*-Δ^3-penten-2-one are typical (Noack & Jones, 1961a)

	s-cis	*s*-trans
$\nu_{C=O}$	1693	1674
$\nu_{C=C}$	1630	1643

Interestingly, however, when Bowles et al. (1970a) studied possible *s*-cis and *s*-trans isomerization in an α,β-unsaturated ester they found no evidence for differences of ≥3 cm^{-1} in $\nu_{C=C}$ and $\nu_{C=O}$ for the *s*-trans form and a second isomer they termed non-*s*-trans (possible *s*-cis). They rationalized their result in terms of an earlier observation by Grigg et al. (1965) on the differences in vibrational band frequencies for certain isomers of analogous ketones and esters.

For some esters in certain solvents and by use of 350.7-nm excitation for the RR spectra, we observed two features in the $\nu_{C=C}$ region. For example, the 1637-cm^{-1} peak for 5-MeFAOMe in CCl_4 (Figure 8) has a shoulder on the low wavenumber side. By use of a rotating sample cell or a flow system, this shoulder was clearly resolved for the ester in CH_3CN and appeared at 1625 cm^{-1} with the major feature centered at 1638 cm^{-1}. However, at higher concentrations in CH_3CN and and by use of 514.5-nm excitation, the 1625-cm^{-1} peak was greatly reduced in relative intensity compared to the main 1638-cm^{-1} band. This suggests that by use of UV excitation a photostationary population of isomers is rapidly set up with an increased population of isomer bearing the 1625-cm^{-1} signature. On the basis of the work on α,β-unsaturated ketones (Noack & Jones, 1961a,b), the 1625-cm^{-1} feature is assigned to an isomer *s*-cis about one, or possibly both, of the ethylenic single bonds. The 1625-cm^{-1}, photoinduced band does not fit the ⟨$\nu_{C=C}$⟩–⟨λ_{max}⟩ correlation (Figure 9) since the species giving rise to this RR band is present to a much lesser extent under the conditions of recording absorption spectra. The facts that in the majority of cases a ⟨$\nu_{C=C}$⟩–⟨λ_{max}⟩ correlation does hold and that $\nu_{C=C}$ band profiles in RR spectra are excitation wavelength independent are taken as evidence that photoinduced isomers are an exception rather than the rule.

For a given acyl group it is likely that changes in both electron polarization and isomer populations move ⟨$\nu_{C=C}$⟩ ⟨λ_{max}⟩ along the lines in Figure 9. *Thus, it is not possible, in general, to ascribe changes in* ⟨$\nu_{C=C}$⟩ *or* ⟨λ_{max}⟩ *to a single cause.* The energy balance between the various rotational isomers is so subtle that changes in solvent will vary the percentage of each isomer. At the same time differences in solvent bulk dielectric, dipole moment, and hydrogen-bonding capacity will bring about marked changes in the induced dipole in the acyl groups π-electron system. When the acyl group undergoes a major change in charge distribution *internally*, for example, in the $-COO^-$ form of the acid, this will likely be the dominant factor in shifting $\langle \nu_{C=C} \rangle$ and $\langle \lambda_{max} \rangle$.

Model for Conformations of Acyl Groups Bound to Chymotrypsin. Several factors must be kept in mind when attempting to explain the changes in spectral properties observed when the acyl groups bind to the active site. Firstly, many of the free acyl groups in solution exist as an equilibrium population involving *s*-cis–*s*-trans isomerization about the ethylenic single bonds. Secondly, as discussed above, the carbonyl region in the RR spectra of the bound acyl groups reveal two distinct environments for the C=O group. Thirdly, the changes in $\langle \nu_{C=C} \rangle$ and $\langle \lambda_{max} \rangle$ upon binding to the active site are small. For example, compared to the $NaDodSO_4$-treated acyl enzymes or the methyl esters in aqueous solution, $\langle \nu_{C=C} \rangle$ in the intact 5-MeFA- or 5-EtFA-chymotrypsins drops by 5 cm^{-1} and $\langle \lambda_{max} \rangle$ increases by 15–20 nm. The small change in $\langle \nu_{C=C} \rangle$ is the most important piece of datum, since RR peak positions involve the electronic ground state only.

The X-ray data for (indoleacryloyl)chymotrypsin (Henderson, 1970) at pH 4.0 showed that this acyl group was bound in a single conformation with a planar configuration, *s*-trans about the =C–C=O single bond and with the carbonyl strongly hydrogen bonded. It is probable that one of the forms of the acyl groups detected here also adopts this conformation. The strong hydrogen bonding to the carbonyl and the *s*-trans conformation will reduce $\nu_{C=O}$, accounting for the broad 1700-cm^{-1} band. At the same time, the hydrogen bonding will polarize the π-electron system reducing $\nu_{C=C}$ and red shifting λ_{max}. The *s*-trans conformation about =C–C=O may increase $\nu_{C=C}$ slightly [by $\sim$3 cm^{-1}, on the basis of Bowles and co-workers (Bowles et al., 1970a) work on α,β-unsaturated esters], but this effect will be counterbalanced and possibly offset by the charge polarization effect of the hydrogen bonding. The second conformation for the bound acyl groups involves a nonbonded carbonyl with $\nu_{C=O}$ at 1720 cm^{-1}; the observation of this feature in the RR spectrum means that good π overlap is retained between the carbonyl and other π electrons. Thus, like the first conformer the second bound conformer must be essentially planar. It may be *s*-cis about the =C–C=O single bond, resulting a slight reduction in $\nu_{C=C}$ compared to *s*-trans. Furthermore, isomerism about the ring to ethylenic single bond cannot be ruled out.

In summary, the slight reduction in $\langle \nu_{C=C} \rangle$ and red shift in $\langle \lambda_{max} \rangle$ are due to strong, sterically well-defined hydrogen bonds to the carbonyl for the conformer bound *s*-trans about the =C–C=O single bond, while the second conformer probably binds *s*-cis about this linkage and thus reinforces the trend to a slightly lower $\langle \nu_{C=C} \rangle$. Since the free chromophores in solution prefer the *s*-trans conformation about the ring to ethylenic single bond, an isomerization about this bond to *s*-cis upon binding may also bring about a reduction in $\langle \nu_{C=C} \rangle$. It is also possible that the potential-energy wells for rotation about the C–C single bonds in the acryloyl group are shallow. This would result in torsionally nonplanar excited energy states for the molecules in solution. By the acyl group being "locked" into a planar configuration, increased conjugation with a concomitant reduction in $\langle \nu_{C=C} \rangle$ would follow. Another source of electron polarization is charge effects due to charged protein groups or local (Warshel & Levitt, 1976) or extended (Hol et al., 1978) protein dipoles. Whole charges are unlikely since on the basis of the X-ray data there are no candidates, at low pH, near the active site. moreover, upon the pH being changed (see the following paper), there is no evidence for a nearby titratable group between pH 3.0 and 6.0. Although, on the basis of X-ray studies (Blow, 1976), α-chymotrypsin does not have an α helix near the active site to serve as a candidate for an extended dipole, the role of local protein dipoles deserves attention. Further work is needed to clarify the effect of both local and extended dipoles on the vibrational and electronic spectra of the type of chromophores considered here.

A firm interpretation cannot be offered, at this time, for the appearance of the "new" 1260-cm^{-1} feature in the RR spectra of some of the native acyl enzyme spectra. It occurs in a region devoid of ring modes (assignments, above), and it is thus probably a band associated with an acryloyl vibrational mode. It is also only observed in the intact active site, so it could result from the active site "freezing out" a conformation with consequent intensity gain in a previously weak or broad mode. Studies under cryoenzymological conditions (Douzou, 1977) may be helpful in this regard as well as in investigating the carbonyl profile.

Acknowledgments

We thank H. Seguin for analyzing the compounds used in this work.

References

Baggott, J. E., & Klapper, M. H. (1976) *Biochemistry 15*, 1473–1481.

Bellamy, L. J. (1968) *Advances in Infrared Group Frequencies*, p 152, Methuen, London.

Bernhard, S. A., & Lau, S.-J. (1972) *Cold Spring Harbor Symp. Quant. Biol. 36*, 75–83.

Bernhard, S. A., Lau, S. J., & Noller, H. (1965) *Biochemistry 4*, 1108–1118.

Blow, D. M. (1976) *Acc. Chem. Res. 9*, 145–152.

Bowles, A. J., George, W. O., & Maddams, W. F. (1969) *J. Chem. Soc. B*, 810–818.

Bowles, A. J., George, W. O., & Cunliffe-Jones, D. B. (1970a) *J. Chem. Soc. B*, 1070–1075.

Bowles, A. J., George, W. O., & Cunliffe-Jones, D. B. (1970b) *J. Chem. Soc., Chem. Commun.*, 103–104.

Carey, P. R. (1978) *Q. Rev. Biophys. 11*, 309–370.

Carey, P. R., & Schneider, H. (1974) *Biochem. Biophys. Res. Commun. 57*, 831–837.

Carey, P. R., & Schneider, H. (1976) *J. Mol. Biol. 102*, 679–693.

Carey, P. R., Froese, A., & Schneider, H. (1973) *Biochemistry 12*, 2198–2208.

Carey, P. R., Carriere, R. G., Phelps, D. J., & Schneider, H. (1978) *Biochemistry 17*, 1081–1087.

Douzou, P. (1977) *Cryobiochemistry: An Introduction*, Academic Press, New York.

Erskine, R. L., & Waight, E. S. (1960) *J. Chem. Soc.*, 3425–3431.

Fersht, A. R. (1977) *Enzyme Structure and Mechanism*, Freeman, Reading, United Kingdom.

Filippakis, S. E., & Schmidt, G. M. J. (1967) *J. Chem. Soc. B*, 229–232.

Gilman, H., & Wright, G. F. (1930) *J. Am. Chem. Soc. 52*, 2550–2554.

Grigg, R., Sargent, M. V., & Knight, J. A. (1965) *Tetrahedron Lett. 19*, 1381–1384.

Henderson, R. (1970) *J. Mol. Biol. 54*, 341–354.

Heyde, M. E., Gill, D., Kilponen, R. C., & Rimai, L. (1971) *J. Am. Chem. Soc. 93*, 6776–6780.

Hol, W. G. J., van Duijnen, P. T., & Berendsen, H. J. C., (1978) *Nature* (*London*) *273*, 443–446.

Janda, M., & Radouch, J. (1967) *Collect. Czech. Chem. Commun. 32*, 2672–2675.

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York.

Kai, F., & Ogawa, H. (1963) *Chem. Pharm. Bull. 11*, 1205–1207.

Katritzky, A. R., & Lagowski, J. M. (1959) *J. Chem. Soc.*, 657–660.

King, W. J., & Nord, F. F. (1949) *J. Org. Chem. 14*, 405–410.

Koo, J., Fish, M. S., Walker, G. N., & Blake, J. (1963) *Organic Syntheses, Collect. Vol. IV*, pp 327–328, Wiley, New York.

Leiswerowitz, L., & Schmidt, G. M. J. (1965) *Acta Crystallogr. 18*, 1058–1067.

Leiserowitz, L., & Rabinovich, D. (1969) *J. Chem. Soc. A*, 2367–2370.

Maddox, M. L., Roe, D. C., & Gerig, J. T. (1975) *J. Chem. Soc., Chem. Commun.*, 902–903.

Noack, K., & Jones, R. N. (1961a) *Can. J. Chem. 39*, 2201–2213.

Noack, K., & Jones, R. N. (1961b) *Can. J. Chem. 39*, 2225–2235.

Pandit, M. W., & Rao, M. S. N. (1975) *Biochemistry 14*, 4106–4110.

Parr, W. J. E., Wasylishen, R. E., & Schaeffer, T. (1976) *Can. J. Chem. 54*, 3216–3223.

Phelps, D. J., Schneider, H., & Carey, P. R. (1981) *Biochemistry* (second of three papers in this issue).

Reitz, A. W. (1938) *Z. Phys. Chem. Abt. B 38*, 381–392.

Rico, M., Orza, J. M., & Morcillo, J. (1965) *Spectrochim. Acta 21*, 689–719.

Rico, M., Barrachina, M., & Orza, J. M. (1967) *J. Mol. Spectrosc. 24*, 133–148.

Salares, V. R., Young, N. M., Bernstein, H. J., & Carey, P. R. (1979) *Biochim. Biophys. Acta 576*, 176–191.

Schmidt, J., Benecky, M., Katina, M., Watters, K. L., & McFarland, J. T. (1978) *FEBS Lett. 96*, 263–268.

Storer, A. C., Murphy, W. F., & Carey, P. R. (1979) *J. Biol. Chem. 254*, 3163–3165.

Storer, A. C., Phelps, D. J., & Carey, P. R. (1981) *Biochemistry* (third of three papers in this issue).

Thompson, H. W., & Temple, R. B. (1945) *Trans. Faraday Soc. 41*, 27–34.

Warshel, A., & Levitt, M. (1976) *J. Mol. Biol. 103*, 227–249.

Yu, T.-J., & Peticolas, W. (1974) *Pept., Polypeptides Proteins, Proc. Rehovot Symp., 2nd*, 370–378.

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

D. J. Phelps, H. Schneider, and P. R. Carey*

ABSTRACT: The resonance Raman (RR) spectra, excited by 350.7-nm irradiation, of 4-NH_2-3-NO_2-cinnamoyl- and 5-CH_3-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 $\nu_{C=C}$ 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-CH_3-thienylacryloyl-, α-CH_3-5-CH_3-thienylacryloyl-, and α-CH_3-5-CH_3-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 $\langle\nu_{C=C}\rangle$ 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-CH_3 or α-CH_3 groups, leads to low rates of deacylation. Good quality RR spectra permitted a careful comparison of the RR spectra of 5-CH_3-thienylacryloylchymotrypsin in H_2O and D_2O. 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

---

[†] From the Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A 0R6. N.R.C.C. No. 19394. *Received October 27, 1980.*

0006-2960/81/0420-3447$01.25/0