Kishore, R., Kumar, A., & Balaram, P. (1985) *J. Am. Chem. Soc. 107*, 8019-8023.

Kishore, R., Raghothama, S., & Balaram, P. (1987) *Biopolymers 26*, 873-891.

Marshall, G. R., Bosshard, H. E., Kendrick, N. C. E., Turk, J., Balasubramanian, T. M., Cobb, S. M. H., Moore, M., Leduc, L., & Needleman, P. (1976) in *Peptides 1976* (Loffet, A., Ed.) pp 361-369, Editions de l'Université de Bruxelles, Brussels, Belgium.

Mathew, M. K., & Balaram, P. (1983a) *FEBS Lett. 157*, 1-5.

Mathew, M. K., & Balaram, P. (1983b) *Mol. Cell. Biochem. 50*, 47-64.

Menestrina, G., Voges, K. P., Jung, G., & Boheim, G. (1986) *J. Membr. Biol. 93*, 111-132.

Mutter, M. (1988) *Trends Biochem. Sci.* (*Pers. Ed.*) *13*, 260-265.

Mutter, M., Altmann, K.-H., Tuchscherer, G., & Vuilleumier, S. (1988) *Tetrahedron 44*, 771-785.

Nagaraj, R., & Balaram, P. (1981) *Acc. Chem. Res. 14*, 356-362.

Osterman, D. G, & Kaiser, E. T. (1985) *J. Cell. Biochem. 29*, 57-72.

Prasad, B. V. V., & Balaram, P. (1984) *CRC Crit. Rev. Biochem. 16*, 307-348.

Ravi, A., & Balaram, P. (1984) *Tetrahedron 40*, 2577-2583.

Regan, L., & Degrado, W. F. (1988) *Science 241*, 976-978.

Richardson, J. S., & Richardson, D. C. (1987) in *Protein Engineering* (Oxender, D. L., & Fox, C. F., Eds.) pp 149-163, Alan R. Liss, New York.

Satyshur, K. A., Rao, S. T., Pyzalska, D., Drendel, W., Greaser, M., & Sundaralingam, M. (1988) *J. Biol. Chem. 263*, 1628-1647.

Toniolo, C., Bonora, G. M. Bavoso, A., Benedetti, E., Di Blasio, B., Pavone, V., & Pedone, C. (1983) *Biopolymers 22*, 205-215.

Direct Observation of the Titration of Substrate Carbonyl Groups in the Active Site of α-Chymotrypsin by Resonance Raman Spectroscopy[†]

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ABSTRACT: By use of resonance Raman (RR) spectroscopy, the population of the reactive carbonyl group in active acylchymotrypsins has been characterized and correlated with acyl-enzyme reactivity. RR spectra have been obtained, with a flow system and 324- and 337.5-nm excitation, at low and active pH for six acylchymotrypsins, viz., (indoleacryloyl)-, (4-amino-3-nitrocinnamoyl)-, (furylacryloyl)-, [(5-ethylfuryl)-acryloyl]-, (thienylacryloyl)-, and [(5-methylthienyl)acryloyl]chymotrypsin. These acyl-enzymes represent a 100-fold range of deacylation rate constants. Good RR spectral quality has enabled us to obtain the vibrational spectrum of the carbonyl group at low and active pH in each acyl-enzyme. The measured pK_a of the spectroscopic changes in the carbonyl region is identical with that for the deacylation kinetics, showing that the RR carbonyl features reflect the ionization state of His-57. A carbonyl population has been observed in the active acyl-enzymes in which the carbonyl oxygen atom of the reactive acyl linkage is hydrogen-bonded in the active site. The proportion of this hydrogen-bonded population, with respect to other observed non-hydrogen-bonded species, together with the degree of polarization of the carbonyl bond, as monitored by $\nu_{C=O}$, has been correlated with the deacylation rate constants of the acyl-enzymes. It is proposed that the hydrogen-bonded carbonyl species is located at or near the oxyanion hole and represents the ground state from which deacylation occurs. An increase in the proportion of the hydrogen-bonded population and an increase in polarization of the carbonyl bond result in an increase in deacylation rate constant. For the first time we have direct RR data on the catalytically transformed region which relate to the difference in acyl-enzyme reactivity.

In order to completely describe the reaction catalyzed by serine proteases, it is important to understand the chemistry of the acyl-enzyme carbonyl group. Here we show that resonance Raman (RR) spectroscopy is a good probe of the structure and environment of the acyl carbonyl group in *O*-(arylacryloyl)chymotrypsins. The RR carbonyl stretching band profile is used to monitor changes in the carbonyl group as the catalytic mechanism is activated. Using this approach, we identify an "active" carbonyl population in which the acyl carbonyl oxygen atom is hydrogen-bonded in the oxyanion hole. The quantity of this hydrogen-bonded species as well as its degree of polarization can then be related to the relative rates of deacylation of each acyl-enzyme.

Direct vibrational spectroscopic analysis of the reactive carbonyl group is limited to intermediates which accumulate on the reaction pathway. During the hydrolysis of substrates by serine proteases, a covalently bound acyl-enzyme intermediate is formed. In general, for ester substrates acylation (acyl-enzyme formation) is much faster than deacylation (acyl-enzyme breakdown), and the acyl-enzyme intermediate accumulates (Kraut, 1977). In order to obtain the vibrational spectrum of the acyl-enzyme carbonyl by RR spectroscopy, a chromophoric acyl group is required whose electronic absorption spectrum includes a contribution from the carbonyl group. In the present study we use β-substituted arylacryloyl compounds, which commonly absorb in the 300–400-nm region. This class of compounds has been used extensively as

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active site directed spectroscopic probes for serine and thiol proteases (Bender et al., 1962; Bernhard et al., 1965; Brubacher & Bender, 1966; Hinkle & Kirsch, 1970). Previous RR studies in this laboratory have included acylchymotrypsins generated from thienylacrylic and furylacrylic acids and their derivatives (MacClement et al., 1981) as well as from the substituted cinnamic acid 4-amino-3-nitrocinnamic acid (Carey & Schneider, 1974, 1976; Phelps et al., 1981). Furthermore Peticolas and co-workers have obtained the RR spectrum of [4-(dimethylamino)cinnamoyl]chymotrypsin (Weber et al., 1986). In both sets of studies good carbonyl profiles were obtained at low pH where the acyl-enzymes were stable. RR spectra have also been obtained previously in this laboratory from several of the above-mentioned acyl-enzymes at active pH; however, the signal to noise ratio was not good enough to permit observation of the reactive carbonyl group (Phelps et al., 1981). The present study employing multichannel photon detection enables the carbonyl profile to be monitored for unstable acyl-enzymes at active pH for the first time.

The work presented in this paper is based on RR spectra obtained at both inactive and active pH of six acyl-enzymes, viz., (indoleacryloyl)-, (4-amino-3-nitrocinnamoyl)-, (furylacryloyl)-, [(5-ethylfuryl)acryloyl]-, (thienylacryloyl)-, and [(5-methylthienyl)acryloyl]chymotrypsin. Of particular interest is (indoleacryloyl)chymotrypsin, an acyl-enzyme for which an X-ray crystallographic structure has previously been determined (Henderson, 1970). This has enabled a direct spectra–structure assignment to be made for this acyl-enzyme at low pH. For each acyl-enzyme the carbonyl profile at active pH is related to its relative reactivity. In each case a hydrogen-bonded carbonyl population is observed at high pH, and it is proposed that this is the *active* carbonyl species from which deacylation occurs. We postulate that the proportion of the hydrogen-bonded population relative to other nonproductively bound carbonyl species observed as well as the degree of polarization of the hydrogen-bonded carbonyl directly reflects the individual reactivity of each acyl-enzyme.

EXPERIMENTAL PROCEDURES

Materials

4-Acetamidobenzaldehyde, 2-ethylfuran, 5-methyl-2-thiophenecarboxaldehyde, thienylacrylic acid, furylacrylic acid, indoleacrylic acid, and 1,1′-carbonyldiimidazole were from Aldrich Chemical Co. α-Chymotrypsin (3× crystallized, lot 87F-8195) and *N-trans*-cinnamoylimidazole were from Sigma Chemical Co. Buffer salts and solvents (spectrophotometric grade) were from usual sources.

Synthesis

4-Acetamido-3-nitrobenzaldehyde. This compound was synthesized from 4-acetamidobenzaldehyde by the method of Ganapati (1938). A total of 12 g (0.0736 mol) of 4-acetamidobenzaldehyde yielded 6.28 g (0.030 mol, 41%) of 4-acetamido-3-nitrobenzaldehyde (yellow crystals) after recrystallization from H_2O.

4-Acetamido-3-nitrocinnamic Acid. 4-Acetamido-3-nitrobenzaldehyde was converted into 4-acetamido-3-nitrocinnamic acid by the method of Koo et al. (1963). A total of 5.5 g (0.0264 mol) of aldehyde yielded 5.25 g (0.021 mol, 79.6%) of 4-acetamido-3-nitrocinnamic acid after recrystallization from H_2O. Anal. Calcd for $C_{11}H_{10}O_5N_2$ (250): C, 52.80; H, 4.00; N, 11.20. Found: C, 50.88; H, 3.92; N, 11.56.

4-Amino-3-nitrocinnamic Acid. 4-Acetamido-3-nitrocinnamic acid was hydrolyzed to 4-amino-3-nitrocinnamic acid essentially by the method of Gabriel and Herzberg (1883). A total of 5.15 g (0.0206 mol) of 4-acetamido-3-nitrocinnamic acid was mixed with 100 mL of 1 M NaOH and maintained at 50 °C for 15 min. The dark red solution that was obtained was acidified with 2 M HCl, and the yellow solid that formed was removed by filtration, washed with 3 × 100 mL of H_2O, and partially dried. Recrystallization from methanol yielded 2.85 g (0.0137 mol, 66.5%) of 4-amino-3-nitrocinnamic acid.

(4-Amino-3-nitrocinnamoyl)imidazole. A total of 1.2 g (0.0074 mol) of 1,1′-carbonyldiimidazole was added to a solution of 1.54 g (0.0074 mol) of 4-amino-3-nitrocinnamic acid in 40 mL of dry tetrahydrofuran (THF) kept under N_2 and cooled in a cold-water bath. After the 1,1′-carbonyldiimidazole had dissolved, the reaction mixture was allowed to stir at RT for 3 h. The dark brown solid that formed was filtered, washed with 3 × 20 mL of THF and recrystallized from dimethylformamide (DMF) to yield 1.32 g (0.0051 mol, 68.9%) of (4-amino-3-nitrocinnamoyl)imidazole. Anal. Calcd for $C_{12}H_{10}O_3N_4$ (258): C, 55.81; H, 3.88; N, 21.71. Found: C, 55.61; H, 3.94; N, 21.83.

4-Amino-3-nitrocinnamic Acid Methyl Ester. One gram (0.0048 mol) of 4-amino-3-nitrocinnamic acid was converted into 0.56 g (0.0025 mol, 52.1%) of 4-amino-3-nitrocinnamic acid methyl ester by the method of MacClement et al. (1981).

5-Ethyl-2-furaldehyde. A total of 10 g (0.104 mol) of 2-ethylfuran was converted into 5-ethyl-2-furaldehyde by the method of Traynelis et al. (1957). Vacuum distillation of the product using a Vigreux column gave 7.09 g (0.057 mol, 54.8%) of 5-ethyl-2-furaldehyde, bp 81 °C (lit. bp 79–81 °C, 12 mmHg). Anal. Calcd for $C_7H_8O_2$ (124): C, 67.74; H, 6.45. Found: C, 67.53; H, 6.52.

(5-Ethyl-2-furyl)acrylic Acid. A total of 6.2 g (0.050 mol) of 5-ethyl-2-furaldehyde was converted into (5-ethyl-2-furyl)acrylic acid by the method of Koo et al. (1963). Recrystallization from H_2O/ethanol gave 6 g (0.036 mol, 72.0%) of (5-ethyl-2-furyl)acrylic acid. Anal. Calcd for $C_9H_{10}O_3$ (166): C, 65.06; H, 6.02. Found: C, 65.34; H, 6.00.

[(5-Ethyl-2-furyl)acryloyl]imidazole. This compound was prepared from (5-ethyl-2-furyl)acrylic acid by the method of MacClement et al. (1981). The resultant solid was recrystallized from 2-propanol. Anal. Calcd for $C_{12}H_{12}O_2N_2$ (216): C, 66.67; H, 5.56; N, 12.96. Found: C, 66.80; H, 5.47; N, 12.91.

(5-Methyl-2-thienyl)acrylic Acid. This compound was prepared from 5-methyl-2-thiophenecarboxaldehyde by a method identical with that for (5-ethyl-2-furyl)acrylic acid.

The other methyl esters and imidazole esters used in this work were prepared in good yield from the corresponding acids by the methods detailed above. In all cases calculated and found elemental analyses were in good agreement.

Preparation of Acylchymotrypsins

α-Chymotrypsin was found to be 94% active by titration with *N-trans*-cinnamoylimidazole (Bender et al., 1966), $\epsilon_{280} = 5 \times 10^4\ M^{-1}\ cm^{-1}$ being used for the enzyme. Ion-exchange chromatography on Amberlite CG-50 resin (Hamilton & Zerner, 1981) failed to improve the activity. RR spectroscopic and kinetic studies on the chromatographed material were identical in all respects with those of the unpurified enzyme.

Acylchymotrypsins were generated by addition of a solution of the (arylacryloyl)imidazole esters in DMF to a solution of the enzyme in 50 mM pH 6.0 phosphate buffer. After 30 s the pH of the reaction mixture was adjusted to 3.0 with 1 M HCl, and the solution was filtered through 0.2-μm Minisart (Sartorius) filters. Unreacted substrate and product were removed either by dialysis (3500 MW cutoff membrane) in 0.2 M NaCl, pH 3.0 (HCl), or by chromatography on G-15 Sephadex equilibrated with 0.2 M NaCl, pH 3.0 (HCl). For chromatography on G-15 the column dimensions were 2 × 40 cm, and the sample size was 10 mL. (Furylacryloyl)- (FA-), [(5-ethylfuryl)acryloyl]- (5-EtFA-), (thienylacryloyl)- (TA-), and [(5-methylthienyl)acryloyl]- (5-MeTA-) chymotrypsins were prepared by addition of 3 mL of 50 mM substrate solution in DMF to 50 mL of phosphate buffer containing 500 mg of chymotrypsin to give final concentrations of 2.83 mM substrate and 9.4 mg/mL protein. (Indoleacryloyl)- (IA-) chymotrypsin was prepared by addition of 6 mL of 10 mM substrate in DMF to 44 mL of phosphate buffer containing 450 mg of chymotrypsin, giving final concentrations of 1.2 mM substrate and 9 mg/mL protein. (4-Amino-3-nitrocinnamoyl)- ($4-NH_2-3-NO_2$-cinn-) chymotrypsin was prepared by addition of 4 mL of 2.5 mM substrate solution in DMF to 16 mL of phosphate buffer containing 100 mg of chymotrypsin, giving final concentrations of 0.5 mM substrate and 5 mg/mL protein. In the case of the latter acyl-enzyme, the pH of the reaction mixture was immediately adjusted to 3.0. At pH 3.0, λ_{max} of the acyl-enzymes were as follows [acyl group, λ_{max} (nm)]: IA, 356; $4-NH_2-3-NO_2$-cinn, 340; TA, 322; 5-MeTA, 341; FA, 324; 5-EtFA, 338. These values were in good agreement with published values (Bernhard & Tashjian, 1965; Phelps et al., 1981; MacClement et al., 1981). The ratios of the absorbance at the chromophore's λ_{max} [$A(\lambda_{max})$] to A_{280} for each acyl-enzyme were as follows: TA, 0.31; IA, 0.33; FA, 0.35; 5-MeTA, 0.36; 5-EtFA, $4-NH_2-3-NO_2$-cinn, 0.38. With 2×10^4 M^{-1} cm^{-1} as an approximate value for ϵ_{max} of the chromophore in each acyl-enzyme [Bernhard et al. (1965) give $\epsilon_{max} = 1.8 \times 10^4$ for FA-chymotrypsin and 2×10^4 for IA-chymotrypsin], values of $A(\lambda_{max})/A_{280}$ of 0.31–0.38 for the acyl-enzymes studied here are equivalent to approximate acylation percentages of 78–95% [assuming that only protein contributes to A_{280} and only chromophore contributes to $A(\lambda_{max})$].

In order to investigate possibilities of enzyme aggregation during preparation of the acyl-enzyme intermediates, acyl-enzymes were also generated from enzyme solutions containing ca. 1 mg/mL protein. Acyl-enzymes prepared in this manner were spectroscopically and kinetically identical with acyl-enzymes prepared from ca. 10 mg/mL protein solutions.

Kinetics

Deacylation rate constants (k_{obs}) were determined on a Cary 219 spectrophotometer interfaced to a Hewlett-Packard computer (HP 9000, Series 200) for data collection and analysis. Deacylation reactions were initiated by the addition of 50 μL of acyl-enzyme (0.1–0.4 mM acyl-enzyme, pH 3.0, 0.2 M NaCl) to 1 mL of buffer (0.105 M buffer, 0.095 M NaCl) equilibrated at 25 ± 0.1 °C in the sample compartment of the spectrophotometer. Deacylation was monitored at the following wavelengths [acyl group, wavelength (nm)]: IA, 380; $4-NH_2-3-NO_2$-cinn, 360; FA, 335; 5-EtFA, 360; TA, 340; 5-MeTA, 360. Sephadex G-15 chromatography of the reaction mixtures at t_∞ indicated that no chromophore remained bound to the enzyme. Buffers used were acetate (pH 4.5–5.5), citrate (pH 5.5–6.5), phosphate (pH 6.5–7.5), Hepes (pH 7.5–8.5), borate (pH 8.5–9.5), and carbonate (pH 9.5–11.0). [Initial experiments in the pH range 7.5–8.5 were performed with Tris-HCl; however, buffer catalysis was observed for this buffer. Analysis of the reaction mixture at t_∞ was consistent with nucleophilic attack by Tris on the acyl-enzyme, generating an arylacryloylamide derivative.] For $4-NH_2-3-NO_2$-cinn-chymotrypsin, deacylation rates at pH >8.0 were determined with a hand-operated stopped-flow apparatus (Hi-Tech, Salisbury, England) with the Cary 219. At least five separate determinations were made at each pH value. [H^+] was routinely varied from $10^{-2}K_a$ to $10K_a$. The rates of base-catalyzed hydrolyses [$k_{(OH^-)}$] of the (arylacryloyl)imidazole esters were determined in 0.1 M carbonate buffer/0.1 M NaCl, pH 10.50, 25 ± 0.1 °C. Hydrolysis was monitored at the following wavelengths [acyl imidazole, wavelength (nm)]. Absorbance decrease followed: $4-NH_2-3-NO_2$-cinn-, 361. Absorbance increase followed: IA, 312; FA, 290; 5-EtFA, 306; TA, 300; 5-MeTA, 310. First-order rate constants were calculated by nonlinear least-squares regression analysis on the Hewlett-Packard computer. Deacylation pK_a values were determined by linear least-squares regression analysis of plots of k_{obs} vs $k_{obs}[H^+]$ (Fersht, 1985a).

Raman Instrumentation

Raman spectra were obtained with 514.5-nm Ar^+ laser excitation and a Jarrell-Ash 0.75-m double monochromator with photon-counting detection described elsewhere (Kumar & Carey, 1975).

Resonance Raman (RR) Instrumentation

RR spectra were obtained with 324- or 337.5-nm Kr^+ laser excitation, a Spex Triplemate, and a Tracor Northern UV-enhanced diode array (Carey & Sans Cartier, 1983). For flow experiments the sample cell consisted of a 1 mm i.d. square Suprasil capillary coupled to a two-jet mixer and Harvard Model 935 infusion pump. A flow-through stirred cell between the mixing block and capillary ensured complete mixing. Flow rates were adjusted such that the acyl-enzymes were observed before significant (>3%) deacylation had occurred. In most cases, flow rates of 5 mL/min were sufficient. For $4-NH_2-3-NO_2$-cinn-chymotrypsin at pH >8.0 the flow rate was 20 mL/min, and the stirred cell was removed as the flow rate was sufficient to ensure complete mixing. At this flow rate we were able to observe the acyl-enzyme ca. 150 ms after mixing. Using $k_{obs(lim)} = 2.2 \times 10^{-1}$ s^{-1}, it can be calculated that after 150 ms the acyl-enzyme should be 97% acylated at the point of observation, ca. 3 cm from the mixing block (Sans Cartier et al., 1988). For the mixing experiments one syringe contained 0.2 M buffer while the other contained acyl-enzyme solution (pH 3.0 0.2 M NaCl). Buffer salts used were as detailed under Kinetics. The pH of the buffer solution was such that after mixing 1:1 with acyl-enzyme solution the desired pH was obtained. Final concentration of acyl-enzyme was 0.1–0.2 mM, and the absorbance at the laser excitation wavelength was ca. 0.8–2.0 (1-cm path length). To examine the contribution of the ca. 1640-cm^{-1} HOH bending vibration to the acyl-enzyme $\nu_{C=O}$ profiles, buffer spectra were recorded. In some cases subtraction was performed for which the 1375-cm^{-1} acetonitrile band (final concentration 5% acetonitrile) was used as an internal standard. pK_a values for the observed pH-dependent changes in $\nu_{C=O}$ were determined by linear least-squares regression analysis of plots of log $(1/y - 1)$ vs pH. For 5-MeTA- and $4-NH_2-3-NO_2$-cinn-chymotrypsins $y = \Delta\nu_{C=O}/\Delta\nu_{C=O,max}$, while for IA-chymotrypsin y = (peak intensity of 1729-cm^{-1} band)/(maximum peak intensity of 1729-cm^{-1} band).

RESULTS AND DISCUSSION

(*i*) *Types of Carbonyl Group Observed in the Active Site of O-(Arylacryloyl)chymotrypsins*. Previous RR studies of *O*-(arylacryloyl)chymotrypsins from this laboratory in which the acyl group was based on furylacrylic acid, thienylacrylic acid, or derivatives thereof indicated the presence of two main carbonyl populations in the active site of acylchymotrypsins at low pH (MacClement et al., 1981). The $\nu_{C=O}$ for these acyl-enzymes is characterized by a narrow band at ca.

Table I: Vibrational Spectroscopic Data for *O*-(Arylacryloyl)chymotrypsins

acylchymotrypsin	pH	$\nu_{C=O}$ (cm^{-1})	$\nu_{C=C}$ (cm^{-1})
IA	3.0	1702	1615
	10.0	1729, 1695	1621
5-MeTA	3.0	1725, 1696	1613
	9.0	1725, 1681	1613
4-NH$_2$-3-NO$_2$-cinn	3.0	1703	1626
	9.0	1691	1626
FA	3.0	1728, 1702	1630
	10.0	1728, 1699	1630
TA	3.0	1723, 1696	1612
	10.0	1726, 1696	1612
5-EtFA	3.0	1713–1680	1623
	10.0	1728–1680	1623

Table II: Vibrational Spectroscopic Data for Arylacrylic Acid Methyl Esters

compd	solvent	$\nu_{C=O}$ (cm^{-1})	$\nu_{1/2}$(C=O) (cm^{-1})^a	$\nu_{C=C}$ (cm^{-1})	$\nu_{1/2}$(C=C) (cm^{-1})^b
IA	hexane	1721	25	1630	28
	acetonitrile	1708	27	1624	28
	H$_2$O	1685^c	35^c	1620	nd^d
5-MeTA	hexane	1722	22	1626	31
	acetonitrile	1709	30	1623	30
	H$_2$O	1686^c	nd	1619	nd
4-NH$_2$-3-NO$_2$-cinn	hexane	1730	27	1629	29
	acetonitrile	1710	29	1627	30
	H$_2$O	1685^c	nd	1623	nd

[a] Full width at half-height for $\nu_{C=O}$. [b] Full width at half-height for $\nu_{C=C}$. [c] $\nu_{C=O}$ band shape and position determined in acetonitrile/^2H_2O (5:95) by FT-IR spectroscopy. Other spectral parameters were determined by Raman spectroscopy. [d] nd, not determined.

1720–1725 cm^{-1} and a broader band near 1700 cm^{-1} (Table I). On the basis of spectroscopic studies of arylacrylic acid methyl esters in various solvents (Table II; MacClement et al., 1981), $\nu_{C=O}$ at 1720–1725 cm^{-1} is consistent with a carbonyl group in an environment in which there are minimal solute–solvent interactions. In contrast $\nu_{C=O}$ at ca. 1700 cm^{-1} is consistent with a carbonyl group in a polar environment in which there are significant solute–solvent interactions (which may include hydrogen bonds) which result in a decrease in double bond character of the carbonyl bond. An additional observation, which is useful when the acyl-enzyme data are considered, is that the position of the $\nu_{C=O}$ band for model esters (Table II) is sensitive to solvent but insensitive to the acyl moiety.

In this study the $\nu_{C=O}$ profiles for three acyl-enzymes at low pH are characterized by two main carbonyl populations with $\nu_{C=O}$'s at 1720–1725 and 1700 cm^{-1} (FA-, TA- and 5-MeTA-chymotrypsin; Table I). In contrast $\nu_{C=O}$ for IA- and 4-NH$_2$-3-NO$_2$-cinn-chymotrypsin give rise to a single broad feature at 1702 and 1703 cm^{-1}, respectively, while $\nu_{C=O}$ for 5-EtFA-chymotrypsin indicates the presence of multiple carbonyl populations at low pH (Table I).

The X-ray structure of IA-chymotrypsin at pH 4.0 has been determined by Henderson (1970). Although the indoleacryloyl moiety is bound in the P$_1$ specificity pocket, the reactive carbonyl group is nonproductively bound in the active site. Instead of being located in the oxyanion hole, the carbonyl oxygen is hydrogen-bonded to two water molecules. One water molecule is strongly hydrogen-bonded between the arylacryloyl carbonyl oxygen and the positively charged imidazole side chain of His-57. The second water molecule is hydrogen-bonded to the carbonyl group of Phe-41 and forms only a weak hydrogen bond with the arylacryloyl carbonyl oxygen atom. In the RR spectrum of IA-chymotrypsin $\nu_{C=O}$ is a broad feature centered at 1702 cm^{-1} (Figure 1). The position and band shape of $\nu_{C=O}$ is consistent with a single hydrogen-bonded population of carbonyl groups. It is probable that the hydrogen-bonded feature seen near this frequency in some or all of the other acyl-enzymes at low pH is also due to a "Henderson-type" structure.

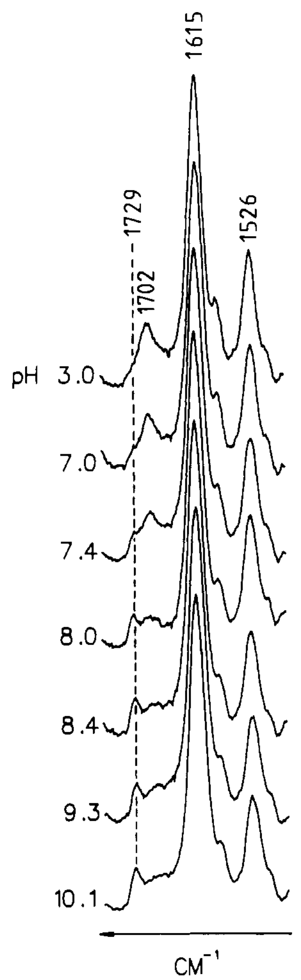

FIGURE 1: 324 nm excited RR spectra of (indoleacryloyl)chymotrypsin obtained in a flow system as a function of pH. Spectral conditions: 100-mW laser power; spectral slit 12 cm^{-1}.

At high pH the acyl-enzymes are active, and deacylation occurs. Deacylation from the Henderson structure requires rotation of the carbonyl group by about 45° and hydrogen bonding in the oxyanion hole (Henderson, 1970; Fersht, 1985b). We postulate that the hydrogen-bonded carbonyl population observed in the acyl-enzymes at high pH is bound more or less tightly in the oxyanion hole. Binding of the arylacryloyl carbonyl group at or near the oxyanion hole is necessary for (a) correct positioning of the attacking water molecule coupled to its general-base catalyst, the imidazole side chain of His-57, and (b) transition state stabilization by the oxyanion hole (Fersht, 1985b).

(*ii*) *$\nu_{C=O}$ Titrates with the pK_a of His-57.* For three acyl-enzymes, IA-, 5-MeTA-, and 4-NH$_2$-3-NO$_2$-cinn-chymotrypsin, $\nu_{C=O}$ in the RR spectrum has been measured as a function of pH. In each case the observed pK_a is the same as that found for the deacylation rate constant of the acyl-enzyme (Table III). Therefore, the change in $\nu_{C=O}$ is directly related to the protonation state of the His-57 imidazole residue and hence to the activity of the acyl-enzyme.

(*A*) *IA-chymotrypsin.* The RR spectrum of IA-chymotrypsin at pH 3.0 in the region 1500–1800 cm^{-1} is shown in Figure 1 (top). The intense band at 1615 cm^{-1} is assigned to the arylacryloyl ethylenic stretching vibration $\nu_{C=C}$, and the band at 1702 cm^{-1} is assigned to the arylacryloyl carbonyl

Table III: pK_a Values for k_{obs} and $\nu_{C=O}$

acylchymotrypsin	pK_a(k_{obs})	pK_a($\nu_{C=O}$)
IA	7.82 ± 0.05	7.70 ± 0.07[a]
5-MeTA	7.51 ± 0.10	7.38 ± 0.05[b]
4-NH_2-3-NO_2-cinn	7.43 ± 0.12	7.43 ± 0.11[c]

[a] Intensity change of 1729-cm^{-1} band followed relative to the 1431-cm^{-1} band (not shown). [b] pK_a for alteration in $\nu_{C=O}$ position; 1696–1681 cm^{-1}. [c] pK_a for alteration in $\nu_{C=O}$ position; 1703–1691 cm^{-1}.

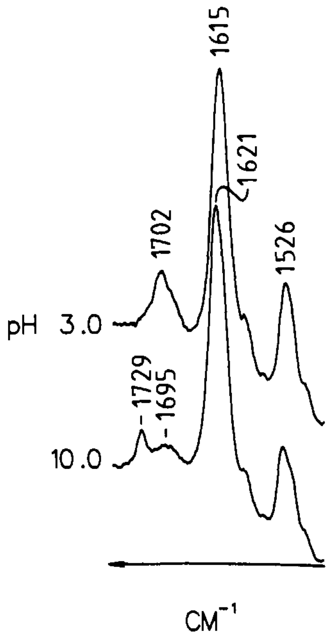

FIGURE 2: 337.5 nm excited RR spectra of (indoleacryloyl)chymotrypsin obtained in a flow system at pH 3.0 and 10.0. Spectral conditions as in legend to Figure 1.

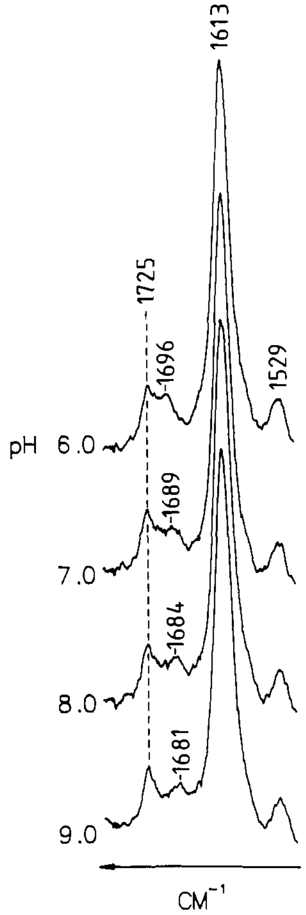

FIGURE 3: 324 nm excited RR spectra of [(5-methylthienyl)acryloyl]chymotrypsin in a flow system as a function of pH. Spectral conditions as in legend to Figure 1.

group $\nu_{C=O}$. As the pH is raised (Figure 1), there is a major alteration in the $\nu_{C=O}$ profile characterized by the appearance of a peak at 1729 cm^{-1}. The intensity of the 1729-cm^{-1} band titrates with a pK_a of 7.70 (±0.07, Table III). As this pK_a is the same, within experimental error, as the value obtained from the pH titration of kinetic activity [Table III, pK_a = 7.82 ± 0.05; Bernhard and Tashjian (1965), pK_a = 7.70], we conclude that the change in $\nu_{C=O}$ reflects the protonation state of the residue which controls the rate of deacylation, namely, the imidazole residue of His-57 (Polgâr & Halâsz, 1982). It can be seen that residual intensity remains at high pH between the band at 1729 cm^{-1} and $\nu_{C=C}$ (Figure 1). The spectra in Figure 1 were obtained with 324-nm excitation. With 337.5-nm laser excitation, the spectral resolution can be improved, and it can be seen in Figure 2 that at high pH an additional band is present at ca. 1695 cm^{-1}. Thus upon deprotonation of the imidazole residue, the single hydrogen-bonded Henderson-type carbonyl population is replaced by two populations of carbonyl groups, one with $\nu_{C=O}$ = 1729 cm^{-1} characteristic of a carbonyl in a nonbonding environment and the other with $\nu_{C=O}$ = 1695 cm^{-1} characteristic of a carbonyl in a hydrogen-bonded environment.

The pK_a of the pH-dependent change in $\nu_{C=O}$ has been determined from the change in intensity of the 1729-cm^{-1} band, which we believe to result from a nonproductively bound acyl carbonyl species (see below). However in all likelihood, generation of the productive carbonyl population with $\nu_{C=O}$ = 1695 cm^{-1} also occurs with the same pK_a as that measured for the 1729-cm^{-1} feature.

One additional pH-dependent change in the RR spectra is that $\nu_{C=C}$ shifts from 1615 cm^{-1} at pH 3.0 to 1621 cm^{-1} at pH 10.0. This indicates that there is more ground-state π-electron polarization in the indoleacryloyl moiety at low compared to high pH (MacClement et al., 1981).

(*B*) *5-MeTA-chymotrypsin.* The RR spectrum of 5-MeTA-chymotrypsin in the region 1500–1800 cm^{-1} at pH 6.0 is shown in Figure 3 (top). This spectrum is identical with that observed at pH 3.0 (data not shown). The intense band at 1613 cm^{-1} is assigned to $\nu_{C=C}$, the ethylenic stretching vibration (MacClement et al., 1981). The $\nu_{C=O}$ profile for 5-MeTA-chymotrypsin is comprised of two bands at 1725 and 1696 cm^{-1} (Figure 3; Carey & Phelps, 1983). This is evidence that in 5-MeTA-chymotrypsin at low pH the acyl group is bound in two conformations. The carbonyl band at 1725 cm^{-1} has the same position and band shape as $\nu_{C=O}$ for (5-methylthienyl)acrylic acid methyl ester in hexane (Table II; MacClement et al., 1981). Thus, the 1725-cm^{-1} acyl-enzyme peak is interpreted as being due to a carbonyl group in a nonbonding environment. The second acyl-enzyme peak in the RR spectrum is broader and appears at 1696 cm^{-1}. On the basis of $\nu_{C=O}$ profiles for (5-methylthienyl)acrylic acid methyl ester in various solvents (Table II), the 1696-cm^{-1} band is consistent with a hydrogen-bonded population of carbonyl groups. It is possible that the hydrogen-bonded conformer is due to a Henderson-type structure as observed in IA-chymotrypsin.

5-MeTA-chymotrypsin has previously been studied at both low and active pH with RR spectroscopy (Phelps et al., 1981). Improvements in spectral signal to noise now enable us to observe $\nu_{C=O}$ as a function of pH. Spectra of 5-MeTA-chymotrypsin at several pH values are shown in Figure 3. As the pH is raised, the carbonyl band at 1696 cm^{-1} (pH 6.0) de-

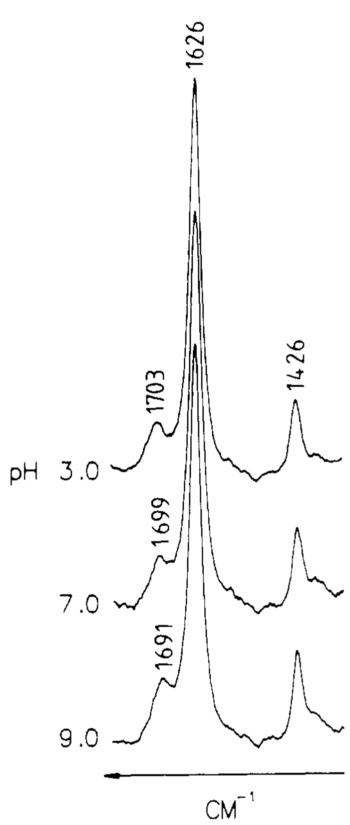

FIGURE 4: 324 nm excited RR spectra of (4-amino-3-nitrocinnamoyl)chymotrypsin in a flow system as a function of pH. Spectral conditions as in legend to Figure 1.

creases in frequency to 1681 cm^{-1} (pH 9.0). The intensity of this band also decreases slightly as the pH is increased. There are no other pH-dependent alterations detected in the RR spectrum. The pH-dependent decrease in $\nu_{C=O}$ occurs with pK_a = 7.51 (±0.10), which is the same within experimental error as the deacylation pK_a (7.38 ± 0.05, Table III). Thus the change in $\nu_{C=O}$ is directly coupled to the protonation state of the His-57 imidazole residue and provides evidence that the "new" feature appearing at 1681 cm^{-1} is associated with the active form of the intermediate from which deacylation occurs. Assignment of the 1681-cm^{-1} band to an active species is further strengthened by the fact that its appearance at 1681 cm^{-1} is fully consistent with hydrogen bonding in an oxyanion hole like environment. An additional source of polarization for $\nu_{C=O}$ is the approach of the water molecule near the point of nucleophilic attack.

There is also a significant population of nonbonded carbonyl groups in the active site with $\nu_{C=O}$ = 1725 cm^{-1}. As this band is not affected upon activation of the acyl-enzyme (Figure 3), it is likely that this population is not attacked in deacylation and represents a nonproductive conformer of the acyl-enzyme. Due to the similarities in band shape and peak position, it is possible that the nonbonded carbonyl observed at 1725 cm^{-1} in the spectrum of 5-MeTA-chymotrypsin (Figure 3) results from a similar structure to that giving rise to the 1729-cm^{-1} band in the high-pH spectrum of IA-chymotrypsin (Figures 1 and 2). As no time dependence after mixing was detected in the carbonyl profiles of IA-chymotrypsin and 5-MeTA-chymotrypsin at high pH (data not shown), interconversion between the nonproductive and productive carbonyl species in these two acyl-enzymes must occur faster than the rate of deacylation.

(*C*) *4-NH$_2$-3-NO$_2$-cinn-chymotrypsin*. This acyl-enzyme has previously been studied at both low and active pH with RR spectroscopy (Carey & Schneider, 1976; Phelps et al., 1981). As for 5-MeTA-chymotrypsin, improvements in signal to noise ratio in the RR spectra now permit us to study $\nu_{C=O}$ for 4-NH$_2$-3-NO$_2$-cinn-chymotrypsin at both low and active pH.

Table IV: Reactivity Coefficients for Acyl-Enzymes

compd	$10^2 k_{obs(lim)}$ (s^{-1})^a	$10^2 k_{(OH^-)}$ (s^{-1})^b	$k_{obs(lim)}/k_{(OH^-)}$
4-NH$_2$-3-NO$_2$-cinn	22.0 ± 0.2	2.0 ± 0.1	11.00 ± 0.05
5-MeTA	3.0 ± 0.2	1.3 ± 0.1	2.31 ± 0.10
IA	0.21 ± 0.02	0.22 ± 0.02	0.96 ± 0.13
5-EtFA	1.1 ± 0.1	1.1 ± 0.1	1.00 ± 0.13
FA	0.29 ± 0.02	1.9 ± 0.1	0.15 ± 0.09
TA	0.51 ± 0.02	2.4 ± 0.1	0.21 ± 0.06

[a] Observed deacylation rate constants for acyl-enzymes: pH 10.5, 0.1 M NaCl, 0.1 M Na$_2$CO$_3$, and 25 ± 0.1 °C. [b] Base-catalyzed hydrolysis rate constants for imidazole esters: pH 10.5, 0.1 M NaCl, 0.1 M Na$_2$CO$_3$, and 25 ± 0.1 °C.

The RR spectrum of 4-NH$_2$-3-NO$_2$-cinn-chymotrypsin at pH 3.0 in the region 1500–1800 cm^{-1} is shown in Figure 4 (top). The intense band at 1626 cm^{-1} is assigned to the ethylenic stretching vibration $\nu_{C=C}$ (Phelps et al., 1981). The $\nu_{C=O}$ for 4-NH$_2$-3-NO$_2$-cinn-chymotrypsin is a single feature at 1703 cm^{-1}. The position of this feature is similar to that observed for $\nu_{C=O}$ of IA-chymotrypsin at low pH (Figures 1 and 2; Table I). On this basis it seems likely that 4-NH$_2$-3-NO$_2$-cinn-chymotrypsin and IA-chymotrypsin have similar structures at low pH in which the arylacryloyl carbonyl oxygen is hydrogen-bonded to one or more water molecules, one of those water molecules being in turn hydrogen-bonded to the positively charged imidazole residue of His-57.

Spectra of 4-NH$_2$-3-NO$_2$-cinn-chymotrypsin at three pH values are shown in Figure 4. It can be seen that as the pH is raised $\nu_{C=O}$ decreases in requency from 1703 (pH 3.0) to 1691 cm^{-1} (pH 9.0). The pH-dependent change in $\nu_{C=O}$ occurs with pK_a = 7.43 (±0.11), which is identical with the pK_a of deacylation, 7.43 ± 0.12 (Table III). We may conclude that the pH-dependent change in $\nu_{C=O}$ is directly linked to the ionization state of the His-57 imidazole side chain. This is the only pH-dependent change observed in the RR spectrum of 4-NH$_2$-3-NO$_2$-cinn-chymotrypsin. RR spectra were also obtained for this acyl-enzyme at pH 3.0 and 10.0 with 337.5-nm laser excitation (data not shown). The improved resolution of these spectra confirmed that $\nu_{C=O}$ remained a single band over the entire pH range studied.

There is no evidence for a "nonbonded" population of carbonyl groups in the active site of 4-NH$_2$-3-NO$_2$-cinn-chymotrypsin at either low or high pH. As the carbonyl must be at or near the oxyanion hole in order for deacylation to occur, we assign $\nu_{C=O}$ at 1691 cm^{-1} in the high-pH spectrum of 4-NH$_2$-3-NO$_2$-cinn-chymotrypsin to a population of carbonyl groups hydrogen-bonded in the oxyanion hole. As discussed above, this assignment also applies to the hydrogen-bonded carbonyl populations seen at high pH in the RR spectra of IA-chymotrypsin (Figure 2, 1695 cm^{-1}) and 5-MeTA-chymotrypsin (Figure 3, 1681 cm^{-1}).

(*iii*) *Acyl-Enzyme Reactivity Is Related to the Population of Hydrogen-Bonded Carbonyl Groups at High pH*. We make the assumption that for any acyl-enzyme the ratio of peak intensities for the non-hydrogen-bonded and hydrogen-bonded features gives the ratio of the populations of the two species. Since RR peak intensities are affected by additional factors, this assumption can only be regarded as a working hypothesis.

The observed deacylation rate constant (k_{obs}) for each acyl-enzyme was essentially pH independent over the pH range 9.5–11.0. Maximal deacylation rate constants [$k_{obs(lim)}$] determined at pH 10.5 are given in Table IV. For comparative purposes $k_{(\mathrm{OH}^-)}$ for the corresponding imidazole esters at pH 10.5 are also given. $k_{(\mathrm{OH}^-)}$ reflects the electron-withdrawing or -donating power of the acyl groups, and in general, the trends in $k_{\mathrm{obs(lim)}}$ and $k_{\mathrm{obs(lim)}}/k_{(\mathrm{OH}^-)}$ are the same. We propose that the reactivity of each acyl-enzyme is related to the amount of hydrogen-bonded carbonyl present at high pH as determined by RR spectroscopy and also to the degree of polarization of this hydrogen-bonded population. With regard to the postulate regarding the dependence of rate on the amount of active acyl-enzyme present, it can be shown that k_{obs} is related to the ratio of active and inactive species by the expression $k_{\mathrm{obs}} = k_3/(1 + 1/K_{\mathrm{eq}})$, where k_3 is the deacylation rate constant for the active acyl-enzyme and K_{eq} = [active]/[inactive] species.

In our model the active acyl group conformer at high pH is one in which the carbonyl group is hydrogen-bonded in the oxyanion hole. According to transition-state theory, there are two sources of rate acceleration: (a) raising the energy of the ground state (in our case the acyl-enzyme); (b) lowering the energy of the transition state (in our case approximating to the tetrahedral intermediate for deacylation). Both a and b decrease the energy gap between ground and transition state and give rise to rate acceleration. The evidence from the present work is that the reactive acyl-enzyme has a more polarized carbonyl compared to inactive species such as a Henderson structure or one in which C=O is in a non-hydrogen-bonding environment. We cannot say from our data if the effect of polarizing C=O destabilizes the overall acyl-enzyme energy. However, it is apparent that the carbonyl is being placed in an environment where favorable hydrogen bonds can occur and which will be of high utility in stabilizing $-\mathrm{C{-}O}^-$ in the tetrahedral intermediate. Thus it is likely that the modest change in $\nu_{\mathrm{C=O}}$ we see in the acyl-enzymes at active pH presages very favorable tetrahedral intermediate–oxyanion hole interactions which themselves are the main source of rate acceleration. This conclusion is consonant with existing views on the source of catalytic rate enhancement (Kraut, 1988).

4-$\mathrm{NH_2}$-3-$\mathrm{NO_2}$-cinn-chymotrypsin has the highest deacylation rate constant (2.2×10^{-1} s^{-1}, Table IV). This results from the fact that all the reactive carbonyl groups in the active site are hydrogen-bonded in the oxyanion hole at high pH. There is no second population of nonproductively bound carbonyl groups. In contrast, both IA-chymotrypsin and 5-MeTA-chymotrypsin have significant populations of nonproductively bound carbonyl groups at high pH. This is reflected in the lower deacylation rate constants for these two acyl-enzymes, viz., 2.1×10^{-3} s^{-1} for IA-chymotrypsin and 3.0×10^{-2} s^{-1} for 5-MeTA-chymotrypsin (Table IV). The fact that $k_{\mathrm{obs(lim)}}$ for 5-MeTA-chymotrypsin is higher than that for IA-chymotrypsin may be ascribed to the fact that the reactive hydrogen-bonded carbonyl population in 5-MeTA-chymotrypsin is significantly more polarized ($\nu_{\mathrm{C=O}}$ = 1681 cm^{-1}) than that in IA-chymotrypsin ($\nu_{\mathrm{C=O}}$ = 1695 cm^{-1}).

(*iv*) *Other Acyl-Enzymes.* (*A*) *FA-chymotrypsin.* RR spectra of FA-chymotrypsin at pH 3.0 and 10.0 in the region 1500–1800 cm^{-1} are shown in Figure 5. The $\nu_{\mathrm{C=C}}$ for this acyl-enzyme is located at 1630 cm^{-1}. At low pH the carbonyl profile is comprised of two features, a band at 1728 cm^{-1} characteristic of a carbonyl population in a nonbonding environment and a band at 1702 cm^{-1} characteristic of a hydrogen-bonded carbonyl species. On going to pH 10.0 (Figure 5), there is a reduction in intensity in the region of the 1702-cm^{-1} band and a concomitant decrease in the apparent peak position to 1699 cm^{-1} probably resulting from improved resolution. However, the frequency and intensity of the band at 1728 cm^{-1} remains the same. The $k_{\mathrm{obs(lim)}}$ for this acyl-enzyme is 2.9×10^{-3} s^{-1}, which is ca. 75-fold lower than $k_{\mathrm{obs(lim)}}$ for 4-$\mathrm{NH_2}$-3-$\mathrm{NO_2}$-cinn-chymotrypsin (Table IV). This results from (a) a large population of nonproductively bound carbonyl groups at high pH and (b) a productive carbonyl population only relatively weakly hydrogen-bonded in the oxyanion hole as delineated by $\nu_{\mathrm{C=O}}$ = 1699 cm^{-1}.

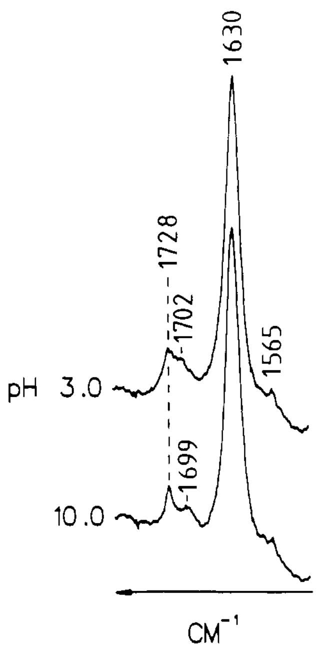

FIGURE 5: 324 nm excited RR spectra of (furylacryloyl)chymotrypsin obtained in a flow system at pH 3.0 and 10.0. Spectral conditions as in legend to Figure 1.

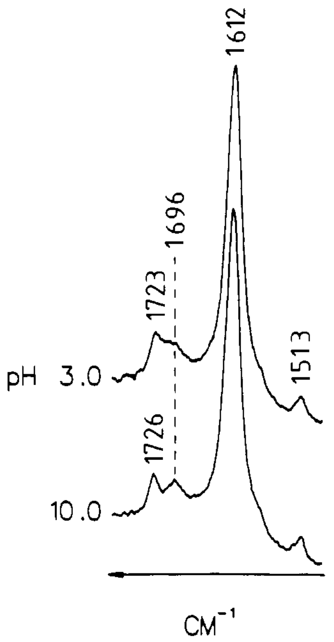

FIGURE 6: 337.5 nm excited RR spectra of (thienylacryloyl)chymotrypsin obtained in a flow system at pH 3.0 and 10.0. Spectral conditions as in legend to Figure 1.

(*B*) *TA-chymotrypsin.* RR spectra of TA-chymotrypsin at pH 3.0 and 10.0 in the region 1500–1800 cm^{-1} are shown in Figure 6. The $\nu_{\mathrm{C=C}}$ for this acyl-enzyme is located at 1612 cm^{-1} (MacClement et al., 1981). The carbonyl profile for TA-chymotrypsin at low pH is similar to that of FA-chymotrypsin with bands at 1723 and 1696 cm^{-1}. Again, these may be ascribed to carbonyl species in nonpolar and hydrogen-

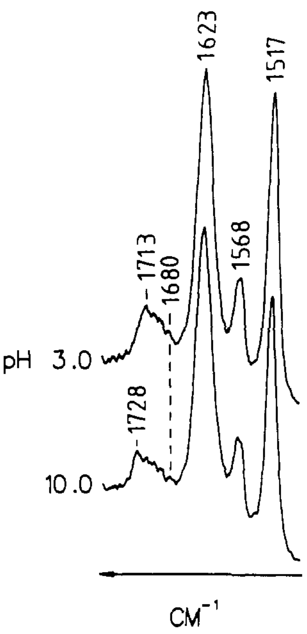

FIGURE 7: 337.5 nm excited RR spectra of [(5-ethylfuryl)acryloyl]chymotrypsin obtained in a flow system at pH 3.0 and 10.0. Spectral conditions as in legend to Figure 1.

bonding environments, respectively. Transition to high pH is characterized by improved resolution of the two carbonyl bands near 1726 and 1696 cm^{-1}. $k_{obs(lim)}$ for TA-chymotrypsin is 5.1×10^{-3} s^{-1}, which is similar to that observed for FA- and IA-chymotrypsin (Table IV). Again, the low relative reactivity of TA-chymotrypsin may be rationalized by the significant population of nonproductively bound carbonyl groups with $\nu_{C=O}$ = 1726 cm^{-1} and a relatively weakly polarized "active" carbonyl population with $\nu_{C=O}$ = 1696 cm^{-1}.

(*C*) *5-EtFA-chymotrypsin.* RR spectra of 5-EtFA-chymotrypsin at pH 3.0 and 10.0 in the region 1500–1800 cm^{-1} are shown in Figure 7. The intense band at 1623 cm^{-1} is assigned to $\nu_{C=C}$ (MacClement et al., 1981). The $\nu_{C=O}$ at low pH is a complex feature with bands from 1713 to 1680 cm^{-1}. At high pH a band appears at 1728 cm^{-1} characteristic in shape and position of a carbonyl group in a nonbonding environment. At high pH the region 1720–1680 cm^{-1} is poorly defined and cannot be used to make semiquantitative statements about reactive carbonyl populations. However, there is certainly intensity in the 1680–1695-cm^{-1} region, and this can account for the "intermediate type" reactivity of 5-EtFA-chymotrypsin seen in Table IV.

(*v*) *Relative Reactivities.* It can be argued that the ratio of $k_{obs(lim)}/k_{(OH^-)}$ for each acyl-enzyme (Table IV) is a more accurate measure of relative reactivity since it takes into account the intrinsic electronic property of each acyl group to stabilize the transition state for base-catalyzed hydrolysis. Thus $k_{obs(lim)}/k_{(OH^-)}$ provides a better measure of the rate acceleration due to the enzyme.

From Table IV it can be seen that the ratios of $k_{obs(lim)}/k_{(OH^-)}$ for the acyl groups generally follow the trend in $k_{obs(lim)}$ seen for the acyl-enzymes. The exception is IA-chymotrypsin, which has $k_{obs(lim)} = 2.1 \times 10^{-3}$ s^{-1}, similar to $k_{obs(lim)}$ for FA- and TA-chymotrypsin but with $k_{obs(lim)}/k_{(OH^-)} = 0.96$ (Table IV). Comparison of the RR carbonyl profiles at high pH for IA- (Figure 2), FA- (Figure 5), and TA- (Figure 6) chymotrypsin suggests similar reactivities for these three acyl-enzymes. Thus on the basis of $k_{obs(lim)}/k_{(OH^-)}$, IA-chymotrypsin is about five times more reactive than might be expected. An explanation for this apparent anomaly is found in the fact that among the six acyl-enzymes studied here IA-chymotrypsin is the only one whose $\nu_{C=C}$ changes upon going to high pH. It can be seen in Figure 2 (Table I) that $\nu_{C=C}$ increases by 6 cm^{-1} upon going from pH 3.0 to pH 10.0. This means that electron polarization through the π-electron chain is reduced at active pH (MacClement et al., 1981; Carey, 1982). This can be caused by, for example, twisting about one of the ethylenic C–C single bonds and has the effect of raising the ground-state energy level of the acyl moiety. If the energy of the rest of the system is little affected, then release of this strain in the transition state will cause rate acceleration. In any event IA-chymotrypsin is in a class of its own since it is the only acyl-enzyme to undergo a major change in π-electron distribution in the ground electronic state upon going to active pH.

The highest $k_{obs(lim)}$ observed in this work is 2.2×10^{-1} s^{-1} for 4-NH$_2$-3-NO$_2$-cinn-chymotrypsin (Table IV). This is similar to k_3 for the nonspecific acyl-enzyme (*N*-acetylglycyl)chymotrypsin (1.1×10^{-1} s^{-1}, pH 7.8; Berezin et al., 1971) but ca. 1000-fold lower than k_3 for the very good acyl-enzyme (*N*-acetyl-L-tyrosyl)chymotrypsin (2.0×10^{2} s^{-1}, pH 7.8; Berezin et al., 1971). Thus, the relative nonspecificity of the acyl-enzymes studied in this work must be noted. Future work in this laboratory is directed at developing a new generation of specific chromophoric substrates with substantially higher $k_{obs(lim)}$ values than those for the acyl-enzymes presented here.

(*vi*) *[4-(Dimethylamino)cinnamoyl]chymotrypsin and the Aldehyde Question.* The RR spectrum of [4-(dimethylamino)cinnamoyl]chymotrypsin (4-DMA-cinn-chymotrypsin) has previously been determined at low pH by Peticolas and co-workers (Weber et al., 1986). The $\nu_{C=O}$ for this acyl-enzyme is a relatively intense feature at 1677 cm^{-1}. The position of $\nu_{C=O}$ was used as evidence for an "aldehyde-like" carbonyl group in the active site possibly resulting from out of plane displacement of the alkyl ester oxygen atom. This hypothesis was advanced as support for the theory proposed by Bernhard and co-workers that the red shift in λ_{max} for (arylacryloyl)acyl-enzymes resulted from an aldehyde-like carbonyl group (Bernhard & Malhotra, 1974). In this regard λ_{max} for *O*-[(arylacryloyl)acyl]-enzymes is similar in each case to λ_{max} for the respective aldehyde model compounds in H$_2$O (Bernhard & Lau, 1972; Breux & Bender, 1976), while $\nu_{C=O}$ at 1677 cm^{-1} for 4-DMA-cinn-chymotrypsin is similar to $\nu_{C=O}$ for 4-(dimethylamino)cinnamaldehyde in a nonpolar environment (Weber et al., 1986). As an extension of the work presented in the present paper we obtained the RR spectrum of 4-DMA-cinn-chymotrypsin at high pH (data not shown). Upon going to high pH there is a slight intensity decrease in $\nu_{C=O}$ for 4-DMA-cinn-chymotrypsin and a modest change in position to 1673 cm^{-1}. On the basis of the position of $\nu_{C=O}$ and the discussion in section v, we might expect 4-DMA-cinn-chymotrypsin to be highly reactive. However, this acyl-enzyme is in fact very unreactive with $k_{obs(lim)} = 1.1 \times 10^{-3}$ s^{-1} and $k_{obs(lim)}/k_{(OH^-)} = 0.2$ (data not shown).

Polarization of the carbonyl group in 4-DMA-cinn-chymotrypsin thus results from some mechanism other than hydrogen bonding in the oxyanion hole. Additionally, the atypically low position of $\nu_{C=O}$ seen at pH 4.0 suggests that this acyl-enzyme is not in a Henderson-type structure. We believe that the acyl moiety in 4-DMA-cinn-chymotrypsin adopts a substantially different conformation in the active site compared to the other acyl-enzymes studied in this paper. Polarization of the carbonyl group in 4-DMA-cinn-chymotrypsin could indeed result from the type of structural deformation discussed by Peticolas and co-workers. Furthermore, it is also possible that the red shift in λ_{max} observed for 4-

DMA-cinn-chymotrypsin is a direct result of this ground-state structural perturbation. We believe however that 4-DMA-cinn-chymotrypsin is an atypical acyl-enzyme. The evidence for the other acyl-enzymes studied here is that the acyl groups are planar (i.e., ester-like) and are not electronically equivalent to aldehydes at low pH. Thus, in all likelihood the red shift in λ_{max} observed for [(arylacryloyl)acyl]-enzymes usually results from excited-state stabilization as predicted by Warshel (Warshel & Russell, 1984). Crucially, whatever the source of RR and absorption spectral perturbations seen for 4-DMA-cinn-chymotrypsin, it is certain that it does not lead to marked rate acceleration.

REFERENCES

Bender, M. L., Schonbaum, G. R., & Zerner, B. (1962) *J. Am. Chem. Soc. 84*, 2540–2550.

Bender, M. L., Beguê-Cantôn, M. L., Blakely, R. L., Brubacher, L. J., Feder, J., Gunter, C. R., Kēzdy, F. J., Killhefer, J. V., Marshall, T. H., Miller, C. G., Roeske, R. W., & Stoops, J. K. (1966) *J. Am. Chem. Soc. 88*, 5890–5913.

Berezin, I. V., Kazanskaya, N. F., & Klyosov, A. A. (1971) *FEBS Lett. 15*, 121–124.

Bernhard, S. A., & Tashjian, Z. H. (1965) *J. Am. Chem. Soc. 87*, 1806–1807.

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

Bernhard, S. A., & Malhotra, O. P. (1974) *Isr. J. Chem. 12*, 471–481.

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

Breaux, E. J., & Bender, M. L. (1976) *Biochem. Biophys. Res. Commun. 70*, 235–240.

Brubacher, L. J., & Bender, M. L. (1966) *J. Am. Chem. Soc. 88*, 5871–5880.

Carey, P. R. (1982) *Biochemical Applications of Raman and Resonance Raman Spectroscopies*, pp 169–177, Academic Press, New York.

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., & Phelps, D. J. (1983) *Can. J. Chem. 61*, 2590–2595.

Carey, P. R., & Sans Cartier, L. R. (1983) *J. Raman Spectrosc. 14*, 271–275.

Fersht, A. R. (1985a) *Enzyme Structure and Mechanism*, 2nd ed., pp 166–167, Freeman, New York.

Fersht, A. R. (1985b) *Enzyme Structure and Mechanism*, 2nd ed., pp 405–413, Freeman, New York.

Gabriel, S., & Herzberg, M. (1883) *Ber. Dtsch. Chem. Ges. 16*, 2036–2043.

Ganapati, K. (1938) *J. Indian Chem. Soc. 15*, 121–128.

Hamilton, S. E., & Zerner, B. (1981) *J. Am. Chem. Soc. 103*, 1827–1831.

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

Hinkle, P. M., & Kirsch, J. F. (1970) *Biochemistry 9*, 4633–4644.

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

Kraut, J. (1977) *Annu. Rev. Biochem. 46*, 331–358.

Kraut, J. (1988) *Science 242*, 533–540.

Kumar, K., & Carey, P. R. (1975) *J. Chem. Phys. 63*, 3697–3707.

MacClement, B. A. E., Carriere, R. G., Phelps, D. J., & Carey, P. R. (1981) *Biochemistry 20*, 3438–3447.

Phelps, D. J., Schneider, H., & Carey, P. R. (1981) *Biochemistry 20*, 3447–3454.

Polgār, L., & Halāsz, P. (1982) *Biochem. J. 207*, 1–10.

Sans Cartier, L. R., Storer, A. C., & Carey, P. R. (1988) *J. Raman Spectrosc. 19*, 117–121.

Traynelis, V. J., Miskel, J. J., & Sowa, J. R. (1957) *J. Org. Chem. 22*, 1269–1270.

Warshel, A., & Russell, S. T. (1984) *Q. Rev. Biophys. 17*, 283–422.

Weber, J. A., Turpin, P. Y., Bernhard, S. A., & Peticolas, W. L. (1986) *Biochemistry 25*, 1912–1917.