Resonance Raman and Fourier Transform Infrared Spectroscopic Studies of the Acyl Carbonyl Group in [3-(5-Methyl-2-thienyl)acryloyl]chymotrypsin: Evidence for Artifacts in the Spectra Obtained by Both Techniques†

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ABSTRACT: The acyl carbonyl group of [3-(5-methyl-2-thienyl)acryloyl]chymotrypsin (5MeTA-chymotrypsin) has been investigated by using both resonance Raman (RR) and Fourier transform infrared (FTIR) spectroscopies. The spectrum of the acyl-enzyme carbonyl group has been obtained as a function of pH over the range 3.0–10.0 in the RR experiments and over the range 3.4–7.6 (p^2H) in the FTIR experiments. The carbonyl spectral profiles obtained by using FTIR spectroscopy are substantially different from the carbonyl profiles obtained by using RR spectroscopy. The FTIR spectra were obtained by subtracting the spectrum of the free enzyme from that of the acyl-enzyme. Use of the active-site inhibitor phenylmethanesulfonyl fluoride demonstrates that part of the intensity observed in the FTIR spectra of 5MeTA-chymotrypsin is due to a subtraction artifact giving rise to enzyme-associated bands, probably from peptide groups perturbed by substrate binding. The enzyme bands can be removed by subtracting the FTIR spectrum of $^{13}C{=}O$ acyl-enzyme from that of $^{12}C{=}O$ acyl-enzyme. Additionally, this procedure reveals that one of the acyl-enzyme carbonyl bands observed at 1727 cm^{-1} using RR spectroscopy is absent in the FTIR acyl-enzyme spectrum. However, a feature near 1720 cm^{-1} can be induced in the FTIR spectrum by actinic light in the near-UV region. Thus, it is proposed that the 1727 cm^{-1} RR carbonyl band results from a population of acyl-enzymes which is generated by exposure to the laser beam during RR data collection. When both the RR and FTIR data are adjusted to remove artifacts, they provide essentially identical carbonyl stretching profiles.

α,β-Unsaturated arylacryloyl compounds have been used for a number of years as active-site-directed resonance Raman (RR) probes of serine and cysteine proteases (Carey & Schneider, 1974; Weber et al., 1986; Carey, 1987). Recent interest has centered on the RR signature of the acyl carbonyl group ($\nu_{C=O}$) in acyl-serine proteases, this being the group that is attacked in the deacylation reaction (Tonge & Carey, 1989). Improvements in instrumentation and sample preparation have permitted the RR spectrum of the acyl carbonyl group to be obtained at alkaline pH where the acyl-enzyme is active, and for a series of acylchymotrypsins and acylsubtilisins, a correlation has been obtained between the frequency of the carbonyl vibration and the rate of deacylation (Tonge & Carey, 1990).

While α,β-unsaturated arylacryloyl compounds are elegant RR probes, they suffer from the drawback that they are not natural protease substrates and consequently react with the enzymes at rates ca. 10–1000-fold lower than specific peptide substrates. However, acyl-enzymes generated from amino acid based ester substrates do not have absorption bands above 300 nm, and this essentially obviates their investigation by RR spectroscopy. In contrast, FTIR difference spectroscopy may have the potential to investigate both chromophoric and nonchromophoric acyl-enzymes.

FTIR spectroscopy has previously been used to characterize substrates bound to triosephosphate isomerase (Belasco & Knowles, 1980), aldolase (Belasco & Knowles, 1983), and citrate synthase (Kurz & Drysdale, 1987). In each case, the investigations centered on the carbonyl groups of the bound substrate molecules. Furthermore acyl-enzymes of chymotrypsin have been under investigation using FTIR spectroscopy by Wharton and co-workers (Wharton et al., 1989). However, their preliminary FTIR studies on α,β-unsaturated (arylacryloyl)acylchymotrypsins revealed differences in the number of carbonyl features and their response to pH changes compared to the data obtained by Carey and co-workers using RR spectroscopy (MacClement et al., 1981; Tonge & Carey, 1989).

This paper presents the results of a combined RR and FTIR investigation of a single acyl-enzyme, [3-(5-methyl-2-thienyl)acryloyl]chymotrypsin (5MeTA-chymotrypsin). The study reveals artifacts present in the spectra derived from both techniques. The consequences of these findings for the use of both RR and FTIR spectroscopies in the study of enzyme-bound intermediates are discussed.

Experimental Procedures

Materials. α-Chymotrypsin (lot 127F-8005) was from Sigma Chemical Co. ^2H_2O (99.8% ^2H) and malonic-*1,3*-$^{13}C_2$ acid (99% ^{13}C) were from MSD Isotopes (Merck Frosst Canada Inc.). 5-Methyl-2-thiophenecarboxaldehyde and 1,1′-carbonyldiimidazole were from Aldrich Chemical Co.

Synthesis. 3-(5-Methyl-2-thienyl)acrylic acid was synthesized from 5-methyl-2-thiophenecarboxaldehyde and malonic acid by the method of Koo et al. (1963). 3-(5-Methyl-2-thienyl)acrylic acid was converted into [3-(5-methyl-2-thienyl)acryloyl]imidazole (5MeTA-imidazole) using 1,1′-carbonyldiimidazole (MacClement et al., 1981). In order to generate substrate labeled with ^{13}C in the carbonyl group, malonic-*1,3*-$^{13}C_2$ acid was used.

Preparation of [3-(5-Methyl-2-thienyl)acryloyl]chymotrypsin. For the RR experiments, [3-(5-methyl-2-thienyl)-

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acryloyl]chymotrypsin (5MeTA-chymotrypsin) was prepared as described in Tonge and Carey (1989) by addition of an excess of imidazole ester substrate dissolved in dimethylformamide to a solution of α-chymotrypsin in H_2O or ^2H_2O. The acyl-enzyme was purified by chromatography on G-25 fine Sephadex at pH 3.0 (H_2O/0.2 M NaCl) or p^{2}H 3.4 (^2H_2O/0.2 M NaCl). The reported p^{2}H values for ^2H_2O solutions were calculated by adding 0.4 to the observed pH meter reading (Glasoe & Long, 1960).

For the FTIR experiments, chymotrypsin was dissolved in ^2H_2O to a concentration of 100 mg/mL. After 72 h, the p^{2}H was adjusted to 3.4 by using ^{2}HCl, and the solution was chromatographed on Sephadex G-25 using 0.2 M NaCl/^2H_2O. Following concentration of the enzyme fractions (Centricon-10, Amicon), active-site assay with *N-trans*-cinnamoylimidazole (Bender et al., 1966) indicated an active enzyme concentration of 3.1 mM (96% active sites/mol of protein). 5MeTA-chymotrypsin was prepared by addition of a quantitative amount of 5MeTA-imidazole dissolved in acetonitrile to an aliquot of the enzyme. For each experiment, the stock enzyme solution was diluted 2-fold either with ^2H_2O or with buffer to raise the p^{2}H. Buffers used were 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), p^{2}H 6.4, 0.2 M MES, p^{2}H 6.8, and 0.2 M phosphate, p^{2}H 7.9 (the p^{2}H after mixing with enzyme is given in Figure 3). In each case, sufficient enzyme was prepared to provide both the sample and reference. In a typical experiment, 0.25 mL of 3.1 mM enzyme was added to 0.25 mL of buffer or ^2H_2O, and two 0.2-mL aliquots were taken and placed in separate vials. To one aliquot (the sample) was added 5 μL of 60 mM 5MeTA-imidazole in acetonitrile, while to the second aliquot (the reference) was added 5 μL of 60 mM imidazole in acetonitrile. "Burst" experiments (see below) on the acyl-enzyme with *p*-nitrophenyl acetate at pH 5.0 revealed that even at p^{2}H 3.4 quantitative acylation had occurred. For the inhibition experiments with phenylmethanesulfonyl fluoride (PMSF), a slight (1.1-fold) excess of the inhibitor was used, and the enzyme (0.2 mL) was added to 11 μL of 30 mM PMSF in acetonitrile (or to 11 μL of acetonitrile), in order to promote inhibitor solubility. In all the experiments, duplicate samples were prepared, and the p^{2}H was monitored throughout the time course of the FTIR experiments to ensure that no variation in p^{2}H occurred during FTIR data collection.

In order to determine the extent of chymotrypsin acylation at p^{2}H 3.4, aliquots of acyl-enzyme were titrated with *p*-nitrophenyl acetate. This reaction was performed at pH 5.0 (0.1 M acetate buffer), and the liberation of *p*-nitrophenol was monitored at 317.5 nm. For this particular assay, formation of the *p*-nitrophenolate anion at ca. 400–420 nm (pH 7.0) could not be easily monitored as any excess 5MeTA-imidazole present in the acyl-enzyme preparation contributed significantly to the absorbance at this wavelength. At pH 5.0, acylation of free enzyme by *p*-nitrophenyl acetate was much more rapid than the deacylation of 5MeTA-chymotrypsin (or of acetylchymotrypsin) which permitted the concentration of free enzyme to be estimated. However, the assay was complicated by the slow increase in absorbance at 317.5 nm due to the slow hydrolysis of the acyl-enzyme at pH 5.0 and formation of 3-(5-methyl-2-thienyl)acrylic acid. To compensate for the slow absorbance change, a double-beam experiment was performed using tandem cells, each tandem cell having a partition separating two compartments. In a typical experiment, 40 μL of acyl-enzyme (ca. 1 mM, p^{2}H 3.4) was added to 2 mL of 0.1 M acetate buffer, pH 5.0, and two 0.9-mL aliquots were pipetted into one compartment of each tandem cell. Simultaneously, 40 μL of 55 mM *p*-nitrophenyl acetate (acetonitrile) was added to 2 mL of the buffer, and two 0.9-mL aliquots of this solution were pipetted into the second compartment of each tandem cell. The two cells were then placed in a double-beam spectrophotometer (Cary 219) thermostated at 25 ± 0.1 °C, the absorbance was zeroed at 317.5 nm, and the sample cell was then inverted. Reaction of *p*-nitrophenyl acetate with free chymotrypsin was characterized by a "fast" burst phase, complete within 150 s, followed by a slow steady-state linear increase in absorbance. Extrapolation of the latter linear phase to $t = 0$ gave the concentration of free enzyme using $\Delta\epsilon_{317.5} = 8130\ M^{-1}\ cm^{-1}$, where $\Delta\epsilon_{317.5} = \epsilon_{317.5}$(*p*-nitrophenol) (9630 $M^{-1}\ cm^{-1}$) − $\epsilon_{317.5}$(*p*-nitrophenyl acetate) (1500 $M^{-1}\ cm^{-1}$) at pH 5.0. By use of this method, the calculated concentration of active enzyme in the absence of 5MeTA-imidazole was within 4% of the values obtained by titration with *p*-nitrophenyl acetate at pH 7.0 and monitoring release of the *p*-nitrophenolate anion at 400 nm or by titration with *N-trans*-cinnamoylimidazole at 335 nm and pH 7.0 (Bender et al., 1966).

Resonance Raman (RR) Instrumentation. RR spectra were obtained at room temperature with 337.5-nm excitation from a Kr^+ laser (Coherent Radiation 3000 K), a Spex 1877 Triplemate, and a Princeton Applied Research OMA III data collection system (1460) interfaced to an intensified photodiode array (1421B-1024-HG). The experimental resolution was 7 cm^{-1}. RR data were collected by using a stirred cuvette or capillary flow system with 90° illumination/collection geometry. For the flow experiments, the sample cell consisted of a 1 mm^2 Suprasil capillary connected to a flow-through stirred cell and two-jet mixer (Tonge & Carey, 1989).

The use of 337.5-nm laser excitation as compared to 324-nm excitation previously used to generate RR spectra of 5MeTA-chymotrypsin (Tonge & Carey, 1989) results in improved resolution of the carbonyl features in the RR spectra. This obtains in part from a decrease in the cm^{-1}/nm coverage at 337.5 nm compared to 324 nm. Thus, at 1650 cm^{-1} from the exciting line with 324-nm excitation, there are 85 cm^{-1}/nm while with 337.5 nm there are 78 cm^{-1}/nm. Additionally, the increase in signal to noise observed with 337.5-nm excitation results from the laser line being closer to the 0–0 vibronic transition of the chromophore (λ_{max} 341 nm, Figure 1) compared to 324-nm laser excitation.

FTIR Instrumentation. FTIR spectra were obtained at room temperature using a Digilab FTS-60 spectrometer with a liquid nitrogen cooled mercury cadmium telluride detector. The sample cell was equipped with CaF_2 windows and a 0.05-nm spacer. Commonly the instrument was purged with N_2 for 30 min prior to data collection, and 256 scans were co-added at a spectral resolution of 2 cm^{-1}. For the acyl-enzyme experiment at p^{2}H 7.6, scanning (32 scans) commenced immediately after sample preparation, and water vapor "noise" in the spectrum was interactively removed with a water vapor spectrum. This allowed the acyl-enzyme spectrum to be acquired within 50 s of mixing ($t_{1/2}$ of the acyl-enzyme in ^2H_2O at p^{2}H 7.6 is ca. 150 s) at which time it was estimated that ca. 80% of the acyl-enzyme remained. In order to avoid the possibility of subtraction artifacts, great care was taken to ensure that both sample and reference solutions contained identical protein concentrations as detailed above. This permitted the difference spectra (acyl-enzyme minus enzyme or $^{12}C{=}O$ acyl-enzyme minus $^{13}C{=}O$ acyl-enzyme) to be obtained by using a subtraction factor of 1.0. For experiments in which the sample and reference were illuminated with UV light prior to or during FTIR data accumulation, a 100-W

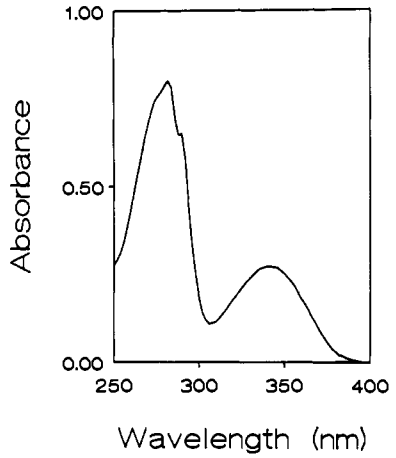

FIGURE 1: UV–visible absorption spectrum of [3-(5-methyl-2-thienyl)acryloyl]chymotrypsin at pH 3.0 (0.1 M NaCl).

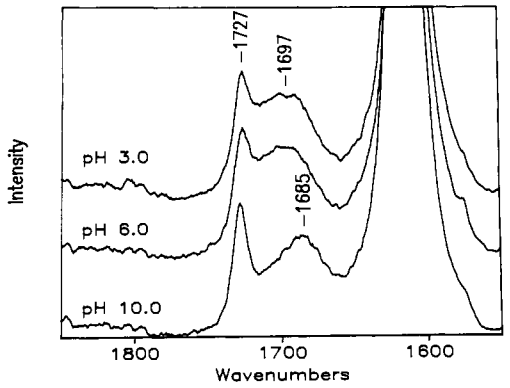

FIGURE 2: Resonance Raman spectra of [3-(5-methyl-2-thienyl)-acryloyl]chymotrypsin as a function of pH in H_2O. At pH 3.0, the acyl-enzyme solution contains 0.1 M NaCl, while at pH 6.0 and 10.0 the acyl-enzyme solution after mixing contains 0.1 M NaCl and 0.1 M phosphate or borate, respectively. Spectra were obtained in a flow system as described in Tonge and Carey (1989) except that 337.5-nm excitation (100 mW) was used. The experimental resolution was 7 cm^{-1}, and the data acquisition time was 10 × 10 s.

mercury arc lamp (Ealing, MA) was used. Illumination of the sample/reference was achieved by fitting the sample compartment door with a quartz window and using an off-axis mirror in the sample compartment. An optical filter was used to remove all light below 300 nm.

Results and Discussion

Resonance Raman Spectra of $\nu_{C=O}$ for 5MeTA-chymotrypsin as a Function of pH. The UV–visible spectrum of 5MeTA-chymotrypsin at pH 3.0 in H_2O is shown in Figure 1; 337.5-nm laser excitation into the 5MeTA π–π^* electronic transition centered at 341 nm (Figure 1) resulted in the RR spectrum shown in Figure 2. In the RR spectrum, the carbonyl region of 5MeTA-chymotrypsin is characterized by a narrow band at 1727 cm^{-1} and a broader feature centered around 1697 cm^{-1}. The band at 1727 cm^{-1} is assigned to a population of carbonyl groups in a nonbonding environment, such as is found for model compounds in carbon tetrachloride or hexane (MacClement et al., 1981; Tonge & Carey, 1989), where there are very weak solute–solvent interactions. In contrast, the broad feature at 1697 cm^{-1} is consistent with a population of carbonyl groups in a hydrogen-bonding environment. The effect of altering the pH on $\nu_{C=O}$ can be seen in Figure 2. The band at 1727 cm^{-1} is essentially unaffected by alteration in pH; however, the apparent maximum of the band at 1697 cm^{-1} (pH 3.0) shifts to 1685 cm^{-1} upon raising the pH. This result has been reported previously (Tonge & Carey, 1989); however, as discussed under Experimental Procedures, the spectra shown herein are of higher resolution due to the use of 337.5-nm excitation as opposed to 324-nm excitation. As discussed before (Tonge & Carey, 1989), the pH-dependent change in $\nu_{C=O}$ reflects the ionization state of the His-57 imidazole residue in the active site, since the pK_a for the change in $\nu_{C=O}$ (7.38 ± 0.05) is the same as that for activation of the catalytic mechanism (7.51 ± 0.10).

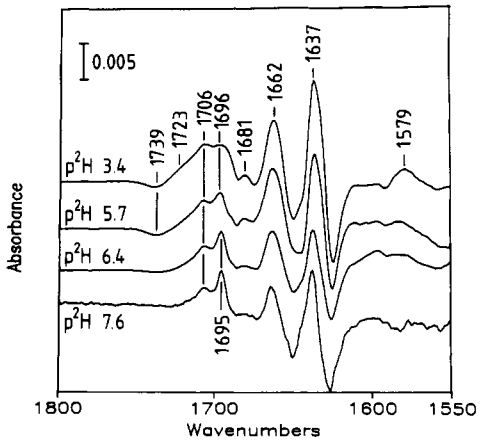

FIGURE 3: FTIR difference spectra of ca. 1.5 mM [3-(5-methyl-2-thienyl)acryloyl]chymotrypsin in ^2H_2O at p^2H 3.4, 5.7, 6.4, and 7.6. The acyl-enzyme (sample) and enzyme (reference) solutions were prepared as described under Experimental Procedures.

FTIR Spectrum of 5MeTA-chymotrypsin in ^2H_2O at p^2H 3.4. The FTIR difference spectrum of 5MeTA-chymotrypsin in ^2H_2O at p^2H 3.4 in the region 1550–1800 cm^{-1} is shown in Figure 3 (top). This spectrum was obtained by subtracting the spectrum of free enzyme plus imidazole from the spectrum of 5MeTA-chymotrypsin. On the basis of RR studies, bands due to the acyl carbonyl portion of the acyl-enzyme would be expected between 1680 and 1730 cm^{-1} ($\nu_{C=O}$). In this region of the FTIR spectrum, there is a broad composite feature comprised of bands at 1696 and 1706 cm^{-1} with a shoulder at ca. 1723 cm^{-1}. At first sight, this profile appears similar to that obtained for the acyl carbonyl group using RR spectroscopy except that in the latter spectrum the band at 1727 cm^{-1} (RR) is more intense. However, that this is not the case will be shown below. Interestingly, major positive bands are also observed at 1637 and 1662 cm^{-1} and negative bands at 1625 and 1649 cm^{-1}. These features were exactly reproduced in six consecutive experiments. It is proposed that binding of the substrate to the enzyme causes a perturbation in the conformation of the protein which results in a small change in the amide I bands from the enzyme's peptide linkages. This results in the incomplete subtraction of amide features when the FTIR spectrum of the enzyme is subtracted from that of the acyl-enzyme.

Finally, there is a positive band at 1579 cm^{-1} in Figure 3. This band may be due to the appearance of COO^- from an enzyme carboxyl group whose pK_a changes upon substrate binding such that in the acyl-enzyme it is ionized (COO^-) while in the free enzyme it is protonated (COO^2H). Further support for this idea comes from the negative peak at ca. 1739 cm^{-1} which may correspond to the disappearance of COO^2H in the acyl-enzyme, since this is in the region where a protonated carboxyl group is expected to occur (Rothschild et al., 1990).

FTIR Spectra of $\nu_{C=O}$ for 5MeTA-chymotrypsin as a Function of pH. The minor differences between the RR and FTIR "carbonyl" profiles in the 1680–1730 cm^{-1} region seen near pH 3.0 become major differences near neutral pH. FTIR difference spectra of 5MeTA-chymotrypsin in ^2H_2O in the p^2H

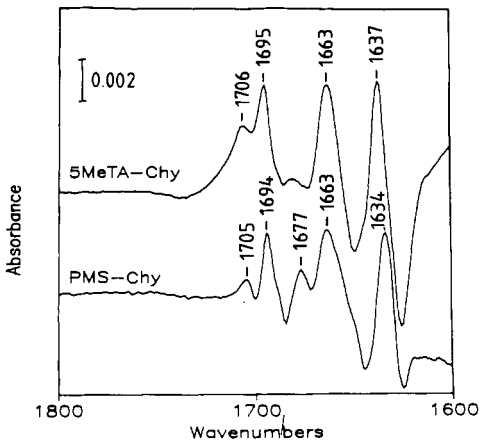

FIGURE 4: FTIR difference spectra of [3-(5-methyl-2-thienyl)-acryloyl]chymotrypsin (5MeTA-Chy) and (phenylmethanesulfonyl)chymotrypsin (PMS-Chy) at p^{2}H 6.4 in ^2H_2O. The acyl-enzyme or inhibitor–enzyme (sample) and enzyme (reference) solutions were prepared as described under Experimental Procedures.

range 3.4–7.6 are shown in Figure 3. The response to the FTIR bands at 1696 and 1706 cm^{-1} to changes in p^{2}H in the region 3.4–6.4 contrasts sharply with the RR data (Figure 2). There is no evidence for a change in the RR profile over this pH range. However, in the FTIR data, as the p^{2}H is raised, the band at 1696 cm^{-1} is seen to increase in relative intensity. Additionally, comparison of the FTIR spectra at p^{2}H 6.4 and 7.6 indicates that there is no evidence for a change in band position which could correspond to the change in band position seen in the RR spectra and which occurs with a pK_a of 7.4 (Tonge & Carey, 1989). However, due to the need to record the FTIR data before significant deacylation occurred, the highest pH possible using FTIR was p^{2}H 7.6, at which pH the His-57 imidazole side-chain population is expected to be only approximately half-ionized.

The bands at 1579 (positive) and 1739 cm^{-1} (negative) also disappear as the p^{2}H is raised from 3.4 to 6.4. This is consistent with the assignment of these bands to a carboxyl group with a pK_a(^2H_2O) < 3.4 in the acyl-enzyme and a pK_a(^2H_2O) of between 3.4 and 6.4 in the free enzyme. One possible assignment of this band is to the carboxyl side chain of Asp-194 which forms a salt bridge with the α-amino group of Ile-16 in the "active" conformation of the enzyme (Hess, 1971). If the pK_a of this group is ca. 4–5 in the free enzyme, then at p^{2}H 3.4 this group will be protonated and consequently the protein will be in an inactive conformation. It is possible that binding of the substrate induces the active conformation in which the carboxyl is ionized and, if this is the case at p^{2}H 3.4, must therefore have a pK_a(^2H_2O) < 3.4 in the acyl-enzyme. This substrate-induced alteration in the equilibrium between active and inactive conformations is analogous to that observed at alkaline pH where the Ile-16 α-amino group (pK_a = 8.5) is deprotonated (Hess, 1971).

FTIR Spectra of (Phenylmethanesulfonyl)chymotrypsin. The FTIR difference spectra of chymotrypsin inhibited with phenylmethanesulfonyl fluoride (PMSF) minus free enzyme at p^{2}H 6.4 are shown in Figure 4. As seen in Figure 4, there is a remarkable correspondence between the FTIR difference spectra of (phenylmethanesulfonyl)chymotrypsin (PMS-chymotrypsin) and 5MeTA-chymotrypsin. In particular, the difference spectra of PMS-chymotrypsin show bands at 1694 and 1705 cm^{-1}. However, the bands in the PMS-chymotrypsin spectra cannot be assigned to $\nu_{C=O}$ for the inhibitor as PMSF possesses no carbonyl groups. Indeed, FTIR spectra of PMSF free in solution show no bands in the region 1450–1850 cm^{-1}

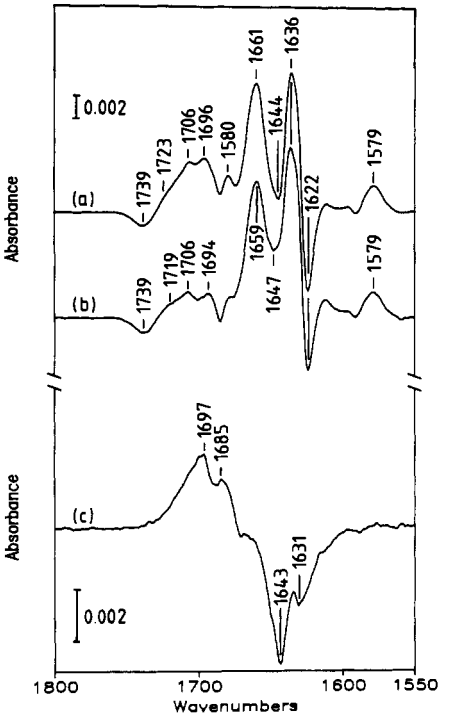

FIGURE 5: FTIR difference spectra of [3-(5-methyl-2-thienyl)-acryloyl]chymotrypsin (5MeTA-chymotrypsin) in ^2H_2O at p^{2}H 3.4. (a) FTIR spectrum of enzyme subtracted from the spectrum of unlabeled 5MeTA-chymotrypsin. (b) FTIR spectrum of enzyme subtracted from the spectrum of ^{13}C=O-labeled 5MeTA-chymotrypsin. (c) FTIR spectrum of ^{13}C=O-labeled 5MeTA-chymotrypsin subtracted from the spectrum of unlabeled 5MeTA-chymotrypsin. The ordinate scale of spectrum c is different from that in spectra a and b. The composition of the acyl-enzyme and enzyme solutions is described under Experimental Procedures.

(data not shown). Thus, the bands in the protein difference spectra must result from modes associated with the enzyme rather than the inhibitor. In Figure 4, the spectrum of PMS-chymotrypsin at p^{2}H 6.4 is shown with the spectrum of 5MeTA-chymotrypsin obtained at the same pH and concentration. It can be seen that the integrated intensity in the 1690–1720 cm^{-1} region is greater for the enzyme–substrate complex compared to the enzyme–inhibitor complex. This suggests that for 5MeTA-chymotrypsin there are additional bands contributing to the intensity in this region of the spectrum, i.e., from the acyl carbonyl group. In order to remove the enzyme-associated features and to reveal the true acyl carbonyl FTIR profile, difference spectroscopy using ^{13}C=O-labeled substrate has been performed.

FTIR Spectra of $\nu_{C=O}$ for 5MeTA-chymotrypsin with the Enzyme-Associated Bands Removed. Figures 5 and 6 show the difference spectra of 5MeTA-chymotrypsin with (a) ^{12}C=O-labeled acyl-enzyme minus free enzyme, (b) ^{13}C=O-labeled acyl-enzyme minus free enzyme, and (c) ^{12}C=O-labeled acyl-enzyme minus ^{13}C=O-labeled acyl-enzyme, at p^{2}H 3.4 and 6.5, respectively. At p^{2}H 6.5, the difference spectrum generated by subtracting the spectrum of free enzyme from that of the ^{13}C=O-labeled acyl-enzyme (Figure 6b) is qualitatively similar to that of PMS-chymotrypsin (Figure 4) in the 1690–1715 cm^{-1} region. In fact, as might be predicted, the ^{13}C=O acyl-enzyme data resemble PMS-chymotrypsin even more closely than the ^{12}C=O acyl-enzyme data. In order to reveal the acyl carbonyl data in the 1680–1720 cm^{-1} region uncluttered by peptide modes, the FTIR spectrum of ^{13}C=O-labeled acyl-enzyme has been subtracted from that of the unlabeled acyl-enzyme. The resulting spectra at both p^{2}H 3.4 (Figure 5c) and 6.5 (Figure 6c) are both characterized by

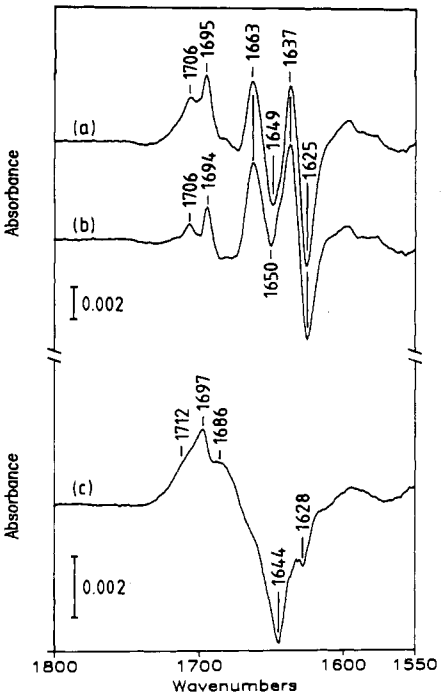

FIGURE 6: FTIR difference spectra of [3-(5-methyl-2-thienyl)-acryloyl]chymotrypsin (5MeTA-chymotrypsin) in ^2H_2O at p^{2}H 6.5. (a) FTIR spectrum of enzyme subtracted from the spectrum of unlabeled 5MeTA-chymotrypsin. (b) FTIR spectrum of enzyme subtracted from the spectrum of $^{13}C{=}O$-labeled 5MeTA-chymotrypsin. (c) FTIR spectrum of $^{13}C{=}O$-labeled 5MeTA-chymotrypsin subtracted from the spectrum of unlabeled 5MeTA-chymotrypsin. The ordinate scale of spectrum c is different from that in spectra a and b. The composition of the acyl-enzyme and enzyme solutions is described under Experimental Procedures.

positive bands centered around 1697 cm^{-1} and a negative band at ca. 1643 cm^{-1}. The positive band can be assigned to $\nu_{C=O}$ for the $^{12}C{=}O$ acyl-enzyme. The negative band at 1643 cm^{-1} corresponds in part to the intensity from the acyl carbonyl groups containing ^{13}C atoms; however, other experiments have shown that analysis of protein difference spectra in the region 1670–1600 cm^{-1} is unwise due to improper subtraction of the protein amide I band centered at 1636 cm^{-1} (where the absorbance is ca. 0.9). There is also evidence for a shoulder at ca. 1685 cm^{-1} although this band is on the edge of the negative feature due to $^{13}C{=}O$. Finally, there is evidence for a shoulder around 1712 cm^{-1} which is more apparent in the spectrum obtained at p^{2}H 6.5. Interestingly, the $^{12}C{=}O$ minus $^{13}C{=}O$ FTIR profiles in the range 1680–1720 cm^{-1} at p^{2}H 3.4 and 6.5 are now very similar (Figures 5c and 6c) and do not show the variation with pH observed in the $^{12}C{=}O$ acyl-enzyme minus enzyme spectra (Figure 3). Thus, the pH dependence of the FTIR data is now consonant with that of the RR data seen in Figure 2.

Comparison of $\nu_{C=O}$ for 5MeTA-chymotrypsin Obtained Using FTIR and RR Spectroscopies: Evidence for a Laser-Induced Isomer in the RR Spectra. Figure 7 contains the RR spectrum of 5MeTA-chymotrypsin at p^{2}H 3.4 obtained in ^2H_2O. Also included is a $^{12}C{=}O$ minus $^{13}C{=}O$ acyl-enzyme FTIR difference spectrum obtained at p^{2}H 3.4 (Figure 7, FTIR DARK). There is now better agreement in the $\nu_{C=O}$ profiles obtained by using the two techniques. Thus, both sets of spectra are characterized by a broad band near 1697 cm^{-1}. In addition, it can be seen that the $\nu_{C=O}$ profile obtained by using RR has a shoulder near 1685 cm^{-1} which is analogous to the shoulder observed near this position in the FTIR spectra (Figures 5–7). However, the band at 1727 cm^{-1} observed in the RR spectra is absent in the FTIR difference spectra.

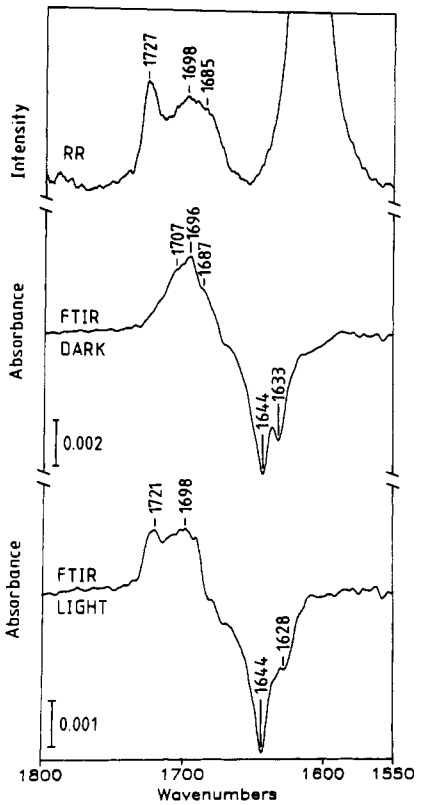

FIGURE 7: Comparison of the FTIR spectra before and after illumination and the RR spectrum of [3-(5-methyl-2-thienyl)acryloyl]chymotrypsin (5MeTA-chymotrypsin) at p^{2}H 3.4 in ^2H_2O (0.1 M NaCl). (Top) RR, RR spectrum of 5MeTA-chymotrypsin obtained by using a stirred cuvette with 100 mW of 337.5-nm laser excitation and an acquisition time of 10 × 10 s. (Center) FTIR DARK, FTIR difference spectrum of 5MeTA-chymotrypsin obtained by subtracting the spectrum of $^{13}C{=}O$-labeled 5MeTA-chymotrypsin from the spectrum of unlabeled 5MeTA-chymotrypsin. (Bottom) FTIR LIGHT, FTIR difference spectrum of 5MeTA-chymotrypsin after exposure with the mercury arc lamp. The spectrum of $^{13}C{=}O$-labeled 5MeTA-chymotrypsin scanned after exposing the acyl-enzyme solution to 1-min irradiation with the mercury arc lamp, as described under Experimental Procedures, was subtracted from the corresponding spectrum of unlabeled 5MeTA-chymotrypsin scanned after 1-min irradiation. The composition of the acyl-enzyme and enzyme solutions is described under Experimental Procedures. Spectra of the same acyl-enzyme solutions scanned before and after illumination were used to obtain the "FTIR DARK" and "FTIR LIGHT" difference spectra. The "FTIR DARK" and "FTIR LIGHT" spectra are plotted with different ordinate scales.

In order to test whether the 1727 cm^{-1} band originated from a light-induced acyl-enzyme population, two experiments were performed. In the first, the laser power used to generate the RR spectra was varied from 5 to 100 mW. However, no power dependency of the RR spectra was observed (data not shown). In the second experiment, the light dependency of the FTIR difference spectra was monitored. Experiments were performed using a mercury arc lamp and an optical filter to illuminate the acyl-enzyme solutions in the FTIR sample cell with light >300 nm while in the FTIR instruments' sample compartment. The results of this experiment are shown in Figure 7 (FTIR LIGHT), where in the FTIR spectrum of $^{13}C{=}O$ 5MeTA-chymotrypsin after 1-min illumination with the mercury lamp has been subtracted from the corresponding FTIR spectrum of $^{12}C{=}O$ 5MeTA-chymotrypsin after 1-min illumination with the mercury lamp. The "FTIR LIGHT" spectrum clearly shows the presence of a "new" carbonyl feature near 1721 cm^{-1}. This band is not present in the "FTIR DARK" difference spectrum obtained by subtracting the FTIR spectra of the same $^{12}C{=}O$ and $^{13}C{=}O$ acyl-enzyme solutions

scanned prior to illumination. Thus, we have direct evidence for the light-induced formation of an acyl-enzyme population having a carbonyl band with $\nu_{C=O}$ ($^{12}C{=}O$) around 1720 cm^{-1}. On the basis of this result, we assign the 1727 cm^{-1} carbonyl band in the RR spectra to a laser-induced population of acyl groups in the active site.

The "FTIR LIGHT" difference spectrum shown in Figure 7 was obtained after 1-min illumination of both the $^{12}C{=}O$ and $^{13}C{=}O$ acyl-enzyme solutions. Further illumination produced no further change in the difference spectrum. Additionally, the light-induced population remained after illumination had been stopped and indeed did not change at all after a further hour in the FTIR instrument in "dark" conditions. Thus, during the 1-min illumination period, a photoinduced equilibrium was established between the "dark" and "light" acyl-enzyme populations. The energy barrier to interconversion between the dark and light species must be relatively larger in order to hinder relaxation of the light-induced population back into the dark form in the absence of light.

On the basis of the above observations, it is hypothesized that the illumination of the acyl-enzyme causes trans to cis isomerization about the acyl groups' C=C ethylenic bond. The energy barrier for this interconversion is expected to be sufficiently high to trap the cis conformer. There is ample precedence in the literature for such a hypothesis. The trans-cis photoisomerization of related arylacryloyl compounds, such as cinnamic acid, is well documented (Wyman, 1955; Hocking, 1969; Shindo et al., 1984). Additionally, workers have studied the reactivity of the geometric isomers of substituted cinnamic acids with serine proteases and their trans-cis interconversion with light while bound in the enzymes' active site (Martinek & Berezin, 1979; Van Deynse et al., 1987; Turner et al., 1988; Porter & Bruhnke, 1989; Stoddard et al., 1990a,b). Since the FTIR and RR experiments documented above indicate that a photoequilibrium is established between the two acyl-enzyme populations, illumination must also be able to cause the back-reaction, viz., cis to trans isomerization. The properties and photochemistry of these isomers are under active investigation.

Conclusion

The appearance of enzyme-based modes in the 1680–1740 cm^{-1} region, wherein $\nu_{C=O}$ for the acyl carbonyl group is expected, limits the application of FTIR spectroscopy to the study of acyl carbonyl groups in acylchymotrypsins where only $^{12}C{=}O$ acyl groups are used. These difficulties can be largely overcome by subtracting the spectrum of $^{13}C{=}O$-labeled acyl-enzyme from the spectrum of unlabeled acyl-enzyme. However, the region below 1680 cm^{-1} where $^{13}C{=}O$ features are expected is hard to probe due to difficulty in performing an exact subtraction of the high optical densities from amide I modes.

Comparison of the RR and FTIR data reveals the presence of a feature at 1727 cm^{-1} unique to the RR experiment. On the basis of FTIR experiments performed using an actinic mercury arc source, the 1727 cm^{-1} carbonyl band in the RR spectra is assigned to a laser beam induced isomer. While this complicates the interpretation of the RR data, its discovery introduces possibilities for using time-resolved spectroscopy to probe temporal events in the active site and for modulating the deacylation process by light in the near-ultraviolet region.

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