Characterization of *trans*- and *cis*-5-Methylthienylacryloyl Chymotrypsin Using Raman Difference Spectroscopy, NMR, and Kinetics: Carbonyl Environment and Reactivity‖

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Abstract: The interpretation of the resonance Raman (RR) data for chromophoric acyl serine proteases is rendered complex due to the fact that the ≈350-nm laser beam used to generate the RR spectrum causes the substrate to photoisomerize in the active site. As a result, a steady-state population of dark (present before irradiation) and light (photoinduced) conformers is set up. In the present work, 2D NMR experiments are used to confirm that the photoinduced conformer of 5-methylthienylacryloyl chymotrypsin is *cis* about the acryloyl —C=C— linkage, whereas it is *trans* for the dark conformer. Using 488.0- or 514.5-nm excited Raman difference spectroscopy the normal Raman spectra, under nonphotolyzing conditions, of the pure *cis* and *trans* forms of the acyl chymotrypsins are reported for the first time. A number of characteristic bands for the two forms are identified, e.g., the bands at 1616, 1470, and 1534 cm^{-1} for the *trans* form of the intermediate occur at 1611, 1419, and 1527 cm^{-1} in the *cis* form. In particular $\nu_{C=O}$ for the acyl carbonyl occurs at 1727 and 1697 cm^{-1} for the *cis* and *trans* forms, respectively. The two forms have markedly different reactivities with the *trans* isomer deacylating 5000 times faster than the *cis*. These spectroscopic and kinetic properties contrast sharply with the behavior of the model compound 5-methylthienylacryloyl methyl ester. The *cis* and *trans* forms of this ester in CCl_4 have values for $\nu_{C=O}$ at 1720 ± 2 cm^{-1}, and the intrinsic reactivities of the *cis* and *trans* methyl esters to base catalyzed hydrolysis are very similar, with k_{OH^-} of 0.0062 and 0.013 s^{-1} in 1.0 M NaOH, respectively. Thus, the differential reactivities and $\nu_{C=O}$ observed for the *cis* and *trans* acyl chymotrypsins are caused by differential interactions with the enzyme's active site and are not due to the intrinsic properties of the *cis* and *trans* form of the acyl group.

Introduction

The elucidation of the structure and chemistry of enzyme-bound intermediates is a central theme in mechanistic enzymology. Whilst many physical techniques provide detailed information on stable complexes, resonance Raman (RR) spectroscopy due to its sensitivity, selectivity, and rapid data collection permits the characterization, via the vibrational spectrum, of transient enzyme–substrate complexes.[1] Interpretation of the RR spectra is facilitated and quantitated by reference to vibrational studies on simple model compounds. In this way the RR technique can be seen as a vector which carries precise structural and thermodynamic data from well-defined model compounds into the complex active site. This strategy has led to a precise structure–reactivity correlation and an understanding of the factors controlling rate enhancement for a range of aryl-substituted α,β-unsaturated acyl serine proteases.[2]

Investigations of aryl-substituted α,β-unsaturated acyl serine proteases have centered on the acyl carbonyl group, the group undergoing nucleophilic attack in the deacylation reaction. The RR spectra of most α,β-unsaturated acyl chymotrypsins are characterized by two carbonyl features, one around 1725 cm^{-1}, characteristic of a carbonyl population in a nonbonding environment, and the other around 1700–1680 cm^{-1}, characteristic of a carbonyl population in a hydrogen-bonding environment.[3,4] FTIR studies on the acyl enzyme 5-methylthienylacryloyl chymotrypsin using actinic near-UV radiation demonstrated that its 1727-cm^{-1} RR band results from an acyl enzyme population induced by the laser beam used to obtain the RR spectrum.[5]

Clues to the structure of the photoinduced isomer come from the early studies of Berezin and co-workers[6] and the later studies of Porter et al.[7] on the *trans* to *cis* photoisomerization of cinnamoyl chymotrypsins and thrombins. The present work describes the characterization, via a combination of Raman difference spectroscopy, NMR, and kinetics, of the two acyl enzyme populations of 5-methylthienylacryloyl chymotrypsin present in the RR studies. The 2D NMR studies, performed using ^{13}C labeling to "edit" the complex acyl enzyme proton spectrum, are used to determine the structure of the acyl group. Thus, the isomer present before light exposure is *trans* about the ethylenic linkage, whilst the photoinduced isomer is *cis* about the ethylenic linkage. Raman difference spectroscopy, under nonphotolyzing conditions, of the purified *trans*- and *cis*-acyl enzymes permits unambiguous assignment of bands observed in the RR spectrum.

The vibrational spectra and rates of hydrolysis of the *trans*- and *cis*-acyl enzymes have been compared with data obtained for the *trans*- and *cis*-methyl ester model compounds. These studies reveal that the 5000-fold variation in deacylation rate between the *trans*- and *cis*-acyl enzymes results from the difference in

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active site environments of the acyl carbonyl group as revealed by the Raman studies.

Experimental Section

Materials. α-Chymotrypsin (lot 100H8275) was from Sigma Chemical Co. 5-Methylthienylacryloyl imidazole, ^{12}C, $^{13}C{=}O$, and $-C{=}{}^{13}C-C{=}O$ were synthesized as described previously.[5] Malonic-*1,3*-$^{13}C_2$ acid and malonic-*2*-^{13}C acid (each 99% ^{13}C), used to synthesize the $^{13}C{=}O$ and $-C{=}{}^{13}C-C{=}O$ substrate derivatives, respectively, and ^2H_2O (99.8% ^2H) were from MSD Isotopes (Merck Frosst Canada Inc.).

Acyl Enzyme Preparation. For the Raman difference experiments *trans*-5-methylthienylacryloyl chymotrypsin was prepared as described previously for the FTIR experiments by adding slightly less than excess of the *trans* substrate in acetonitrile to a sample of ca. 6 mM chymotrypsin at pH 3.5 in 0.1 M phosphate buffer. The corresponding reference sample was prepared by adding the same volume of acetonitrile to an identical enzyme sample.[5] For the NMR experiments the *trans*-acyl enzyme was prepared by adding excess substrate to enzyme at pH 3.0 followed by chromatography on G-25 to purify the acyl enzyme and subsequent concentration using Amicon centricon-10 concentrators. During the concentration procedure the enzyme was exchanged into ^2H_2O containing 0.3 M KCl and 2 mM ^2HCl. The final concentration of acyl enzyme was ca. 1–2 mM.

Preparation of the photoinduced acyl enzyme was as described in Tonge and Carey, 1992.[2] A sample of *trans*-acyl enzyme at pH 3.0 was irradiated with a 100 W mercury arc lamp using a filter to remove light <300 nm which established a photostationary population of *trans* and photoinduced acyl enzymes. Formation of the photochemical equilibrium was monitored by UV spectroscopy using a Cary 3 spectrometer (Varian). Subsequently the pH of the acyl enzyme solution was raised to 10.0 by addition of solid Na_2CO_3 for ca. 10 min in order to hydrolyze the more labile *trans*-acyl enzyme (k_3 (*trans*) 0.031 s^{-1}). Following this the photoinduced acyl enzyme was separated from free enzyme by chromatography on the affinity resin 4-phenylbutylamine sepharose at pH 8.0. Fractions containing acyl enzyme were adjusted to pH 3.0 and concentrated using a 50-mL Amicon stirred cell (10 kD YM10 membrane) followed by a Centricon-10 concentrator (Amicon). Samples for Raman spectroscopy were exchanged into 0.1 M phosphate buffer pH 3.0 during the concentration procedure whilst samples for NMR spectroscopy were exchanged into ^2H_2O containing 0.3 M KCl and 2 mM ^2HCl. Final concentration of the photoinduced acyl enzyme was 6 and 1 mM for the Raman and NMR experiments, respectively.

Model Compound Characterization. *trans*-5-Methylthienylacryloyl methyl ester (λ_{max} 317 nm, acetonitrile) was synthesized by dissolving *trans*-5-methylthienylacryloyl imidazole in methanol and standing for 4 h. The methyl ester was purified by HPLC (Gilson) on a C18 Waters Delta Pak reverse phase column (1.9 × 30 cm) using acetonitrile/water (90:10) as eluent (3 mL/min). *cis*-5-Methylthienylacryloyl methyl ester (λ_{max} 317 nm, acetonitrile) was prepared by irradiating a sample of the *trans*-methyl ester with a 100-W mercury arc lamp to form a photostationary population of the *trans* and *cis* esters by photoisomerization. The *trans* and *cis* esters were separated by HPLC, the *trans* ester eluting at 32.5 min and the *cis* ester at 30.5 min under the conditions described above. Base catalyzed hydrolysis rates were determined by diluting a sample of each ester into 1.0 M NaOH and monitoring the absorbance decrease at 340 nm. After hydrolysis was complete the reaction mixture was acidified to pH 2 with concentrated HCl and chromatographed on the C18 colmun using methanol/0.1 M HCl/H_2O (80:20) as eluent (3 mL/min). *trans*-5-Methylthienylacrylic acid eluted at 34.0 min and the *cis* acid at 30.5 min.

Vibrational spectra of *trans*- and *cis*-5-methylthienylacryloyl methyl ester were obtained using a Digilab FTS-60 spectrometer with a liquid nitrogen cooled mercury cadmium telluride detector. A 10 mM solution of the *trans* ester in CCl_4 was scanned (64 scans, 2 cm^{-1} resolution) in a 0.1-mm pathlength cell equipped with KBr windows. The sample in the cell was then irradiated for 5 min using a 100-W mercury arc lamp to generate a photostationary population of the *trans*- and *cis*-methyl esters and rescanned. The spectrum of *cis*-5-methylthienylacryloyl methyl ester was obtained by interactively subtracting the spectrum of *trans*-5-methylthienylacryloyl methyl ester obtained before irradiation. Identical spectra were also obtained of the purified *cis*-methyl ester.

Raman Spectroscopy. Raman spectra were obtained using a Spex Triplemate equipped with a Model DIDA-1000 reticon detector connected to a ST-100 detector (Princeton Instruments, Trenton, NJ).[8] Laser excitation at 488.0 or 514.5 nm was provided by an argon ion laser (Model 165, Spectra Physics, Mountain View, CA). Separate spectra for free enzyme and acyl enzyme were measured using a split cell and sample holder with a linear translator. Sample volume was typically 30 μL. A difference spectrum was obtained by numerically subtracting the enzyme spectrum from the acyl enzyme spectrum. Spectral resolution was 6 cm^{-1}. Spectral calibration was performed using the known Raman lines of toluene, and absolute band positions are accurate to within ±2 cm^{-1}.

NMR Spectroscopy. NMR spectra were obtained at 18 °C using Bruker AM500 and AMX500 spectrometers operating at 500.13 MHz for ^1H and 125.97 MHz for ^{13}C. ^{13}C NMR spectra were recorded for acyl enzyme complexes which were enriched with ^{13}C at either the $-CH{=}CH-{}^{13}C{=}O$ or $-CH{=}{}^{13}CH-C{=}O$ ($^{13}C_\alpha$) positions of the acyl moiety. In the ^{13}C observed experiments, typically over 1000 scans were acquired with a relaxation delay of 2 s between the scans and broadband ^1H decoupling was employed throughout the experiments.

To delineate the conformation of the ethylenic bond of the acyl group in the complex, a set of ^{13}C edited ^1H NMR experiments were performed with ^{13}C in the ethylenic bond ($-CH{=}{}^{13}CH-C(=O)-$).[9] The ^{13}C editing allows the observation of protons associated with only the enriched portion of the acyl group without interference from enzyme protons. Through-bond and through-space correlations involving the ethylenic protons were studied using $^{13}C(F_1)$ half-filtered HOHAHA and $^{13}C(F_1)$ half-filtered NOESY 2D ^1H NMR experiments, respectively. In the $^{13}C(F_1)$ half-filtered experiments the protons attached to the enriched ^{13}C nucleus were selected by replacing the first 90° ^1H pulse of the regular pulse sequence (HOHAHA or NOESY) with the pulse sequence element:

$$90°_x\,{}^1H\text{–}\tau\text{–}90°_x\,{}^{13}C\,180°_x\,{}^1H\,90°_{(x/-x)}\,{}^{13}C\text{–}\tau$$

where $\tau = {}^1/_2{}^1J_{CH} \approx 3$ ms. During the chemical shift evolution (t_1, t_2) and mixing periods (HOHAHA or NOESY) no ^{13}C pulses (or ^{13}C decoupling) were applied in order to observe long-range ^{13}C–^1H spin–spin couplings (cf. E.COSY type cross peak patterns[10,11]). HOHAHA transfer was achieved using a MLEV sequence at a field strength of 7.3 kHz for 15 ms. NOESY spectra were recorded with a 60 ms mixing time. All 2D data sets were acquired with 2048 data points in t_2 and typically 160–256 real data points in t_1. Processing was done with standard Bruker software.

Results and Discussion

Generation and Characterization of the Photoinduced Acyl-Enzyme. Figure 1 contains the absorption spectrum of 5-methylthienylacryloyl chymotrypsin before and after exposure to light >300 nm. Before light exposure the acyl enzyme has λ_{max} 341 nm arising from the 5-methylthienylacryloyl acyl group. Irradiation causes a 3-nm blue shift in the λ_{max} to 338 nm and a decrease in overall absorption intensity. After 4 × 1 min cycles of irradiation no further change in the spectrum occurs, and a photochemical equilibrium has been reached. The changes in the absorption spectrum result from the fact that the photoinduced acyl enzyme has a smaller extinction coefficient and blue shifted λ_{max} compared to the *trans*-acyl enzyme. The purified photoinduced isomer of 5-methylthienylacryloyl chymotrypsin is characterized by λ_{max} 335 nm at pH 3.0 and ϵ_{335} 13 000 M^{-1} cm^{-1}.[2] This compares with ϵ_{341} ca. 20 000 M^{-1} cm^{-1} for the *trans*-acyl enzyme.

Deacylation kinetics of both the *trans* and photoinduced 5-methylthienylacryloyl chymotrypsins are commonly followed by monitoring absorption decrease at 355-360 nm upon dilution of stable acyl enzyme at pH 3.0 into high pH buffer. Hydrolysis of *trans*-5-methylthienylacryloyl chymotrypsin at pH 10.0 followed at 360 nm is characterized by k_3 0.031 s^{-1} and a final absorption (A_∞) ca. 5% that of the initial absorption (A_0). In contrast hydrolysis of a photoequilibrium mixture of *trans* and photoinduced acyl enzymes is characterized by k_3 0.031 s^{-1} and A_∞ ca. 20% of the A_0 at 360 nm, where A_∞ is the absorbance after 5 min (13 half-lives of the *trans*-acyl enzyme). The remaining absorbance at 360 nm is due to unhydrolyzed photoinduced acyl

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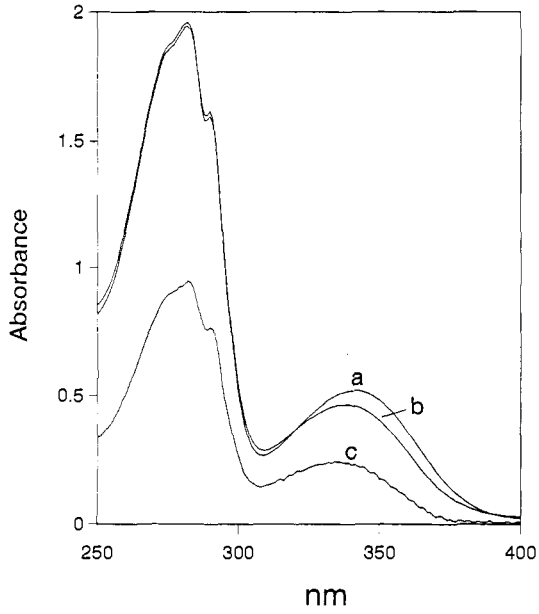

Figure 1. UV–vis absorption spectra of 5-methylthienylacryloyl chymotrypsin at pH 3.0 in 1 mM HCl, 0.3 M KCl. (a) *trans*-Acyl enzyme (40 μM), before light irradiation. (b) Photoequilirium mixture of *trans* and photoinduced acyl enzymes formed by irradiation of the *trans*-acyl enzyme solution (40 μM) for 5 min with a 100-W mercury arc lamp. (c) Purified photoinduced (*cis*) acyl enzyme (20 μM).

enzyme. In order to obtain k_3 for the photoinduced acyl enzyme the absorbance at 360 nm was monitored over a period of 150 h ($t_{1/2}$ = 30 h). This gave k_3 6.0 × 10^{-6} s^{-1} at pH 10.0 for the photoinduced 5-methylthienylacryloyl chymotrypsin. The 5-methylthienylacrylic acid product from the deacylation reaction was characterized by HPLC[2] and was found to have the same retention time as *cis*-5-methylthienylacrylic acid (see Experimental Section). k_3 determined by following production of the acid product by HPLC as a function of time from the purified photoinduced acyl enzyme agreed with k_3 determined spectroscopically (k_3 = 6.0 × 10^{-6} s^{-1}, see above and Tonge and Carey, 1992[2]).

NMR Spectroscopy: Structure of the Photoisomer. The *trans* and photoinduced acyl enzymes were initially characterized using ^{13}C NMR spectroscopy. *trans*-(^{13}C=O)5-Methylthienylacryloyl chymotrypsin (—CH_β=CH_α—^{13}C=O) is characterized by a ^{13}C=O chemical shift of 172.0 ppm (Figure 2a). Upon irradiation of the acyl enzyme two resonances are observed, the 172.0 ppm resonance and a new ^{13}C=O resonance at 168.7 ppm (Figure 2b). Hydrolysis and subsequent purification of the photoinduced isomer yields an acyl enzyme with a single ^{13}C=O resonance at 168.7 ppm (Figure 2c). For 5-methylthienylacryloyl methyl ester in 20% acetonitrile/80% ^2H_2O the *trans* and *cis* isomers have ^{13}C=O chemical shifts at 170 and 169 ppm, respectively. Thus, the 3-ppm difference in ^{13}C=O chemical shift for the two acyl enzyme isomers is larger than for the model compound and reflects the different environment of the acyl carbonyl group in each acyl enzyme (see below).

For ($^{13}C_\alpha$)5-methylthienylacryloyl chymotrypsin (—CH_β=$^{13}CH_\alpha$—C=O) the $^{13}C_\alpha$ chemical shifts are at 118 and 115 ppm for the *trans* and photoinduced isomers, respectively (data not shown). Additionally, for 5-methylthienylacryloyl methyl ester in 20% acetonitrile/80% ^2H_2O the $^{13}C_\alpha$ chemical shift is at 111.5 ppm for the *cis* isomer, 3 ppm upfield of the ^{13}C chemical shift for the *trans* isomer (114.6 ppm). The fact that the $^{13}C_\alpha$ is 3 ppm upfield for the photoinduced acyl enzyme compared to the *trans*-acyl enzyme suggests that the two acyl enzyme differ structurally about the ethylenic bond. However as the chemical shifts are sensitive to both structural (intrinsic) and environmental (extrinsic) factors it is not possible to unequivocally determine the

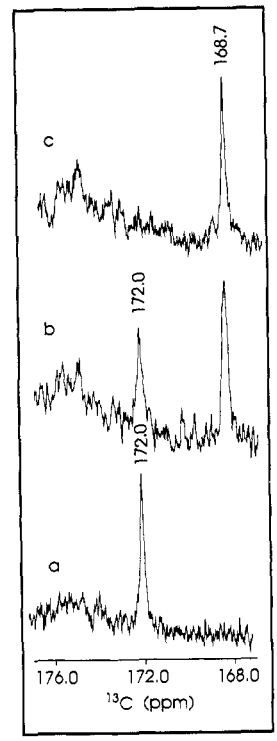

Figure 2. ^{13}C NMR spectra of (^{13}C=O) 5-methylthienylacryloyl chymotrypsin at pD 3.1 in ^2H_2O containing 0.3 M KCl and 2 mM ^2H_2O. (a) *trans*-Acyl enzyme, 1.5 mM. (b) Photoequilibrium mixture of *trans* and photoinduced acyl enzymes formed by irradiation of the *trans*-acyl enzyme solution (1.5 mM) for 30 min with a 100-W mercury arc lamp. (c) Purified photoinduced (*cis*) acyl enzyme (1.5 mM).

structure of the acyl group based solely on the ^{13}C chemical shifts. In order to ascertain the conformation of each acyl enzyme isomer about the ethylenic C=C bond, NOEs were observed between the ethylenic protons for both the *trans* and photoinduced acyl enzymes. These experiments were performed on ($^{13}C_\alpha$)5-methylthienylacryloyl chymotrypsin in order to obtain signals from only the ethylenic protons using ^{13}C edited ^1H NMR.[9]

Initially a HOHAHA experiment was performed in order to identify the expected positions for cross peaks arising from the H_α and H_β (—CH_β=$^{13}CH_\alpha$—C=O) protons of the acyl groups' ethylenic bond. This experiment gives E.COSY type cross peak patterns for protons which are scalar coupled to each other and to the $^{13}C_\alpha$ nucleus.

The $^{13}C(F_1)$ half-filtered HOHAHA 2D proton spectrum of *trans*-5-methylthienylacryloyl chymotrypsin is shown in Figure 3. As the experiment was performed without ^{13}C decoupling, the diagonal peak arising from the $^{13}C_\alpha$ proton is split in F_1 by the large heteronuclear one bond coupling constant ($^1J_{CH}$) giving rise to the diagonal peaks centered at 5.9 ppm. Additionally, a pair of E.COSY type cross peaks can be clearly identified at 7.6 ppm on the F_2 axis which arise from the scalar coupling of the H_α proton with a second proton, which must be H_β (—CH_β=$^{13}CH_\alpha$—C=O). The cross peak is split in F_1 by $^1J_{CH}$ and in F_2 by a small long-range coupling constant $^2J_{CH}$ (4–5 Hz). Finally the diagonal peak for H_β at 7.6 ppm is too weak to be observed since C_β is not enriched. This highlights the importance of doing the HOHAHA experiment in order to aid assignment of any NOE cross peaks that are observed (see below).

The HOHAHA experiment was also performed for the photoinduced acyl enzyme. This experiment is shown in Figure 4. The diagonal peak for the H_α nucleus is again split in F_1 giving rise to the peaks centered at 5.9 ppm. The cross peak between H_α and H_β can be observed at 6.95 ppm on the F_2 axis and is displaced in F_1 by $^1J_{CH}$ ($C_\alpha H_\alpha$ coupling, 167 Hz) and in F_2 by $^2J_{CH}$ ($C_\alpha H_\beta$ coupling). However the displacement in F_2 (2–3 Hz) is smaller than in the *trans* isomer (4–5 Hz).

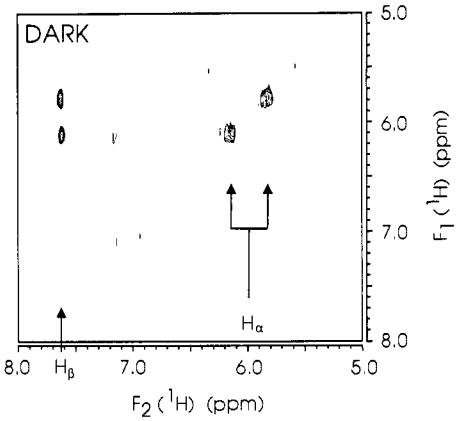

Figure 3. The $^{13}C(F_1)$ half-filtered HOHAHA 2D proton spectrum of 1.5 mM *trans* (DARK) ($^{13}C_\alpha$)5-methylthienylacryloyl chymotrypsin at pD 3.1 in ^2H_2O containing 0.3 M KCl and 2 mM ^2H_2O. The position of the $^{13}C_\alpha$ proton resonance is indicated by "H_α". This resonance is split in F_1 by $^1J_{CH}$. The E.COSY cross peak at ca. 7.6 ppm (F_2) is indicated by "H_β". The cross peak is split in F_1 by $^1J_{CH}$ and in F_2 by $^2J_{CH}$.

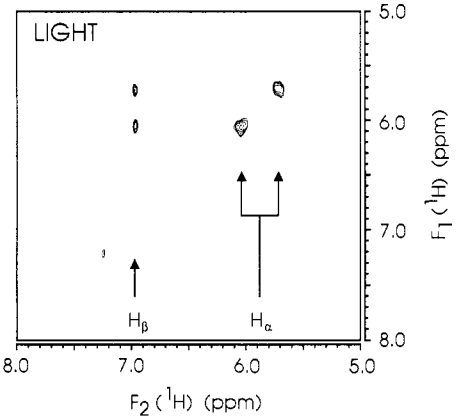

Figure 4. The $^{13}C(F_1)$ half-filtered HOHAHA 2D proton spectrum of the photoinduced (LIGHT) isomer of ($^{13}C_\alpha$)5-methylthienylacryloyl chymotrypsin at pD 3.1. Sample concentration 1.0 mM in ^2H_2O containing 0.3 M KCl and 2 mM ^2H_2O. The position of the $^{13}C_\alpha$ proton resonance is indicated by "H_α". This resonance is split in F_1 by $^1J_{CH}$. The E.COSY cross peak at 6.95 ppm (F_2) is indicated by "H_β". The cross peak is split in F_1 by $^1J_{CH}$ and in F_2 by $^2J_{CH}$.

In the $^{13}C(F_1)$ half-filtered NOESY 2D proton experiment similar E.COSY type cross peak patterns are observed for protons which are close in space and scalar coupled to the same ^{13}C nucleus. For the *trans* complex (Figure 5) no cross peaks between the H_α and H_β protons can be detected. Indeed no cross peak was detected even with a mixing time of 120 ms (data not shown). In contrast, a strong NOESY cross peak is observed for the photoinduced acyl enzyme (Figure 6) at F_1 5.90 ppm and F_2 6.95 ppm. The NOESY cross peak is observed at the same position as the HOHAHA cross peak (Figure 4) and again is displaced in F_2 by the small $^2J_{CH}$ ($C_\alpha H_\beta$ coupling). Correlations from H_α to other spatially close protons would not show a displacement of cross peaks in F_2 as such protons are not scalar coupled to C_α. The displacement of the NOESY cross peak and its overlap with the HOHAHA cross peak supports the hypothesis that the observed NOE is between the H_α and H_β protons of the bound ligand and not from another proton at the same chemical shift.

For the model compound 5-methylthienylacryloyl methyl ester the measured NOE between the H_α and H_β protons is 5–6 times stronger for the *cis* compared to the *trans* isomer (data not shown). This corresponds to a H_α to H_β distance ratio of 1:1.3 for the *cis* and *trans* forms of the model[12] which is in good agreement with literature values for the distances expected for the *cis* (2.3 Å) and

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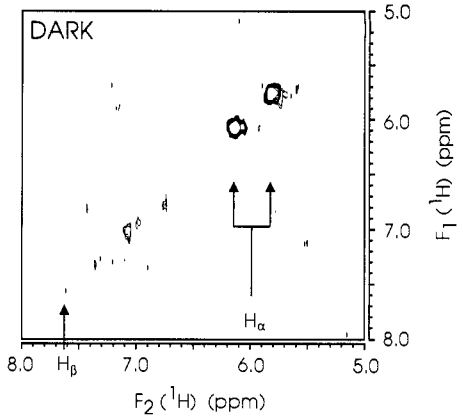

Figure 5. The $^{13}C(F_1)$ half-filtered NOESY 2D proton spectrum of 1.5 mM *trans* (DARK) ($^{13}C_\alpha$)5-methylthienylacryloyl chymotrypsin at pD 3.1 in ^2H_2O containing 0.3 M KCl and 2 mM ^2H_2O. The position of the $^{13}C_\alpha$ proton resonance is indicated by "H_α". This resonance is split in F_1 by $^1J_{CH}$. Based on the HOHAHA data (see Figure 3 and text) the cross peak arising from NOE transfer between H_α and H_β (—$C_\beta H$=$C_\alpha H$—C(=O)—) is predicted at ca. 7.6 ppm on the F_2 axis, indicated by "H_β". However no cross peak is observed.

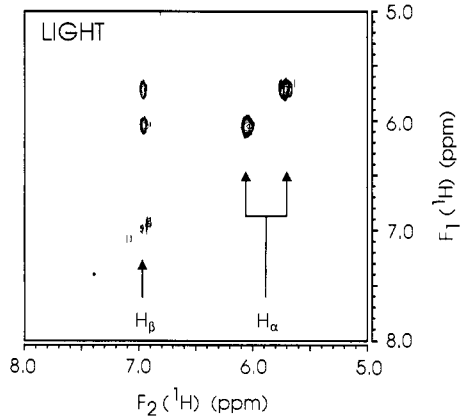

Figure 6. The $^{13}C(F_1)$ half-filtered NOESY 2D proton spectrum of the photoinduced (LIGHT) isomer of ($^{13}C_\alpha$)5-methylthienylacryloyl chymotrypsin at pD 3.1. Sample concentration 1.0 mM in ^2H_2O containing 0.3 M KCl and 2 mM ^2H_2O. The position of the $^{13}C_\alpha$ proton resonance is indicated by "H_α". This resonance is split in F_1 by $^1J_{CH}$. The NOESY cross peak at 6.95 ppm (F_2) is indicated by "H_β". The cross peak is split in F_1 by $^1J_{CH}$ and in F_2 by $^2J_{CH}$.

trans (2.9–3.0 Å) isomers.[13] Since a NOESY cross peak is readily observed for the photoinduced but not for the *trans*-acyl enzyme between the H_α and H_β protons, the distance between H_α and H_β is significantly shorter for the photoinduced acyl enzyme compared to the *trans*-acyl enzyme. Thus the NMR results strongly support the hypothesis that the photoinduced acyl enzyme is *cis* about the C=C ethylenic bond. This is consistent with the 3 ppm difference in ^{13}C=C NMR chemical shift observed between the *trans* and photoinduced (*cis*) acyl enzymes.

Vibrational Assignments, Conformation, and Environment of the Acyl Group in the Active Site. The difference Raman spectra of purified *trans*- and *cis*-5-methylthienylacryloyl chymotrypsin at pH 3.0 are shown in Figure 7. Preliminary band assignments have been made based on isotopic labeling experiments, normal mode calculations, and model studies.

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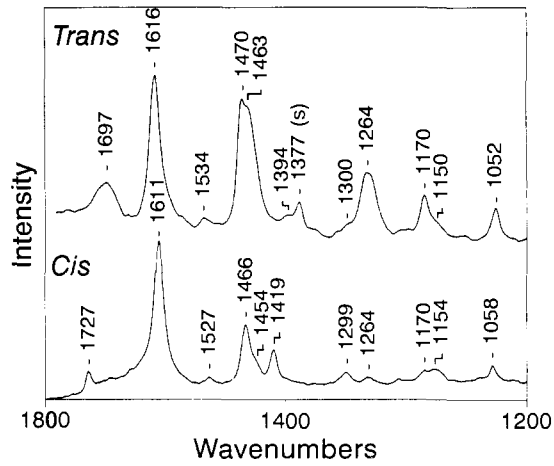

Figure 7. Difference Raman spectra of *trans*- and *cis*-5-methylthienylacryloyl chymotrypsin at pH 3.0. Top: Raman spectrum of *trans*-5-methylthienylacryloyl chymotrypsin obtained by subtracting a spectrum of chymotrypsin, 6 mM in 0.1 M phosphate buffer pH 3.0 from a spectrum of acyl enzyme generated by adding 1 μL of 5-methylthienylacryloyl imidazole, 180 mM in CH_3CN, to 30 μL 6 mM chymotrypsin in 0.1 M phosphate buffer pH 3.0. Bottom: Raman spectrum of *cis* (photoinduced) 5-methylthienylacryloyl chymotrypsin obtained by subtracting a spectrum of chymotrypsin, 6 mM in 0.1 M phosphate buffer pH 3.0 from a spectrum of *cis*-acyl enzyme, 6 mM in 0.1 M phosphate buffer pH 3.0. (s) = features due to solvent.

Unique Vibrational Bands for *cis*- and *trans*-Acyl Enzymes. The Raman spectrum of *trans*-5-methylthienylacryloyl chymotrypsin has an intense band at 1616 cm^{-1} which may be assigned to $\nu_{C=C}$, the ethylenic stretching vibration. Upon *trans* to *cis* isomerization this band shifts slightly and appears at 1611 cm^{-1} in the spectrum of the *cis*-acyl enzyme. In the resonance Raman (RR) spectrum of 5-methylthienylacryloyl chymotrypsin[1] $\nu_{C=C}$ is observed at 1615 cm^{-1} consistent with contributions from both *trans*- and *cis*-acyl enzymes.

In the Raman spectrum of *trans*-5-methylthienylacryloyl chymotrypsin a band is observed at 1470 cm^{-1} with a shoulder at ca. 1463 cm^{-1}. The band at 1470 cm^{-1} is assigned to a feature which contains a major contribution from a totally symmetric ring mode, which is observed for thiophene at 1409 cm^{-1} and which shifts to 1454–1430 cm^{-1} upon 2-substitution of the thiophene ring.[14,15] In the Raman spectrum of *cis*-acyl enzyme a new band is observed at 1419 cm^{-1}. This is assigned to the "ring mode" observed at 1470 cm^{-1} in the spectrum of the *trans*-acyl enzyme. Thus the frequency of this band is likely sensitive to the conformation about the ethylenic bond as a result of vibrational coupling with motions of the ethylenic moiety. Additionally in the *cis*-acyl enzyme spectrum a band is observed at 1466 cm^{-1} with an unresolved shoulder around 1454 cm^{-1}. These bands contribute to the shoulder observed around 1463 cm^{-1} in the Raman spectrum of the *trans*-acyl enzyme and are assigned to asymmetric bending modes of the 5-methyl group.

In the acyl enzyme Raman spectra a weak band appears at 1534 cm^{-1} for the *trans* isomer and at 1527 cm^{-1} for the *cis* isomer. Similarly in the FTIR spectra of 5-methylthienylacryloyl methyl ester in CCl_4 this band appears at 1538 (*trans*) and 1527 cm^{-1} (*cis*) (data not shown). The RR spectrum of 5-methylthienylacryloyl chymotrypsin shows a composite band around 1530 cm^{-1} which can be resolved into two components at 1535 and 1527 cm^{-1}.[1] This band is assigned to a thiophene ring mode, observed at 1534-1514 cm^{-1} for 2-substituted thiophenes,[15] which is coupled to $\nu_{C=C}$ and thus sensitive to the conformation about the ethylenic bond.

Finally in the Raman spectrum of *trans*-5-methylthienylacryloyl chymotrypsin a band of medium intensity is observed at 1264 cm^{-1} which has previously been assigned to ν_{C-O}.[16] Detailed discussion on this band and the other features observed in the 1100–1300-cm^{-1} region will be presented in a future publication.

$\nu_{C=O}$: Environment of the Acyl Carbonyl Group. The resonance Raman spectrum of 5-methylthienylacryloyl chymotrypsin at pH 3.0 is characterized by two bands at 1727 and around 1697 cm^{-1} which are assigned, on the basis of ^{13}C isotopic labeling, to the $\nu_{C=O}$'s for two discrete acyl enzyme populations.[1,3] FTIR experiments using near-UV irradiation have shown that $\nu_{C=O}$ at 1727 cm^{-1} arises from a photoinduced acyl enzyme population generated during RR data collection.[5] In contrast, the broad $\nu_{C=O}$ band around 1697 cm^{-1} in the RR spectrum is assigned to an acyl enzyme population present before light exposure.[5] The NMR experiments described above clearly demonstrate that $\nu_{C=O}$ at 1697 cm^{-1} is due to the *trans*-acyl enzyme population whilst $\nu_{C=O}$ at 1727 cm^{-1} is due to the *cis*-acyl enzyme population. These assignments are in full accord with the results from Raman difference spectroscopy.

The Raman difference spectra (acyl enzyme minus enzyme) of *trans*- and *cis*-5-methylthienylacryloyl chymotrypsin at pH 3.0 are shown in Figure 7. In the region wherein $\nu_{C=O}$ is expected the *cis*-acyl enzyme spectrum shows a single narrow band at 1727 cm^{-1}. In contrast the spectrum of the *trans*-acyl enzyme contains a broad band centered around 1697 cm^{-1}. There is no evidence for a band around 1727 cm^{-1} in the spectrum of the *trans*-acyl enzyme.

FTIR spectra have been obtained for purified *cis*- and *trans*-5-methylthienylacryloyl methyl esters. In CCl_4, $\nu_{C=O}$ is observed at 1718 cm^{-1} for the *cis* isomer. For the *trans* isomer $\nu_{C=O}$ is characterized by a band at 1722 cm^{-1} with a shoulder around 1708 cm^{-1}. In the spectrum of *trans*-(α-CD)5-methylthienylacryloyl methyl ester (—CH=CD—C(=O)—) a single $\nu_{C=O}$ is observed at 1717 cm^{-1}. This suggests that the appearance of two bands in the $\nu_{C=O}$ region for the *trans* isomer results from Fermi resonance of $\nu_{C=O}$ with an overtone or combination band which involves motions of the α-CH proton.

Thus $\nu_{C=O}$ for the *cis* and *trans* methyl esters have very similar frequencies. The difference in $\nu_{C=O}$ observed for the *trans*- and *cis*-acyl enzymes must result from differences in active site environments of the acyl carbonyl groups. Based on model studies $\nu_{C=O}$ for the *trans*-acyl enzyme at 1697 cm^{-1} is assigned to a carbonyl population in a hydrogen bonding environment whilst $\nu_{C=O}$ at 1727 cm^{-1} for the *cis*-acyl enzyme is assigned to a carbonyl population in a nonbonding environment.[3]

The ^{13}C NMR studies described above show that the ^{13}C=O chemical shift for *trans*-5-methylthienylacryloyl chymotrypsin is 172 ppm, 3 ppm downfield of the ^{13}C=O chemical shift for the photoinduced (*cis*) acyl enzyme (169 ppm). An empirical relationship has been observed for acetone, a model carbonyl-containing compound, wherein an increase in hydrogen bonding propensity of the solvent results in a downfield shift in the ^{13}C=O chemical shift.[17] Thus the alteration in carbonyl environment between the *trans*- and *cis*-acyl enzymes revealed by the Raman studies is consistent with the difference in ^{13}C=O chemical shift observed using ^{13}C NMR spectroscopy.

Environment of the Carbonyl Group: Modulation of Reactivity. As discussed above the $\nu_{C=O}$ values for *trans*- and *cis*-5-methylthienylacryloyl methyl esters are very similar, occurring between 1720 ± 2 cm^{-1} for both compounds. Additionally, the intrinsic reactivities of the *trans*- and *cis*-methyl ester model compounds toward base catalyzed hydrolysis are very similar. Recent experiments show that in 1.0 M NaOH k_{OH^-} is 0.013 ± 0.001 s^{-1} for *trans*-5-methylthienylacryloyl methyl ester and 0.0062 ± 0.0002 s^{-1} for *cis*-5-methylthienylacryloyl methyl ester.

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The situation for the *trans*- and *cis*-acyl enzymes is quite different. The spectroscopic studies described above have been performed at pH 3.0 where the acyl enzymes are stable. In order to correlate the spectroscopic parameters with acyl enzyme reactivity vibrational data is obtained at pH 10.0 where the acyl enzymes are maximally active. At pH 10.0 $\nu_{C=O}$ for *trans*- and *cis*-5-methylthienylacryloyl chymotrypsin are at 1685 and 1727 cm^{-1}, respectively.[2] Moreover, *trans*-5-methylthienylacryloyl chymotrypsin (k_3 = 0.031 s^{-1}) deacylates 5000 times faster than the *cis*-acyl enzyme ($k_3 = 6.0 \times 10^{-6}$ s^{-1}).

To summarize the carbonyl frequencies for the *cis*- and *trans*-methyl esters are very similar as are the rate constants for base hydrolysis of the two isomers. In contrast, $\nu_{C=O}$ for the *cis* and *trans* forms of the acyl group in the acyl enzymes differ by 32 cm^{-1} and the deacylation rate constants by a factor of 5000. These data taken together strongly suggest that the active site is modulating acyl enzyme reactivity via the acyl carbonyl group. Compared to the *cis*-acyl enzyme hydrogen bonding interactions between the enzyme and the carbonyl group of the *trans*-acyl enzyme have destabilized the *trans*-acyl enzyme ground state relative to the *cis*-acyl enzyme thus lowering the activation energy barrier for the *trans*-acyl enzyme and causing an increase in deacylation rate.[2]

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