Forces, Bond Lengths, and Reactivity: Fundamental Insight into the Mechanism of Enzyme Catalysis†

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ABSTRACT: Comparison of spectroscopic, kinetic, and thermodynamic data for a series of functioning acyl-serine proteases suggests that the observed variation in deacylation rates can be accounted for by changes in the properties of the acyl-enzyme's ground state. The acyl-enzyme's catalytically crucial acyl carbonyl group is probed by resonance Raman spectroscopy. Its spectral frequency is used to gauge both the carbonyl bond length and the strength of hydrogen bonding (originating from groups making up the oxyanion hole) to the carbonyl oxygen atom. As the deacylation rate increases 16 300-fold through the series, a shift in carbonyl frequency, $\nu_{C=O}$, of -54 cm^{-1} corresponds to a carbonyl bond length increase of 0.025 Å. The decrease in $\nu_{C=O}$ is also consistent with an increase in hydrogen bond donor enthalpy of -27 kJ mol^{-1}. Interestingly, this value resembles closely the decrease in activation energy for deacylation through the series, 24 kJ mol^{-1}, demonstrating that the hydrogen bonds to the carbonyl oxygen atom can provide sufficient energy to account for the observed rate accelerations.

Understanding the mechanism of enzyme-catalyzed reactions represents a fundamental challenge in mechanistic bioorganic chemistry (Kraut, 1988). A combination of enzyme kinetics and protein engineering, aided by crystallographic analysis, has been powerful in characterizing the role of individual enzyme–substrate contacts in enzyme-catalyzed reactions (Fersht, 1987). However, the subtle interplay of distances and forces in an enzyme's active site which brings about catalysis has yet to be fully characterized at the sub-angstrom level. In the present work we show how information of this kind can be elicited from spectroscopic analysis of functioning enzyme–substrate complexes.

In a previous publication we examined the resonance Raman (RR) carbonyl stretching region for 13 acyl-serine proteases (Tonge & Carey, 1990). These included a number of α,β-unsaturated acyl groups bound to chymotrypsin, subtilisin Carlsberg and BPN′, and oxyanion hole mutants of subtilisin BPN′. A linear correlation was observed between $\nu_{C=O}$ and $\log k_3$, where k_3 is the rate constant for acyl-enzyme hydrolysis. The correlation spanned a 500-fold range of reactivity, $\nu_{C=O}$ decreasing as the deacylation rate increased. Moreover, using an empirical correlation between $\nu_{C=O}$ and $r_{C=O}$, the carbonyl bond length, it was possible to quantify the bond length extension of the carbonyl that occurred as reactivity increased.

In the present paper the linear correlation has been extended to cover a 16 300-fold range of acyl-enzyme reactivity. Additionally, detailed hydrogen-bonding studies on model acceptor esters and hydrogen bond donors have allowed us to estimate the hydrogen-bonding enthalpy ($-\Delta H$) required to bring about the decrease in $\nu_{C=O}$ (-54 cm^{-1}) that is observed through the acyl-enzyme series. A stimulating finding is that the value of $-\Delta H$ required to cause a decrease in $\nu_{C=O}$ of 54 cm^{-1} is similar to the change in activation energy calculated from the variation in k_3 through the series. This suggests that sufficient energy is available from interactions in the acyl-enzyme ground state to account for all the observed change in acyl-enzyme reactivity.

EXPERIMENTAL PROCEDURES

Materials. α-Chymotrypsin (lot 100H8275) was from Sigma Chemical Co. [*trans*-3-(5-Methyl-2-thienyl)acryloyl]-imidazole and [*trans*-3-(2-thienyl)acryloyl]imidazole were prepared as described in Tonge and Carey (1989).

Preparation and Characterization of Cis Acyl-Enzymes. α-Chymotrypsin was purified by chromatography on 4-phenylbutylamine–Sepharose by a modification of the procedure described by Stevenson and Landman (1971) (R. Muhandiram, I. Ekiel, H. Deng, R. Callender, P. R. Carey, and P. J. Tonge, unpublished results).

[*cis*-3-(5-Methyl-2-thienyl)acryloyl]chymotrypsin was prepared at pH 3.0 as follows. A 0.2-mL quantity of 60 mM [*trans*-3-(5-methyl-2-thienyl)acryloyl]imidazole in dimethylformamide was added to 2 mL of purified chymotrypsin containing ca. 100 mg of enzyme adjusted to pH 3.0. After 1 min the solution was chromatographed on 5-mL Sephadex G-25 (fine) centrifuge columns (4 × 0.5 mL) equilibrated with 0.3 M NaCl, pH 3.0. The eluted fractions were combined and exposed to near-UV light in 1-min periods using a 100-W mercury arc lamp equipped with a 300-nm cutoff filter. The formation of a photochemical equilibrium mixture of cis and trans acyl-enzymes was monitored by analysis of aliquots of the acyl-enzyme solution using absorption spectroscopy. Production of the cis acyl-enzyme was characterized by a 3-nm blue shift in λ_{max}, from 341 to 338 nm, and a decrease in absorption intensity (Muhandiram et al., unpublished results). Illumination was continued until no further change in the absorption intensity was observed (a total decrease of ca. 10%). Solid Na_2CO_3 was added to raise the pH to ca. 10, as judged by pH paper, in order to hydrolyze the remaining trans acyl-enzyme (k_3 of 0.031 s^{-1}). After 10 min the solution was chromatographed on 5-mL Sephadex G-25 (fine) centrifuge columns (4 × 0.5 mL) equilibrated with 50 mM bicine, pH 8.0. The collected fractions were pooled and applied to a column of 4-phenylbutylamine–Sepharose (2 × 30 cm) equilibrated at pH 8.0 with 50 mM bicine buffer. [*cis*-3-(5-Methyl-2-thienyl)acryloyl]chymotrypsin eluted first from the column while elution of active enzyme, generated by hydrolysis of the trans acyl-enzyme, was retarded. The cis

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acyl-enzyme fractions were pooled, the pH was adjusted to 3.0 with 0.1 M HCl, and the solution was concentrated using a 50-mL Amicon stirred cell. The cis acyl-enzyme was characterized by a λ_{max} of 335 nm and an A_{280}/A_{335} ratio of 3.85. Using 13 000 M^{-1} cm^{-1} as ϵ_{max} for the *cis*-acyl group (unpublished work, this laboratory) and ϵ_{280} of 5×10^4 M^{-1} cm^{-1} for chymotrypsin gave 1 acyl group/mol of protein. [*cis*-3-(2-Thienyl)acryloyl]chymotrypsin was prepared in an identical fashion and had a λ_{max} of 317 nm at pH 3.0.

The deacylation rate constant at 24 °C for [*cis*-3-(5-methyl-2-thienyl)acryloyl]chymotrypsin was determined at pH 8.0 (0.2 M Hepes), 9.0 (0.2 M borate), and 10.0 (0.2 M carbonate) by monitoring the decrease in absorbance at 350 nm as a function of time. In order to confirm that the product of deacylation was the cis acid, product was analyzed at 310 nm by reverse-phase HPLC on a Waters Delta Pak C18 column (3.9 mm × 30 cm). One-tenth-milliliter aliquots of the deacylating reaction mixture (ca. 0.2 mM acyl-enzyme, pH 10.0, 0.2 M carbonate) were taken and added to 0.1 mL of 0.1 M HCl and 0.8 mL of methanol before injection onto the HPLC column. Chromatography was performed using 80% methanol/20% 0.1 M HCl as eluent at 1 mL/min. Experiments using purified acid showed that the cis and trans acids eluted at 29.0 and 30.0 min, respectively.

Deacylation of [*cis*-3-(2-thienyl)acryloyl]chymotrypsin at pH 10.0 in 0.2 M carbonate buffer was monitored at 340 nm. Product analysis was performed by HPLC as described above with the knowledge that purified *cis*- and *trans*-3-(2-thienyl)-acrylic acids had been found to elute at 30.8 and 31.8 min, respectively.

Hydrogen-Bonding Studies. Enthalpies of hydrogen bond formation ($-\Delta H$) between the hydrogen bond donors 3,5-dichlorophenol, phenol, and ethanol and the carbonyl acceptor model compounds 3-(2-thienyl)acrylic acid methyl ester and 3-(5-methyl-2-thienyl)acrylic acid methyl ester were determined in CCl_4. Equilibrium constants (K_{eq}) for complex formation were determined using FTIR spectroscopy to quantify the concentration of free donor in donor–acceptor mixtures using ν_{O-H} around 3600 cm^{-1}. Values of K_{eq} were determined at seven temperatures between 270 and 323 K by varying the concentration of ester from 0 to 200 mM while maintaining the concentration of donor at 3 mM (3,5-dichlorophenol, phenol) or 6 mM (ethanol). Following the van't Hoff equation, plots of log K_{eq} against $1/T$ yielded ΔH and ΔS. In order to observe the shift in $\nu_{C=O}$ ($-\Delta\nu_{C=O}$) induced by hydrogen bond formation, ratios of donor/acceptor were prepared such that ca. 50% of the acceptor was hydrogen bonded.

RESULTS AND DISCUSSION

Deacylation Rates and RR Band Signatures for Individual Acyl-Enzyme Populations: Extension of Correlation Using Cis Acyl-Enzymes. The partial resonance Raman (RR) spectrum of [3-(5-methyl-2-thienyl)acryloyl]chymotrypsin at pH 10.0 is shown in Figure 1. Two bands are observed at 1685 and 1727 cm^{-1} which, on the basis of isotopic labeling studies, are attributed to $\nu_{C=O}$ arising from two discrete acyl-enzyme populations (MacClement et al., 1981; Carey & Phelps, 1983). FTIR studies on [3-(5-methyl-2-thienyl)-acryloyl]chymotrypsin carried out using actinic near-UV irradiation have previously shown that the 1727-cm^{-1} RR band results from photoisomerization of the acyl group in the active site (Tonge et al., 1991). The photoisomerization is brought about by the laser beam used to excite the RR spectrum. Other workers have characterized similar photochemical

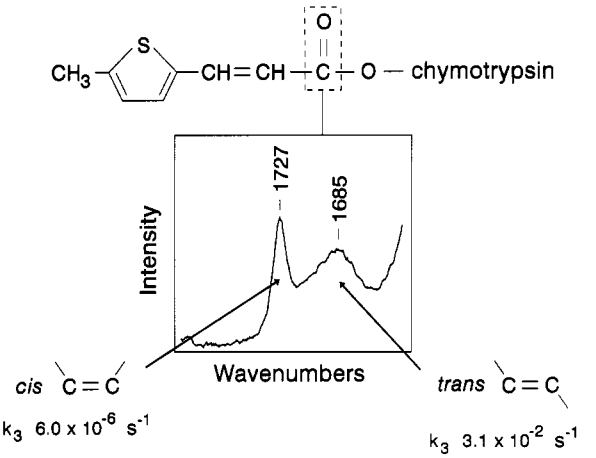

FIGURE 1: Partial resonance Raman spectrum of [3-(5-methyl-2-thienyl)acryloyl]chymotrypsin at pH 10.0. The spectrum was obtained using 337.5-nm laser excitation and a rapid-mixing, rapid-flow system as described in Tonge and Carey (1990). The region of the spectrum shows bands due to two populations of the acyl carbonyl group ($\nu_{C=O}$, see text). Both the trans and cis (photoinduced) acyl-enzymes have been characterized spectroscopically and kinetically (see text and Table I), and the maximal deacylation rates of each are given in the figure.

Table I: Deacylation Kinetics for the Cis Acyl-Enzymes

	k_3 (s^{-1}), spectroscopic[a]	k_3 (s^{-1}), HPLC[b]
cis-5MeTA-chymotrypsin[c]		
pH 8.0	$(6.2 \pm 0.6) \times 10^{-6}$	
pH 9.0	$(6.6 \pm 0.7) \times 10^{-6}$	
pH 10.0	$(6.0 \pm 0.4) \times 10^{-6}$	$(6.8 \pm 1.1) \times 10^{-6}$
cis-TA-chymotrypsin[d]		
pH 10.0	$(2.50 \pm 0.05) \times 10^{-5}$	$(2.9 \pm 0.3) \times 10^{-5}$

[a] k_3 determined by monitoring the decrease in absorbance at 350 nm (5MeTA-chymotrypsin) or 340 nm (TA-chymotrypsin). [b] k_3 determined by monitoring production of the cis acid by HPLC. [c] [*cis*-3-(5-methyl-2-thienyl)acryloyl]chymotrypsin. [d] [*cis*-3-(2-thienyl)acryloyl]chymotrypsin.

events by absorption spectroscopy and have assigned the photogenerated isomer to a cis form about the substrate's C=C double bond (Martinek & Berezin, 1979; Porter & Bruhnke, 1989). We have confirmed this assignment by NMR (Muhandiram et al., unpublished results) and have also confirmed our interpretation of the RR data by obtaining the (nonresonance) Raman spectrum of the bound acyl groups for the pure cis and trans isomers by Raman difference spectroscopy carried out under nonphotolyzing conditions (Muhandiram et al., unpublished results). Thus in the RR spectrum of [3-(5-methyl-2-thienyl)acryloyl]chymotrypsin at pH 10.0, $\nu_{C=O}$ at 1685 cm^{-1} is assigned to the trans acyl-enzyme population while $\nu_{C=O}$ at 1727 cm^{-1} is assigned to the cis acyl-enzyme population.

The limiting deacylation rate constant (k_3) for [3-(5-methyl-2-thienyl)acryloyl]chymotrypsin has been determined previously at pH 10.0. As these kinetics were performed on an acyl-enzyme which had not been exposed to actinic near-UV light, the measured k_3 of 0.031 s^{-1} can be assigned to the trans acyl-enzyme population giving rise to the $\nu_{C=O}$ RR feature at 1685 cm^{-1} (Figure 1). In the present study k_3 for [*cis*-3-(5-methyl-2-thienyl)acryloyl]chymotrypsin has been determined to be 6.0×10^{-6} s^{-1} at pH 10.0. This value appears to be pH independent between pH 8.0 and 10.0 (Table I). Additionally, HPLC analysis has confirmed that the spectroscopically determined kinetic values are due to the formation of cis acid product (see Experimental Procedures). The value of k_3 determined by plotting the integrated area of the cis acid

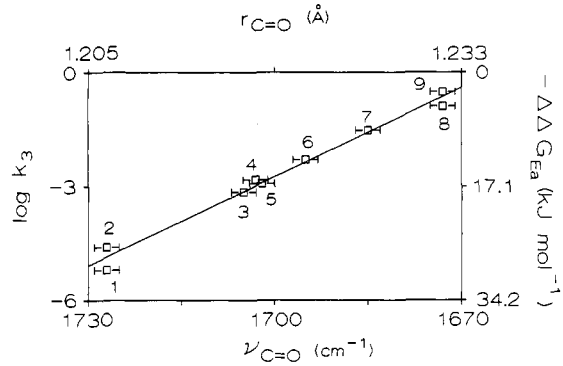

FIGURE 2: Correlation between the carbonyl stretching frequency, $\nu_{C=O}$, and log k_3, where k_3 is the maximal deacylation rate at pH 10, for the series of acyl-serine proteases. The error bars represent the estimated accuracy of determining $\nu_{C=O}$ (±2 cm^{-1}). The value of k_3 represents an average of at least three determinations. Points: (1) [*cis*-3-(5-methyl-2-thienyl)acryloyl]chymotrypsin [$\nu_{C=O}$, 1727 cm^{-1}; k_3, (6.0 ± 0.4) × 10^{-6} s^{-1}]; (2) [*cis*-3-(2-thienyl)acryloyl]chymotrypsin [$\nu_{C=O}$, 1727 cm^{-1}; k_3, (2.50 ± 0.05) × 10^{-5} s^{-1}]; (3) [3-(5-methyl-2-thienyl)acryloyl]subtilisin BPN′ Asn155Leu; (4) [3-(5-methyl-2-thienyl)acryloyl]subtilisin BPN′ Asn155Gln; (5) [3-(5-methyl-2-thienyl)acryloyl]subtilisin BPN′ Asn155Arg; (6) [*trans*-3-(2-thienyl)acryloyl]chymotrypsin; (7) [*trans*-3-(5-methyl-2-thienyl)acryloyl]chymotrypsin; (8) [3-(5-methyl-2-thienyl)acryloyl]subtilisin BPN′ wild type; (9) [3-(5-methyl-2-thienyl)acryloyl]subtilisin Carlsberg. Points 3–9 were taken from Tonge and Carey (1990). The line shown is a linear regression fit to all the data points with $r = 0.993$. The upper abscissa scale, $r_{C=O}$, shows the carbonyl bond length calculated from $\nu_{C=O}$. The right ordinate scale shows the free energy of activation ($\Delta\Delta G_{Ea}$) calculated from k_3 using $\Delta G_{Ea} = -RT \ln k_3$ and using $\Delta G_{Ea} = 0$ when $k_3 = 1$ s^{-1} for obtaining $\Delta\Delta G_{Ea}$.

peak as a function of time agrees with the spectroscopically determined value (Table I). Thus $\nu_{C=O}$ at 1727 cm^{-1} in the RR spectrum (Figure 1) can be attributed to the cis acyl-enzyme population with a k_3 of 6.0 × 10^{-6} s^{-1}.

The RR spectrum of [3-(2-thienyl)acryloyl]chymotrypsin at pH 10.0 also gives rise to two $\nu_{C=O}$ bands, at 1727 and 1695 cm^{-1} (data not shown). By analogy to [3-(5-methyl-2-thienyl)-acryloyl]chymotrypsin, $\nu_{C=O}$ at 1727 cm^{-1} for [3-(2-thienyl)-acryloyl]chymotrypsin is assigned to the cis acyl-enzyme with a measured k_3 of 2.5 × 10^{-5} s^{-1} (Table I). Additionally, $\nu_{C=O}$ at 1695 cm^{-1} can be assigned to the trans acyl-enzyme population with a k_3 of 5.1 × 10^{-3} s^{-1} (Tonge & Carey, 1990). Thus, the two chymotrypsin acyl-enzymes yield four data points for the log k_3 $\nu_{C=O}$ correlation, permitting it to be extended to a 16 300-fold range in k_3 values.

RR and kinetic data have also been previously obtained for a number of subtilisin acyl-enzymes (Tonge & Carey, 1990). In order to use these data here, it is important to be certain that rate constants obtained for nonirradiated acylsubtilisins have not been associated with a photoinduced $\nu_{C=O}$ population in the RR spectrum. FTIR spectroscopic experiments on [3-(5-methyl-2-thienyl)acryloyl]subtilisin Carlsberg and BPN′ (wild type) in D_2O at pD 4.5 reveal that exposure to near-UV light does not generate a significantly new $\nu_{C=O}$ population in these acyl-enzymes (data not shown). Corresponding kinetic experiments have failed to demonstrate that exposure to near-UV radiation generates an acyl-enzyme population which deacylates appreciably faster or slower than the acyl-enzyme population present before light exposure. Thus the $\nu_{C=O}$ and k_3 values obtained previously for the subtilisin acyl-enzymes can be used here without modification.

The above data are incorporated in Figure 2, which demonstrates a linear correlation between $\nu_{C=O}$ and log k_3 ($r = 0.993$). The data indicate that an increase of k_3 of 16 300-fold is associated with a shift in $\nu_{C=O}$ of −54 cm^{-1}. Using an empirical relationship between $\nu_{C=O}$ and $r_{C=O}$, the acyl C=O bond length (Simon & Sasvári, 1975; Simon et al., 1986; Horváth et al., 1987), we can estimate that the rate increase of 16 300-fold from the unreactive *cis*-acylchymotrypsins to the reactive acylsubtilisins is accompanied by a C=O bond length increase of 0.025 Å. This is an important quantitation since it gauges the progress of the C=O group along the reaction coordinate in the acyl-enzyme ground state. An unperturbed ground state will have a formal C=O double bond while in the transition state for deacylation the carbon–oxygen linkage will probably be close to a C—O single bond (Fersht, 1985a). This being the case, a bond length change of 0.025 Å seen in Figure 2 represents approximately 11% of the bond length change expected on going from a formal C=O to a C—O bond. Thus, in this sense we can see that a major change in reactivity is associated with a rather modest C=O distortion in the direction of the reaction coordinate.

Site-directed mutagenesis studies using subtilisin BPN′ in which Asn-155, a component of the catalytically important "oxyanion hole", has been replaced by other amino acids having differing hydrogen-bonding propensities suggest that the decrease in $\nu_{C=O}$ and the accompanying increase in k_3 observed through the acyl-enzyme series can be attributed to stronger hydrogen bonding of the acyl carbonyl in the oxyanion hole (Tonge & Carey, 1990). In the next section we discuss how the observed shift in $\nu_{C=O}$ in the RR spectra can be used to quantitate the change in hydrogen-bonding strength.

Estimation of Hydrogen-Bonding Enthalpy in the Active Site. In addition to providing data on $r_{C=O}$, the acyl carbonyl RR signatures enable us to estimate the magnitude of the forces needed to bring about the observed changes in $\nu_{C=O}$ (Figure 2). The use of vibrational band shifts to quantitate the enthalpies of hydrogen bonds involving small (nonmacromolecular) compounds has a long and distinguished history (Pimentel & McClellan, 1960). The great majority of studies have used the stretching frequency of an –NH or –OH group, acting as a hydrogen bond donor, as the experimental measurable. These bands undergo large shifts (typically −200 cm^{-1}) upon going from a "free" to a hydrogen-bonding situation. In contrast, extensive studies on C=O groups as an acceptor have not been reported due to the much smaller changes in frequency of $\nu_{C=O}$ upon hydrogen bonding. Thus, we have undertaken careful FTIR studies on a number of model interactions with a view of quantitating the enthalpy of hydrogen bonding which would be needed to change $\nu_{C=O}$ by the 54 cm^{-1} seen in Figure 2.

Extensive FTIR data were obtained as a function of temperature (between 273 and 323 K) for hydrogen-bonding pairs in CCl_4 with either 3-(5-methyl-2-thienyl)acrylic acid methyl ester or 3-(2-thienyl)acrylic acid methyl ester as acceptor and phenol, 3,5-dichlorophenol, or ethanol as donor. Using the van't Hoff equation ($\log K = -\Delta H/RT + \Delta S/R$), a $-\Delta H$ vs $-\Delta\nu_{C=O}$ correlation was obtained. This is shown in Figure 3; each point (O) represents the observed $-\Delta\nu_{C=O}$ and calculated $-\Delta H$ for one pair of model compounds. By extrapolation of the $-\Delta H$, $-\Delta\nu_{C=O}$ correlation derived for the model compounds, the value for $-\Delta H$ which would give rise to the observed shift of −54 cm^{-1} for $\nu_{C=O}$ in the acyl-enzyme series is calculated to be 27 kJ mol^{-1}. A stimulating observation is that this amount of energy is similar to the change in activation energy $-\Delta\Delta G_{Ea}$, 24 kJ mol^{-1}, associated with a change in reactivity of 16 300-fold throughout the series. This point is elaborated in Figure 3 where the change in free energies of activation $-\Delta\Delta G_{Ea}$, derived from k_3's, is plotted against

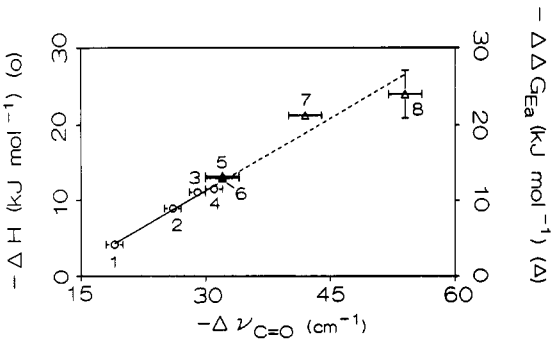

FIGURE 3: (○) Enthalpy of hydrogen bond formation ($-\Delta H$) and corresponding shift in $\nu_{C=O}$ ($-\Delta\nu_{C=O}$) data for model compounds determined in CCl_4. The estimated accuracy of the band position determined by FTIR is ±1 cm^{-1}. Error bars for $-\Delta$H are too small to show up on the graph. Points (1) 3-(2-thienyl)acrylic acid methyl ester and ethanol ($-\Delta H$, 4.14 ± 0.07 kJ mol^{-1}; $-\Delta\nu_{C=O}$, 19 cm^{-1}); (2) 3-(2-thienyl)acrylic acid methyl ester and phenol ($-\Delta H$, 8.90 ± 0.08 kJ mol^{-1}; $-\Delta\nu_{C=O}$, 26 cm^{-1}); (3) 3-(2-thienyl)acrylic acid methyl ester and 3,5-dichlorophenol ($-\Delta H$, 11.10 ± 0.15 kJ mol^{-1}; $-\Delta\nu_{C=O}$, 29 cm^{-1}); (4) 3-(5-methyl-2-thienyl)acrylic acid methyl ester and 3,5-dichlorophenol ($-\Delta H$, 11.50 ± 0.15 kJ mol^{-1}; $-\Delta\nu_{C=O}$, 31 cm^{-1}). (△) Difference in energy of activation ($-\Delta\Delta G_{Ea}$) and corresponding difference in $\nu_{C=O}$ ($-\Delta\nu_{C=O}$) for related pairs of acyl-enzymes taken from the data in Figure 2 as well as the overall $-\Delta\Delta G_{Ea}$ and $-\Delta\nu_{C=O}$ throughout the entire acyl-enzyme series calculated from the linear regression of the data in Figure 2. The estimated accuracy of the band position from RR spectroscopy is ±2 cm^{-1}. Error bars for $-\Delta\Delta G_{Ea}$ are too small to show up on the graph except for (8). The error for (8) is significantly larger than for (5)–(7) because this point results from linear regression to the data in Figure 2. Points: (5) [*cis*- and *trans*-3-(2-thienyl)acryloyl]chymotrypsin ($-\Delta\Delta G_{Ea}$, 13.2 ± 0.1 kJ mol^{-1}; $-\Delta\nu_{C=O}$, 32 cm^{-1}); (6) [3-(5-methyl-2-thienyl)acryloyl]-subtilisin BPN′ wild type and Asn155Leu ($-\Delta\Delta G_{Ea}$, 12.9 ± 0.3 kJ mol^{-1}; $-\Delta\nu_{C=O}$, 32 cm^{-1}); (7) [*cis*- and *trans*-3-(5-methyl-2-thienyl)-acryloyl]chymotrypsin ($-\Delta\Delta G_{Ea}$, 21.3 ± 0.2 kJ mol^{-1}; $-\Delta\nu_{C=O}$, 42 cm^{-1}); (8) $-\Delta\Delta G_{Ea}$ and $-\Delta\nu_{C=O}$ throughout the entire acyl-enzyme series calculated from the linear fit to the data in Figure 2 (for $-\Delta\nu_{C=O}$ of 54 cm^{-1}, $-\Delta\Delta G_{Ea}$ is 24.0 kJ mol^{-1}). The solid line shown is a linear regression fit to data points 1–4 with r = 0.994. The dashed line is an extrapolation of the solid line to $-\Delta\nu_{C=O}$ of 54 cm^{-1}.

$-\Delta\nu_{C=O}$ for three pairs of acyl-enzymes (points 5–7) and for the slope derived in Figure 2 for all acyl-enzymes (point 8). As can be seen, there is a remarkable correlation between the model compound $-\Delta H$ vs $-\Delta\nu_{C=O}$ and the acyl-enzyme $-\Delta\Delta G_{Ea}$ vs $-\Delta\nu_{C=O}$ data. Several conclusions can be drawn from Figure 3: (1) there is sufficient energy available in the change of hydrogen-bonding strengths to account for the change in activation energies in the series, (2) the equivalence of $-\Delta H$ and $-\Delta\Delta G_{Ea}$ supports the intuitive notion that entropy is not an important factor in deacylation, and (3) the fact that the $\Delta\Delta G_{Ea}$ and ΔH vs $-\Delta\nu_{C=O}$ points lie on the same line implies that the observed 16 300-fold modulation of k_3 values is caused by changes in the ground states of the acyl-enzymes and that the transition state throughout the series is invariant. It has to be pointed out that we have assumed that a one-to-one acceptor–donor complex in CCl_4 is a good model for the acyl group C=O binding in the oxyanion hole. This may be reasonable for the acyl C=O in the ground state (Henderson, 1970), but in the transition state two [and for subtilisin three (Braxton & Wells, 1991)] hydrogen bond donors become operative. Nevertheless, the experimental correlations between log k_3 vs $-\Delta\nu_{C=O}$ (Figure 2) and between the $-\Delta H$ vs $-\Delta\nu_{C=O}$ and $-\Delta\Delta G_{Ea}$ vs $-\Delta\nu_{C=O}$ (Figure 3) provide a simple model to explain a wide variation in kinetic rate constants—in terms of both a subtle change in bond length and the forces giving rise to these changes.

Finally, we can comment on whether our results constitute evidence for substrate strain (Jencks, 1975, 1987; Fersht, 1985b). There is clear evidence that interactions, in all likelihood involving the oxyanion hole hydrogen bond donors, distort the C=O bond in the direction of the reaction coordinate. This distortion is modest and reflects the fact that a lot of energy ($\Delta\Delta G_{Ea}/r_{C=O}$ = 960 kJ mol^{-1} Å^{-1} from Figure 2) is required to elongate the C=O linkage. Ultimately, only the energy from hydrogen bonding is available to perturb the C=O moiety in the ground state. In Fersht's terminology (Fersht, 1985b), the C=O is being "stressed" by forces which tend to distort it toward the transition state; however, there is no evidence for cruel or unusual forces being brought into play.

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