Length of the Acyl Carbonyl Bond in Acyl-Serine Proteases Correlates with Reactivity†

Peter J. Tonge and Paul R. Carey*
Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6
Received August 21, 1990; Revised Manuscript Received October 2, 1990

ABSTRACT: Resonance Raman (RR) spectroscopy has been used to obtain the vibrational spectrum of the acyl carbonyl group in a series of acylchymotrypsins and acylsubtilisins at the pH of optimum hydrolysis. The acyl-enzymes, which utilize arylacryloyl acyl groups, include three oxyanion hole mutants of subtilisin BPN′, Asn155Leu, Asn155Gln, and Asn155Arg, and encompass a 500-fold range of deacylation rate constants. For each acyl-enzyme a RR carbonyl band has been identified which arises from a population of carbonyl groups undergoing nucleophilic attack in the active site. As the deacylation rate (k_3) increases through the series of acyl-enzymes, the carbonyl stretching band ($\nu_{C=O}$) is observed to shift to lower frequency, indicating an increase in single bond character of the reactive acyl carbonyl group. Experiments involving the oxyanion hole mutants of subtilisin BPN′ indicate that a shift of $\nu_{C=O}$ to lower frequency results from stronger hydrogen bonding of the acyl carbonyl group in the oxyanion hole. A plot of log k_3 against $\nu_{C=O}$ is linear over the range investigated, demonstrating that the changes in $\nu_{C=O}$ correlate with the free energy of activation for the deacylation reaction. By use of an empirical correlation between carbonyl frequency ($\nu_{C=O}$) and carbonyl bond length ($r_{C=O}$) it is estimated that $r_{C=O}$ increases by 0.015 Å as the deacylation rate increases 500-fold through the series of acyl-enzymes. This change in $r_{C=O}$ is about 7% of that expected for going from a formal C=O double bond in the acyl-enzyme to a formal C–O single bond in the tetrahedral intermediate for deacylation. The data also allow us to estimate the energy needed to extend the acyl carbonyl group along its axis to be 950 kJ mol^{-1} Å^{-1}.

The source of the catalytic power of enzymes is only partially understood. One difficulty lies in the inability of even the most powerful biophysical tools to define the structures of enzyme–substrate intermediates, and changes in these structures, at the level needed to follow important kinetic differences. In chemical reactions, changes in critical bond lengths of 0.01 Å, for example, can have very significant effects on measured rate constants (Jones & Kirby, 1979). Here we show that resonance Raman (RR) spectroscopy can be used to define bond length differences of less than 0.01 Å for the acyl carbonyl group in a series of acyl-serine proteases. The enzymes involved are chymotrypsin, subtilisins Carlsberg and BPN′, and three forms of the latter wherein one of the residues making up the oxyanion hole, Asn155, has been replaced (Bryan et al., 1986; Wells et al., 1986).

In a preceding paper (Tonge & Carey, 1989) we demonstrated that RR spectroscopy could be used to obtain the vibrational spectrum of the acyl-enzyme carbonyl group for a number of acylchymotrypsins at the pH for optimum acyl-enzyme hydrolysis. In each acyl-enzyme's RR spectrum we assigned a carbonyl band to a population of acyl groups undergoing nucleophilic attack in the active site. On the basis of the RR data, we postulated that a more reactive acyl-enzyme gave rise to a carbonyl band at lower frequency. Furthermore, we hypothesized that the decrease in carbonyl frequency resulted from stronger hydrogen bonding of the acyl carbonyl group in the oxyanion hole.

The present paper combines the previous studies on acylchymotrypsins with data obtained for acyl-enzymes involving subtilisin Carlsberg and subtilisin BPN′ as well as site-directed mutants of subtilisin BPN′ in which one of the hydrogen-bond donors comprising the oxyanion hole has been altered. This has permitted extension of the previous observations to more reactive acyl-enzymes involving the wild-type subtilisins as well as to less reactive acyl-enzymes involving the oxyanion hole subtilisin mutants. The series of acyl-enzymes for which RR data have been obtained now spans a 500-fold range of deacylation rate constants. Moreover, RR data for the acyl-enzymes involving the oxyanion hole mutants of subtilisin BPN′ provide evidence that the decrease in $\nu_{C=O}$ observed as acyl-enzyme reactivity increases results from stronger hydrogen bonding of the acyl carbonyl group in the active site. One of the most important aspects of the present work is that we have been able to demonstrate, for the first time, a quantitative relationship between the position of $\nu_{C=O}$ and the corresponding rate constant for deacylation.

Experimental Procedures

Materials. α-Chymotrypsin (lot no. 127F-8005), subtilisin BPN′ (protease type XXVII; Nagarse; lot no. 58F-0098), and subtilisin Carlsberg (protease type VIII; lot no. 68F-0904) were from Sigma Chemical Co. ^2H_2O (99.8% ^2H), malonic-*2*-^{13}C acid (99% ^{13}C), and malonic-*1,3*-$^{13}C_2$ acid (99% ^{13}C) were from MSD Isotopes (Merck Frosst Canada Inc.). Buffer salts and solvents (spectrophotometric grade) were from usual sources. Subtilisin BPN′ in which the oxyanion hole residue Asn155 has been replaced with Leu, Gln, and Arg, was obtained from a strain of *Bacillus subtilis* containing the gene for subtilisin from *Bacillus amyloliquefaciens* (subtilisin BPN′). The clones were a gift from Genex Corp. (Gaithersburg, MD 20877) and Dr. Phil Bryan.

Substrates were synthesized as described previously (MacClement et al., 1981; Tonge & Carey, 1989). [(5-Methyl-2-thienyl)acryloyl]imidazole labeled with ^{13}C in the ethylenic double bond (—C=^{13}C—C=O) or with ^{13}C in the carbonyl bond (—C=C—^{13}C=O) was synthesized by use of malonic-*2*-^{13}C acid and malonic-*1,3*-$^{13}C_2$ acid, respectively.

Expression and Purification of the Subtilisin BPN′ Mutants. Expression and purification of the mutant subtilisins

† Issued as NRCC Publication No. 31907.
* To whom correspondence should be addressed.

0006-2960/90/0429-10723$02.50/0

Table I: $\nu_{C=O}$ and k_3 Values for the Acyl-Serine Proteases

acyl-enzyme	$\nu_{C=O}$ (cm^{-1})[a]	k_3 (s^{-1})[b]
(1) [(5-methylthienyl)acryloyl]subtilisin BPN′ Asn155Leu[c]	1705	0.0007
(2) [(5-methylthienyl)acryloyl]subtilisin BPN′ Asn155Gln[c]	1703	0.0015
(3) [(5-methylthienyl)acryloyl]subtilisin BPN′ Asn155Arg[c]	1702	0.0012
(4) [(5-methylthienyl)acryloyl]subtilisin BPN′ wild type[c]	1673	0.13
(5) (furylacryloyl)chymotrypsin[d]	1699	0.0029
(6) (thienylacryloyl)chymotrypsin[d]	1695	0.0051
(7) (4-methoxycinnamoyl)chymotrypsin[c]	1695	0.0029
(8) [(5-methylthienyl)acryloyl]chymotrypsin	1685[c]	0.031[d]
(9) (4-methoxycinnamoyl)subtilisin Carlsberg[c]	1675	0.35
(10) [(5-methylthienyl)acryloyl]subtilisin Carlsberg[c]	1673	0.31
(11) (4-amino-3-nitrocinnamoyl)chymotrypsin[d]	1691	0.22
(12) (4-amino-3-nitrocinnamoyl)subtilisin BPN′ wild type[c]	1680	0.009
(13) (indolylacryloyl)chymotrypsin[d]	1695	0.0021

[a] RR data were obtained at the pH of the maximal deacylation rate, and the frequency of the lowest recorded carbonyl band ($\nu_{C=O}$) is given. [b] Deacylation rate constants (k_3) were determined in H_2O as described in Tonge and Carey (1989). The rate constant given is the maximum value observed between pH 9 and pH 10.5. [c] This work. [d] Tonge and Carey (1989).

was performed essentially as described in Bryan et al. (1986) except that the DEAE-cellulose treatment and acetone fractionation steps were omitted. Final purification of the proteins was performed by HPLC on a TSK CM-3SW column (7.5 × 75 mm) with 10 mM phosphate buffer, pH 6.2, and a 0–0.2 M NaCl gradient (Willis & Szabo, 1989).

Preparation of Acyl-Enzymes. Acylchymotrypsins were prepared as described in Tonge and Carey (1989). Acylsubtilisins (BPN′ and Carlsberg wild type) were prepared in an analogous fashion except that 20 mM acetate buffer (0.1 M NaCl), pH 4.2, was used and the chromatographic purification of the acyl-enzymes was performed with this buffer at 4 °C. In a typical preparation 1 mL of 60 mM imidazole ester substrate in dimethylformamide was added to 500 mg of subtilisin in 10 mL of pH 4.2 20 mM acetate buffer and 0.1 M NaCl, followed by chromatography at 4 °C on a Sephadex G-25 (fine) column (2 × 40 cm) with the same buffer. The acylsubtilisins were diluted with the pH 4.2 buffer to give a solution with an absorbance of 2.0–3.0 at 324 nm (ca. 0.15 mM acyl-enzyme) and allowed to come to room temperature before use.

[(5-Methylthienyl)acryloyl]subtilisin BPN′ Asn155Leu, Asn155Gln, and Asn155Arg were prepared by adding 0.1 mL of 60 mM [(5-methylthienyl)acryloyl]imidazole in dimethylformamide to 0.5 mL of the engineered enzymes (5 mg/mL) in 10 mM phosphate buffer, pH 6.2. The acyl-enzymes were purified by centrifugation through a 1 × 5 cm Sephadex G-25 (fine) "syringe" column equilibrated with 20 mM acetate buffer and 0.1 M NaCl, pH 4.2.

Resonance Raman Instrumentation. Resonance Raman (RR) spectra were obtained as described previously (Carey & Sans Cartier, 1983; Tonge & Carey, 1989) except that an EG&G Princeton Applied Research 1460 OMA III data collection system and 1421 UV-enhanced diode array were used. RR spectra of the acyl-enzymes were obtained at pH 9.4–10.0 where the deacylation rate (k_3) is maximal by mixing the acyl-enzymes in a 1:1 ratio with 0.2 M borate or carbonate buffer (0.1 M NaCl) of the appropriate pH. In most cases a flow system was used with a two-jet (slow flow) or four-jet (fast flow, 1 mL s^{-1}) mixer (Sans Cartier et al., 1983; Tonge & Carey, 1989). For the acylsubtilisin BPN′ oxyanion hole mutants, 0.2 mL of the acyl-enzyme (A_{324} 2.4) was mixed with 0.2 mL of 0.2 M borate buffer and 0.1 M NaCl in a cuvette to give a final pH of 9.8. No flow system was required for these acyl-enzymes due to the slow rate of hydrolysis ($t_{1/2}$ 8–16 min at pH 9.8). For each of the spectra an enzyme-only background was subtracted. Assignment of bands in the RR spectra to $\nu_{C=O}$ of the acyl carbonyl group was performed with $^{13}C{=}O$-labeled substrate and also by RR spectroscopy of reaction mixtures after >95% deacylation had occurred.

Results and Discussion

Evidence That Downshifts in $\nu_{C=O}$ Are Caused by Hydrogen Bonding to the Oxyanion Hole. The RR spectrum of [(5-methyl-2-thienyl)acryloyl]subtilisin BPN′ wild type at pH 9.8 is shown in Figure 1 (top) between 1600 and 1800 cm^{-1}. It can be seen that the carbonyl profile for this acyl-enzyme is made up of more than one feature. As in the case of acylchymotrypsins at low pH (MacClement et al., 1981; Tonge & Carey, 1989) we ascribe the multiple carbonyl bands to the presence of a heterogeneous carbonyl population wherein each carbonyl group is rapidly fluctuating between sites having differing hydrogen bonding capacities. Since increasing the strength of the hydrogen bonds to a carbonyl oxygen atom decreases its stretching frequency (Pimentel & McClellan, 1960) the peak at 1673 cm^{-1} in Figure 1 is assigned to a carbonyl population strongly bonded to hydrogen-bond donors in the oxyanion hole. This population we designate the productive acyl-enzyme, and for the other subtilisin and chymotrypsin intermediates studied, and lowest recorded $\nu_{C=O}$ has been assigned to the productive acyl-enzyme. Our interpretation receives support from data on acylsubtilisins wherein the oxyanion hole of subtilisin BPN′ has been restructured by protein engineering. Replacement of Asn155, one of the hydrogen-bond donors in subtilisin's oxyanion hole, with residues such as leucine results in a large decrease in the catalytic activity of the enzyme (Table I; Wells et al., 1986; Bryan et al., 1986). As is evident in Figure 1, the RR data for [(5-methyl-2-thienyl)acryloyl]subtilisin BPN′ Asn155Leu consist of a broad unresolved carbonyl feature near 1705 cm^{-1}. Interestingly the profile does not include a low-frequency component below 1700 cm^{-1}, and thus, the enzyme Asn155Leu does not form strong hydrogen bonds to the acyl carbonyl oxygen. Similar RR carbonyl profiles are obtained for [(5-methyl-2-thienyl)acryloyl]subtilisin BPN′ Asn155Gln ($\nu_{C=O}$ 1703 cm^{-1}) and Asn155Arg ($\nu_{C=O}$ 1702 cm^{-1}) (data not shown). Evidently, any of the three changes results in the acyl carbonyl group being placed in a similar environment which only forms weak hydrogen bonds to the carbonyl oxygen atom. The deleterious effect of this environment can be seen by comparing the maximal deacylation rate constants (k_3) for the "mutant" (k_3 0.0007–0.0015 s^{-1}) and wild-type (k_3 0.13 s^{-1}) [(5-methyl-2-thienyl)acryloyl]acyl-enzymes (Table I).

Correlation between log k_3 and $\nu_{C=O}$. As discussed in the introduction, RR spectroscopic and kinetic data for several acylchymotrypsins have revealed a qualitative correlation between a reduction in $\nu_{C=O}$ for the productive acyl carbonyl group and an increase in the rate of deacylation (Tonge &

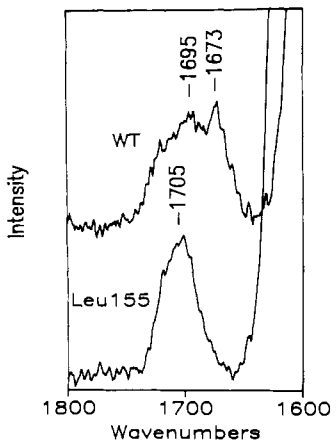


FIGURE 1: Carbonyl stretching region of the RR spectra of [(5-methylthienyl)acryloyl]subtilisin BPN′ obtained with wild-type enzyme (top) and Asn155Leu (bottom). The data were obtained at pH 9.8 with 100-mW 324-nm Kr^+ laser excitation (experimental resolution 7 cm^{-1}) and an acquisition time of 10 × 10 s. Stable acyl-enzyme in 20 mM acetate buffer and 0.1 M NaCl, pH 4.2, was mixed in a 1:1 ratio with 0.2 M borate buffer (0.1 M NaCl) to give a final pH of 9.8. The spectrum of [(5-methylthienyl)acryloyl]subtilisin BPN′ wild type was obtained with substrate labeled with ^{13}C in the ethylenic double bond in order to shift the vibrational frequency of this bond ($\nu_{C=C}$) to lower frequency and hence improve resolution in the carbonyl region of the RR spectrum. To the limits of the signal/noise in our spectra, the observed $\nu_{C=O}$ profile is identical with that obtained with unlabeled substrate.

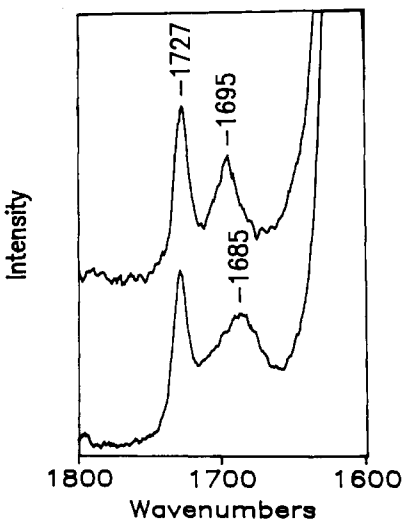


FIGURE 2: Carbonyl stretching region of the RR spectra of (thienylacryloyl)chymotrypsin (top) and [(5-methylthienyl)acryloyl]chymotrypsin (bottom). The spectra were obtained at pH 9.8 in 0.1 M carbonate and 0.1 M NaCl, as described in Tonge and Carey (1989), with 100-mW 337.5-nm Kr^+ laser excitation (experimental resolution 7 cm^{-1}) and 10 × 10-s acquisition time. On the basis of Fourier transform infrared studies, the band observed at 1727 cm^{-1} in both spectra is assigned to a carbonyl group associated with a laser-induced population of acyl-enzymes, present only during RR data collection (Tonge et al., unpublished data).

Carey, 1989). An example of this relationship is shown in Figure 2 wherein RR data are presented for (thienylacryloyl)chymotrypsin and [(5-methyl-2-thienyl)acryloyl]-chymotrypsin at pH 9.8, obtained with 337.5-nm laser excitation. The carbonyl bands at 1695 cm^{-1}, (thienylacryloyl)-chymotrypsin, and 1685 cm^{-1}, [(5-methyl-2-thienyl)acryloyl]chymotrypsin, are assigned in each case to the productive acyl-enzyme complexes, which is consistent with the hypothesis that a more reactive acyl-enzyme gives rise to a carbonyl group at lower frequency as the observed k_3's for the two acyl-enzymes are 0.0051 and 0.031 s^{-1}, respectively (Table I).

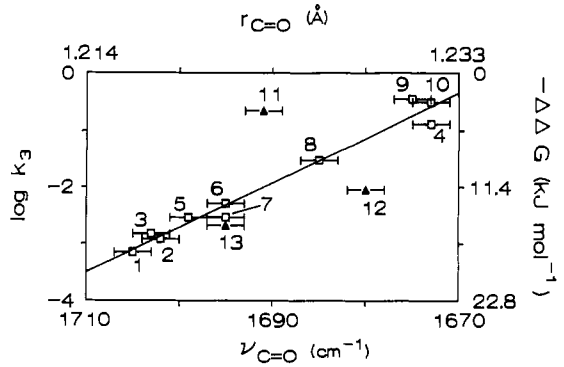
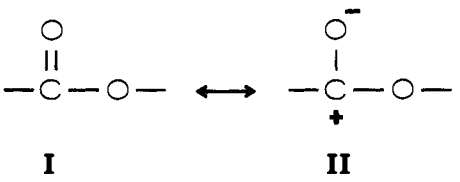


FIGURE 3: Plot of log k_3 against $\nu_{C=O}$ for the acyl-serine proteases. The curve shown was calculated by linear regression to points 1–10 (r 0.986). Data values are taken from Table I. Also shown on the abscissa is the estimated carbonyl bond length ($r_{C=O}$, Å) estimated from $\nu_{C=O}$ with (Horváth et al., 1987) $r_{C=O}$ = 2.00988 − 0.0004653$\nu_{C=O}$. Also shown on the ordinate is the relative free energy of activation ($\Delta\Delta G$, kJ mol^{-1}) calculated at 298 K from k_3 with $\Delta G = -RT \ln (k_3 h/kT)$ (Fersht, 1985) (where h and k are the Planck constant and the Boltzmann constant, respectively) and by arbitrarily setting $\Delta G = 0$ at $k_3 = 1$ s^{-1}. The acyl-enzymes are [(5-methylthienyl)acryloyl]subtilisin BPN′ [(1) Asn155Leu, (2) Asn155Arg, (3) Asn155Gln, and (4) wild type], (5) (furylacryloyl)chymotrypsin, (6) (thienylacryloyl)chymotrypsin, (7) (4-methoxycinnamoyl)chymotrypsin, (8) [(5-methylthienyl)acryloyl]chymotrypsin, (9) (4-methoxycinnamoyl)subtilisin Carlsberg, (10) [(5-methylthienyl)-acryloyl]subtilisin Carlsberg, (11) (4-amino-3-nitrocinnamoyl)chymotrypsin, (12) (4-amino-3-nitrocinnamoyl)subtilisin BPN′, and (13) (indolylacryloyl)chymotrypsin. The error bars represent the estimated accuracy of peak maxima determination (±2 cm^{-1}).

The RR spectroscopic and kinetic data for the acyl-chymotrypsins and acyl-subtilisins have been used to generate a quantitative relationship between the observed decrease in $\nu_{C=O}$ and increase in deacylation rate. This is shown in Figure 3 wherein $\nu_{C=O}$ has been plotted against log k_3. For 10 of the 13 acyl-enzymes studied, a linear correlation (r = 0.986) is observed, demonstrating that a decrease in $\nu_{C=O}$ for the productive acyl-enzyme is accompanied by an increase in reactivity. Of the remaining three acyl-enzymes two, (4-amino-3-nitrocinnamoyl)chymotrypsin and (4-amino-3-nitrocinnamoyl)subtilisin BPN′, show significant deviation from the correlation (Figure 3). However, it is known that the distribution of charge in the π-electron system of the 4-amino-3-nitrocinnamoyl group is far more sensitive to perturbation by enzyme forces than is the case for the other acyl groups. Thus, for example, active site contacts involving the amino or nitro groups could affect the distribution of electrons throughout the acyl group's π-electron chain and perturb $\nu_{C=O}$ (Carey & Salares, 1980). Finally, the data point for (indolylacryloyl)chymotrypsin has been plotted in Figure 3. While this point lies near the line calculated for the other acyl-enzymes, we do not feel confident in including this point in the correlation due to the atypically low base-catalyzed hydrolysis rate of (indolylacryloyl)imidazole (Tonge & Carey, 1989). In contrast, the base-catalyzed hydrolysis rates for the other acyl groups used in this study are similar (Tonge & Carey, 1989), indicating that the variations in k_3 observed for the analogous acyl-enzymes are due to specific enzyme effects and do not, in part, result from variation in the intrinsic reactivity of the acyl groups.

Estimation of Carbonyl Bond Length Extension in the Active Site. Using an empirical correlation (Layton et al., 1956; Horváth et al., 1987) between the frequency of the carbonyl stretching vibration and the carbonyl bond length, $r_{C=O}$, we can also estimate $r_{C=O}$ on the abscissa of Figure 3. The correlation (Horváth et al., 1987) between $\nu_{C=O}$ and $r_{C=O}$

Scheme I



I II

was derived from experimental observations on cyclic and heterocyclic organic compounds, but in all likelihood it can be applied over the limited range of values for the acyl-enzymes considered here. Interestingly, we can now measure, albeit indirectly, small changes in $r_{C=O}$—changes in the region of 0.001–0.01 Å. The 32-cm^{-1} variation in $\nu_{C=O}$ seen through the series in Figure 3 is equivalent to a change in $r_{C=O}$ of approximately 0.015 Å, and this increase in $r_{C=O}$ corresponds to an increase in k_3 of 500-fold.

The observed increase in reactivity can be explained in a number of ways (Belasco & Knowles., 1983), any or all of which can account for the increase in k_3 throughout the series. Experimentally we have evidence for a lengthening of the carbonyl linkage in more reactive acyl-enzymes. This indicates that the carbonyl bond is becoming more single bond in character. Although we do not know the length of the carbon–oxygen bond in the next intermediate on the reaction pathway, $C-O^-$ in the tetrahedral intermediate for deacylation, the increase in length of 0.015 Å in the series is about 7% of the expected change on going from a formal carbonyl double bond in the least reactive acyl-enzyme ($r_{C=O}$ 1.217 Å; Horváth et al., 1987) to a formal C–O single bond (r_{C-O} 1.44 Å; Hanson, 1975). If we assume that the linear relationship seen in Figure 3 extends over a wider range, then for a very good substrate (Karasaki & Ohno, 1978) with k_3 of the order of 100 s^{-1}, Figure 3 enables us to predict that $r_{C=O}$ will lengthen by about 0.03 Å. That is, it will make about 14% of the change needed to go to a typical C–O single bond. Thus, we have evidence for modest distortion of the ground-state structure of part of the acyl group toward the next intermediate on the reaction pathway.

Another perspective on the results, which is probably related to the above model of hydrogen bonding to the carbonyl reducing $\nu_{C=O}$, concerns the canonical forms of the ester group. The observed decrease in $\nu_{C=O}$ is also consistent with an increase of canonical form II shown in Scheme I to the overall ester structure (Callender et al., 1988). We can expect that the increase in the contribution of canonical form II increases reactivity by decreasing the electron density on the carbonyl carbon atom and making it more susceptible to nucleophilic attack. In this regard, other investigators using Fourier transform infrared spectroscopy have identified polarized substrate carbonyl groups in stable enzyme–substrate complexes involving triosephosphate isomerase (Belasco & Knowles, 1980), aldolase (Belasco & Knowles, 1983) and citrate synthase (Kurz & Drysdale, 1987).

Finally, the increase in hydrogen bonding strength to carbonyl we see for reactive acyl-enzymes in their ground state probably presages a catalytically important stabilization of charge buildup in the transition state, although, of course, we have no spectroscopic data for the latter.

An alternative way to consider the data in Figure 3 is to convert log k_3 to the relative value for free energy of activation, that is, the second scale, kJ mol^{-1}, on the ordinate. The slope kJ mol^{-1} $Å^{-1}$ gives the energy needed to extend the carbonyl bond along its axis (Jones & Kirby, 1984). The energy is 950 kJ mol^{-1} $Å^{-1}$, which is consonant with the value of 1050 kJ mol^{-1} $Å^{-1}$ derived by Jones and Kirby (1984) for the extension of a single C–O bond in the unimolecular heterolysis of a series of axial aryl tetrahydropyranyl acetals. While we cannot make a direct comparison between the C–O bond in a "simple" chemical system and our result for a carbonyl linkage in an acyl-enzyme, the correspondence between the two values is intuitively satisfying.

Although we are considering two quite different processes, it is interesting to compare the magnitude of the change in the free energy of activation observed through the acyl-enzyme series (15.4 kJ mol^{-1}) with the enthalpy of a hydrogen bond required to cause a 32-cm^{-1} decrease in $\nu_{C=O}$. The latter value requires a vibrational spectroscopic study on α,β-unsaturated esters which has not yet been undertaken. However, other simple ketones and esters have been extensively studied, and from the work of Thijs and Van Zeegers-Huyskens (1984) we can estimate that for methyl acetate a change in $\nu_{C=O}$ of 32 cm^{-1} would require a hydrogen-bond enthalpy of ca. 30 kJ mol^{-1} while for acetophenone the same change in $\nu_{C=O}$ would require 39 kJ mol^{-1}. Thus, the change in free energy of activation we observe through the series of acyl-enzymes is of the same order as that expected to be required to alter $\nu_{C=O}$ by 32 cm^{-1} via the formation of one or more hydrogen bonds to the carbonyl oxygen.

Summary

By combining RR spectroscopic measurements on functioning acyl-enzyme intermediates with X-ray crystallographic and infrared spectroscopic data on model compounds, we have been able to relate small changes in the critical acyl carbonyl bond to differences in the deacylation rate for a series of acyl-serine proteases. This approach is capable of extension to faster more "natural" substrates (Smolarsky, 1980) and providing some quantitative insight into how proteases achieve rate enhancement.

Acknowledgments

We are grateful to Dr. P. Bryan and Genex Corp. (Gaithersburg, MD 20877) for the gift of the subtilisin BPN′ clones, to Dr. Muhammed Waseem for the expression and purification of the subtilisin clones, and to Dr. Marianne Pusztai and Mrs. Eva Eichler for substrate synthesis.

References

Belasco, J. G., & Knowles, J. R. (1980) *Biochemistry 19*, 472–477.

Belasco, J. G., & Knowles, J. R. (1983) *Biochemistry 22*, 122–129.

Bryan, P., Pantoliano, W., Quill, S. G., Hsiao, H.-Y., & Poulos, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3743–3745.

Callender, R., Chen, D., Lugtenburg, J., Martin, C., Rhee, K. W., Sloan, D., Vandersteen, R., & Yue, K. T. (1988) *Biochemistry 27*, 3672–3681.

Carey, P. R., & Salares, V. R. (1980) *Adv. Infrared Raman Spectrosc. 7*, 1–58.

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

Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., p 49, Freeman, New York.

Hanson, A. W. (1975) *Acta Crystallogr. B31*, 1963–1965.

Horváth, G., Illényi, J., Pusztay, L., & Simon, K. (1987) *Acta Chim. Hung. 124*, 819–822.

Jones, P. G., & Kirby, A. J. (1979) *J. Chem. Soc., Chem. Commun.*, 288–289.

Jones, P. G., & Kirby, A. J. (1984) *J. Am. Chem. Soc. 106*, 6207–6212.

Karasaki, Y., & Ohno, M. (1978) *J. Biochem. 84*, 531–538.

Kurz, L. C., & Drysdale, G. R. (1987) *Biochemistry 26*, 2623–2627.
Layton, E. M., Kross, R. D., & Fassel, V. A. (1956) *J. Chem. Phys. 25*, 135–138.
MacClement, B. A. E., Carriere, R. G., Phelps, D. J., & Carey, P. R. (1981) *Biochemistry 20*, 3438–3447.
Pimentel, G. C., & McClellan, A. L. (1960) *The Hydrogen Bond*, Freeman, London.
Sans Cartier, L. R., Storer, A. C., & Carey, P. R. (1988) *J. Raman Spectrosc. 19*, 117–121.
Smolarsky, M. (1980) *Biochemistry 19*, 478–484.
Thijs, R., & Zeegers-Huyskens, T. (1984) *Spectrochim. Acta 40A*, 307–313.
Tonge, P. J., & Carey, P. R. (1989) *Biochemistry 28*, 6701–6709.
Wells, J. A., Cunningham, B. C., Graycar, T. P., & Estell, D. A. (1986) *Philos. Trans. R. Soc. London, A 317*, 415–423.
Willis, K. J., & Szabo, A. G. (1989) *Biochemistry 28*, 4902–4908.

Articles

Fidelity of DNA Recognition by the *Eco*RV Restriction/Modification System in Vivo†

John D. Taylor, Annette J. Goodall, Christian L. Vermote, and Stephen E. Halford*
Department of Biochemistry, Centre for Molecular Recognition, University of Bristol, Bristol BS8 1TD, U.K.
Received June 28, 1990; Revised Manuscript Received August 22, 1990

ABSTRACT: The *Eco*RV restriction/modification system consists of two enzymes that recognize the DNA sequence GATATC. The *Eco*RV restriction endonuclease cleaves DNA at this site, but the DNA of *Escherichia coli* carrying the *Eco*RV system is protected from this reaction by the *Eco*RV methyltransferase. However, in vitro, the *Eco*RV nuclease also cleaves DNA at most sites that differ from the recognition sequence by one base pair. Though the reaction of the nuclease at these sites is much slower than that at the cognate site, it still appears to be fast enough to cleave the chromosome of the cell into many fragments. The possibility that the *Eco*RV methyltransferase also protects the noncognate sites on the chromosome was examined. The modification enzyme methylated alternate sites in vivo, but these were not the same as the alternate sites for the nuclease. The excess methylation was found at GATC sequences, which are also the targets for the *dam* methyltransferase of *E. coli*, a protein that is homologous to the *Eco*RV methyltransferase. Methylation at these sites gave virtually no protection against the *Eco*RV nuclease: even when the *Eco*RV methyltransferase had been overproduced, the cellular DNA remained sensitive to the *Eco*RV nuclease at its noncognate sites. The viability of *E. coli* carrying the *Eco*RV restriction/modification system was found instead to depend on the activity of DNA ligase. Ligase appears to proofread the *Eco*RV R/M system in vivo: DNA, cut initially in one strand at a noncognate site for the nuclease, is presumably repaired by ligase before the scission of the second strand.

Restriction/modification (R/M)[1] systems possess two enzyme activities: a modification methyltransferase that recognizes a specific DNA sequence and catalyzes the transfer of a methyl group from AdoMet to a particular base within the recognition sequence and a restriction endonuclease that cleaves the DNA provided that neither strand has been methylated (Arber, 1979; Smith, 1979). These systems are widespread in prokaryotes, and their function is to maintain the integrity of the bacterial DNA. DNA that lacks the appropriate pattern of methylation is cleaved by the restriction enzyme, while the cellular DNA is protected by the methyltransferase (Arber, 1979). However, restriction enzymes can also cleave DNA at sequences other than their recognition sites (Bennett & Halford, 1989), often making the double strand break at these sequences by first cutting one strand of the DNA and then the second (Taylor & Halford, 1989; Thielking et al., 1990; Lesser et al., 1990). These reactions could be lethal to the cell unless the bacterium has a mechanism to protect the alternative sequences on its chromosome. One possibility is that the modification enzyme has a less stringent specificity for the recognition site than the nuclease, so that any alternative site for the nuclease is already methylated. This appears to be the case with the *Eco*RI R/M system (Woodbury et al., 1980a,b). An alternative is that another enzyme in the cell, perhaps DNA ligase, acts to proofread the specificity of the nuclease by selectively repairing DNA nicked at noncognate sites. A proofreading scheme for the *Eco*RV restriction enzyme involving DNA ligase has been modeled in vitro (Taylor & Halford, 1989), but it remains to be determined whether or not such a scheme operates in vivo.

*Eco*RI and *Eco*RV are both type II R/M systems (Smith, 1979). With the type II systems, in contrast to type I or III (Bickle, 1987), the restriction and modification activities are due to two separate enzymes [reviewed by Modrich and

†This work was funded by the Science and Engineering Research Council.

[1] Abbreviations: AdoMet, *S*-adenosylmethionine; Ap, ampicillin (with superscripts r and s to denote resistance and sensitivity); bp, base pair(s); BSA, bovine serum albumin; βME, β-mercaptoethanol; Cm, chloramphenicol; DMSO, dimethyl sulfoxide; kb, 1000 bp; Kn, kanamycin; R/M, restriction/modification; Sm, streptomycin; [ ], plasmid carrier state.

0006-2960/90/0429-10727$02.50/0