Unlocking the Secrets of Enzyme Power Using Raman Spectroscopy†

PAUL R. CAREY*,‡ AND PETER J. TONGE§

Department of Biochemistry, University of Ottawa, Ottawa, Canada K1H 8M5, and The Picower Institute for Medical Research, Manhasset, New York 11030

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Introduction

Enzymes have exercised a fascination over the minds of chemists for much of this century. An enzyme's prodigious powers of rate enhancement and selectivity when catalyzing a chemical reaction have even led to speculation that forces unique to a macromolecular environment, and as yet poorly understood, may be at work.[1] However, the contemporary consensus is that enzymes achieve their success by conventional, known chemistry: they are simply superb chemists using every available approach to achieve the twin goals of rate acceleration and specificity.[2] Factors such as favorable electrostatic and entropic effects and conformational distortions undoubtedly play a role in enzymatic catalysis, but their respective contributions are not easily quantitated. What are needed are experimental strategies to place the ideas on the source of enzyme power on a firm quantitative footing. We set out in this Account one such approach.

An important direct question is, How accurately do we need to define enzyme and enzyme–substrate structures to relate quantitatively structure to reactivity? The evidence from chemistry is alarming in this respect. For example, Kirby and co-workers have shown that small changes in bond lengths, e.g., of 0.03 Å for a C–O bond undergoing spontaneous hydrolysis in alkyl aryl acetals, correspond to large changes in reactivity, of 10^6-fold or more in the hydrolysis rate constant.[3,4] Alarm derives from the fact that such an accurate description of structure, possible from small molecule crystallography, is generally beyond the reach of macromolecular crystallography or NMR structural techniques for proteins. However, in this Account we show that Raman spectroscopic studies, coupled with a number of other techniques, can provide some of the very precise structural information on active-site bound substrates needed to relate structure to reactivity. Moreover, the Raman data can also be used to quantitate the strength of some key substrate–active site contacts involving hydrogen bonds. These measurements can be made on functioning enzyme–substrate complexes in solution, and thus critical issues of structure, active-site forces, and reactivity can be addressed.

The first studies of enzyme–substrate complexes by Raman spectroscopy[5,6] involved the examination of chromophoric acyl–enzymes using the resonance Raman (RR) effect. These studies of, e.g., substituted cinnamoyl and thienylacryloyl (TA) chymotrypsins have continued and constitute the bulk of the present Account. The cinnamoyl and TA acyl groups are chromophoric by virtue of their delocalized π electron systems and thus give rise to intense RR spectra when the Raman experiment utilizes an excitation wavelength which lies within the chromophore's absorption band. A second RR approach using dithioacyl–cysteine protease intermediates, e.g., $CH_3OPheNHCH_2C(=S)S$-papain, which gives detailed information on the torsional angles in and about the dithio group, has been discussed in detail elsewhere.[7,8] Recently, advances in technology allow the obtention of Raman data from an enzyme–substrate complex under normal Raman, i.e., nonresonance, conditions.[9] As a result there will be greatly increased activity in applying Raman spectroscopy to a wide range of mechanistic problems in enzymology which until now have been inaccessible.

Obtaining Vibrational Data from Functioning Enzyme–Substrate Complexes

In this section we will be concerned with obtaining vibrational spectroscopic data for key active-site groups

Paul Carey was born in Dartford, England, in 1945. He obtained a B.Sc. in chemistry and a D.Phil. in chemical applications of NMR at the University of Sussex in the U.K. In 1969 he joined the National Research Council of Canada in Ottawa as a postdoctoral fellow with Harold Bernstein and changed his main research activity from NMR to Raman spectroscopy. He became director of the Protein Structure and Design Section in the Institute for Biological Sciences at NRC in 1988 and professor of biochemistry at the University of Ottawa in 1993. He is presently professor of biochemistry at Case Western Reserve University, Cleveland. His research interests focus on the applications of Raman spectroscopy in biochemistry with special emphasis on understanding protein–ligand interactions at a level satisfying to the discriminating chemist.

Peter Tonge was born in Northampton, England, in 1961. He obtained a B.Sc. in biochemistry in 1982 and a Ph.D. in biochemistry in 1986 at the University of Birmingham, England. In 1986 he moved to the National Research Council of Canada in Ottawa, Canada, as a NATO-SERC postdoctoral research fellow. In 1988 he became a research associate at NRC and in 1993 a research officer. The focus of his research at NRC has been on the use of spectroscopic techniques to develop structure–reactivity relationships for enzymes. In 1994 he was appointed staff investigator at The Picower Institute for Medical Research, where he is investigating the molecular basis of Alzheimer's disease.

* Address correspondence to this author: Department of Biochemistry, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106-4935. Fax number: 216 368 4544. Telephone number: 216 368 5302.

‡ University of Ottawa.

§ The Picower Institute for Medical Research.

† Abbreviations: RR, resonance Raman; TA, thienylacryloyl; 5MeTA, (5-methylthienyl)acryloyl.

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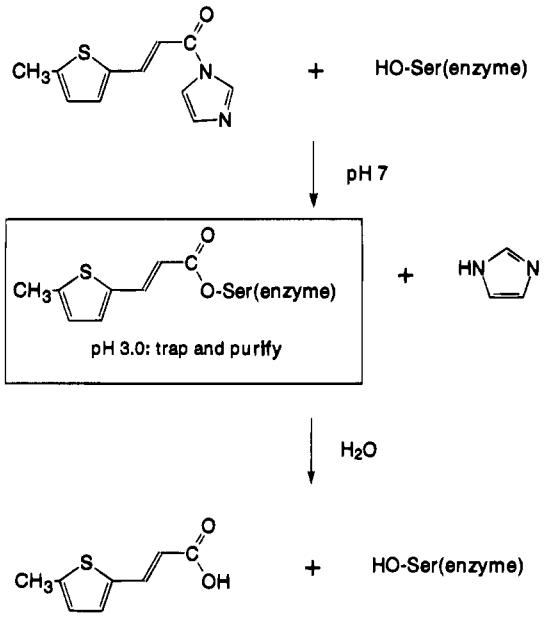
Scheme 1



in enzyme–substrate complexes while catalysis is occurring. The enzymes in question are the serine proteases chymotrypsin and subtilisin, whose biological function is to catalyze the hydrolysis of peptide bonds: $RC(=O)NHR' + H_2O \rightarrow RC(=O)OH + H_2NR'$. The reaction proceeds via a transient covalent intermediate called an acyl enzyme, RC(=O)O–enzyme. Serine proteases also hydrolyze esters and imidazolides, and by using an excess of a chromophoric substrate and by manipulating pH it is possible to trap and purify an acyl–enzyme with a chromophoric acyl group linked to the active site serine (Scheme 1).

The reaction is initiated with an excess of substrate near neutral pH in order to generate a quasi-steady-state population of acyl–enzymes. After a few seconds the pH is lowered to 3, where the acyl–enzyme becomes stable and can be purified by standard biochemical techniques. The stabilization results from the protonation of the side chain of His-57, which is thus unable to act as a general base in the deacylation step. Examination of the stable acyl–enzyme by absorption spectroscopy reveals intense absorbance near 280 nm due to protein and a second intense feature near 340 nm due to the chromophoric acyl moiety. In a RR experiment the acyl–enzyme is irradiated in solution using a laser whose wavelength lies under the chromophore's absorption band,[10] e.g., a Kr^+ laser line at 350.6 nm. The scattered light is then analyzed for frequency and intensity and is found to contain a major contribution from photons scattered from the acyl chromophore. This is due to the RR effect, and the practical outcome is that it is possible to obtain the vibrational spectrum of the chromophore in a complex biological matrix (in this case a protein) without interference from weak protein or solvent modes. Thus, in essence the RR spectrum is the vibrational equivalent of absorption spectroscopy in that it focuses in on a chromophore in a complex biological milieu, but the vibrational spectrum is capable of providing much more detailed chemical information compared to absorption data.

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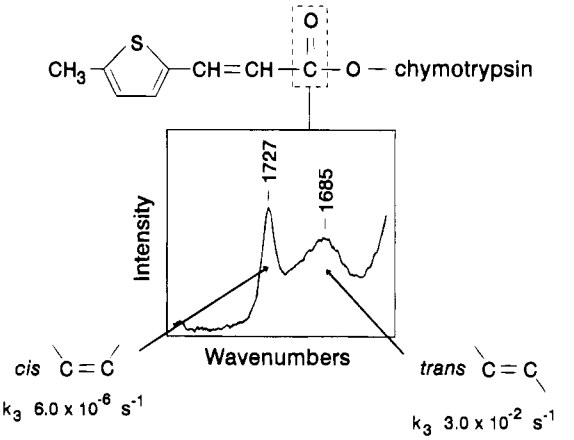

Figure 1. The carbonyl stretching region of the RR spectrum of 5MeTA-chymotrypsin. The 1727 and 1685 cm^{-1} features are from isomers which are *cis* and *trans* about the ethylenic bond, respectively.

The RR spectrum of a purified acyl–enzyme contains many features associated with the normal modes of the active-site bound acyl group. The spectral data can provide information by a number of routes, e.g., by a qualitative group frequency approach or by complete theoretical and normal mode analyses. An alternate approach using precise structural and spectroscopic data on model compounds to "calibrate" the RR data for the enzyme–substrate complexes will be detailed in the next section. The discussion in this Account focuses on RR features found near 1700 cm^{-1}, which are due to stretching motions of the acyl carbonyl group, $\nu_{C=O}$.

In order to obtain RR data from functioning, active acyl–enzymes the pH is "jumped" in a rapid mixing, rapid continuous flow cell and the acyl–enzyme examined prior to deacylation.[11] In this cell, stable acyl–enzyme at pH 3 is efficiently mixed with pH 10 buffer in a four-hole mixing jet, and the stream issuing from the jet is interrogated by the laser beam before more than 5% of the acyl–enzyme is converted to product. The rate constant for deacylation, k_3, is known from kinetic studies using absorption spectral changes as a monitor. For example, for 5MeTA-subtilisin BPN' k_3 is 0.13 s^{-1}. At pH 10 the imidazole side chain of His-57 is neutral and acts as a general base in assisting attack on the acyl C=O group by a water molecule.

A typical C=O stretching region RR profile is seen in Figure 1 for 5MeTA-chymotrypsin. At pH 10 there are two features, a "sharp" band near 1725 cm^{-1} and a broad feature near 1685 cm^{-1}. In fact we are detecting two conformers in the active site.[12] Detailed FTIR, NMR, and Raman difference spectroscopic studies[13,14] have shown that the 1685 cm^{-1} band is due to a C=O group in an acyl conformation which is *trans* about the ethylenic C=C linkage, while the 1725 cm^{-1} band is due to a C=O group in a conformer which is *cis* about C=C. The *cis* conformer is a photoisomer caused by the laser beam used to generate the RR

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Table 1. pK_a values for k_{obs} and $\nu_{C=O}$[a]

acyl–chymotrypsin	pK_a (k_{obs})	pK_a ($\nu_{C=O}$)
indolylacryloyl	7.82 ± 0.05	7.70 ± 0.07
5-MeTA	7.51 ± 0.10	7.38 ± 0.05
4-NH_2-3-NO_2-cinnamoyl	7.43 ± 0.12	7.43 ± 0.11

[a] For experimental conditions, see ref 17.

spectrum. It has important structural and kinetic qualities which will be detailed below. The first important generalization to emerge, however, is that *the RR spectrum of a chromophoric acyl–enzyme provides the vibrational spectrum of the acyl C=O group as it is poised for nucleophilic attack.* We will see that this spectrum can be interpreted to provide novel mechanistic information.

Precise Details of Active Site Bonds and Forces

RR data for the $\nu_{C=O}$ profiles and enzyme kinetic data have been obtained for 15 chromophoric acyl–enzymes involving chymotrypsin and subtilisin, the acyl groups being derivatives of cinnamoyl, furylacryloyl, TA, or indolylacryloyl.[15,16] The findings can be summarized as follows.

(a) Many intermediates show a sharp 1725 cm^{-1} C=O feature due to an isomer *cis* about the acyl group's C=C bond. This isomer is generated by the near-UV laser light used to generate the RR spectrum.[13] The position of the band is essentially invariant with pH and among different acyl–enzymes. The position and band shape suggest that the C=O giving rise to the 1725 cm^{-1} peak is in a hydrophobic, non-H-bonding environment.[12,14]

(b) Pure *cis* acyl–chymotrypsins could be prepared from mixtures of the *cis* and *trans* acyl–enzymes which had been formed by irradiation of a pure *trans* acyl–chymotrypsin population with near-UV light.[16] At pH 10 the *cis* forms deacylate very slowly with a $k_3 \approx 10^{-5}$ s^{-1}.

(c) All acyl–enzymes show the broad C=O stretch seen in Figure 1, which is consistent with a population of hydrogen-bonded carbonyls. The position of the broad C=O feature decreases in cm^{-1} with increasing pH, and a RR spectroscopic pK can be measured by plotting the peak position as a function of pH.[17] Table 1 compares the spectroscopic pK with the pK measured from the deacylation kinetics for three substrates. The observed identity is strong evidence that the RR C=O feature reflects the ionization state of the side chain of His-57 which assists in the attack of the C=O by a water molecule. This important finding can be summarized: *The equivalence of the enzyme kinetic and $\nu_{C=O}$ spectroscopic pK's means that the carbonyl feature reflects activation of the deacylation mechanism and can be used to probe that mechanism.*

(d) An early empirical observation[17] was that more reactive intermediates (with higher deacylation constants, k_3's) had lower $\nu_{C=O}$'s at pH 10. This led to the hypothesis that the acyl C=O is hydrogen bonded at or near the oxyanion hole (as can be seen in Figure 2, this comprises two active-site H-bond donating groups which stabilize charge buildup on the carbonyl oxygen in the transition state) and that more reactive

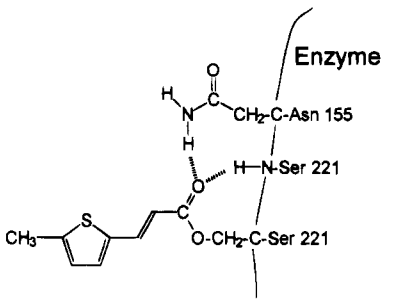

Figure 2. Substrate binding in the oxyanion hole of subtilisin BPN′.

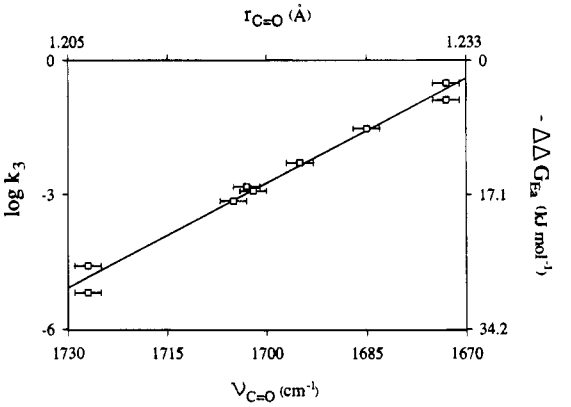

Figure 3. Correlation between the carbonyl stretching frequency, $\nu_{C=O}$, and log k_3, where k_3 is the maximal deacylation rate at pH 10 for the series of acyl–serine proteases. Each point represents an acyl–enzyme. $\nu_{C=O}$ and log k_3 are also recast in the forms of $r_{C=O}$ (carbonyl bond length) and $\Delta\Delta G$ (free energy of activation for deacylation), respectively.

intermediates have more polarized C=O groups in the sense that there is a greater contribution of C^+-O^- to the overall structure.

(e) The hypothesis that the broad, low-cm^{-1} carbonyl feature is due to an oxyanion hole bonded population was tested by forming acyl–enzymes of subtilisin where one of the hydrogen-bonding donors in the oxyanion hole was "knocked out" by protein engineering.[15] As seen in Figure 2 the oxyanion hole of subtilisin consists of a N–H group from the peptide backbone and a second N–H donor from the side chain of Asn-155.

When Asn-155 was changed by site-selected mutagenesis to Leu-155, which lacks a H-bonding donor, profound changes were seen in the RR C=O profile of the acyl–enzyme. In particular, compared to the RR spectrum of the wild-type acyl–enzyme, the low-cm^{-1} "polarized" C=O feature was not seen (the peak had probably shifted and merged with a broad spectral feature at higher cm^{-1}). This lent strong support to the notion that the low-cm^{-1} broad C=O is indeed due to a population of acyl carbonyls H-bonding in the oxyanion hole. Moreover, the k_3 for 5MeTA-subtilisin BPN′ Leu155Asn is 0.007 s^{-1} compared to 0.13 s^{-1} for that of the wild-type intermediate, indicating the deleterious kinetic consequences of crippling the oxyanion hole.

(f) A plot of $\nu_{C=O}$ against log k_3 for each of the intermediates characterized reveals a linear relationship,[16] as can be seen in Figure 3.

This observation takes on added significance when it is recast in the form of carbonyl bond length, $r_{C=O}$ vs log k_3. This is possible because there is an empiri-

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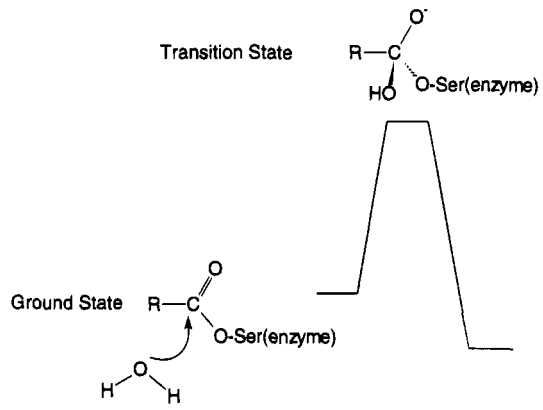

Figure 4. Reaction profile for deacylation.

cal relationship between $\nu_{C=O}$ and $r_{C=O}$ based on X-ray crystallographic and IR spectroscopic measurements on the same crystals of a large series of compounds.[18] The plot of $r_{C=O}$ against log k_3 reveals that, as the reactivity increases by 16300-fold, the carbonyl bond length increases by 0.025 Å. Since the transition state for the deacylation reaction is close to a tetrahedral intermediate (Figure 4), we can see that for the more reactive acyl–enzymes the C=O bond is being distorted in the direction consonant with going from a formal double bond in the ground state to a single bond in the transition state. Indeed, an extension in length of 0.025 Å is about 10% of the change from a C=O double bond, typically 1.22 Å, to a C–O single bond, typically 1.44 Å.[19] The important conclusion is that *it is possible to estimate with great accuracy the length of a catalytically crucial bond in the active site as the reaction is occurring.*

The approach is feasible because extremely accurate bond lengths and vibrational spectroscopic data on small model compounds are used to transform the RR $\nu_{C=O}$ data for each acyl–enzyme to an accurate $r_{C=O}$ value. In this way vibrational spectroscopy can be seen as a vector which carries extremely accurate measurements on model compounds to a functioning active site where such measurements cannot be made directly. It is also worth emphasizing that the small changes in $r_{C=O}$ that we are characterizing here, which correspond to enormous changes in reactivity, are beyond the limits of accuracy achievable by direct X-ray crystallographic measurements on even stable macromolecular complexes.

(g) For hydrogen-bonded C=O groups, shifts in $\nu_{C=O}$ can also be used to calculate the enthalpy, ΔH, of the hydrogen bond(s) formed between the acyl C=O group and the H-bonding donor(s) in the oxyanion hole.[16] Again the approach was to carry out a series of experiments on model compounds to "calibrate" changes in $\nu_{C=O}$ in terms of changes in ΔH and to transfer this information to the active acyl–enzyme intermediates.

The model compounds consisted of 5MeTA methyl ester and TA methyl ester. FTIR was used to monitor shifts in their $\nu_{C=O}$ values in CCl_4 in the presence of one of the H-bonding donors 3,5-dichlorophenol, phenol, or ethanol. In CCl_4 there is an equilibrium between individual ester and alcohol molecules and a complex consisting of a single alcohol OH group hydrogen bonding to an ester carbonyl moiety. An equilibrium constant, K, could be calculated from the H-bonded and non-H-bonded features in the O–H stretching region of the donor. When these experiments were performed at a series of temperatures in the FTIR instrument, ΔH and ΔS for the O–H to C=O hydrogen bond could be calculated using the van't Hoff equation:

$$\log K = -\Delta H/RT + \Delta S/R$$

Thus, it is possible to develop a relationship between ΔH, the enthalpy of the hydrogen bond, and the resulting shift in $\nu_{C=O}$. For the series of acyl–enzymes depicted in Figure 3, the change in $\nu_{C=O}$ is 54 cm^{-1}, which, along with the correlation developed with the model compounds, indicates that there is a change in the hydrogen-bonding strength, throughout the series, of $\Delta\Delta H = -27$ kJ mol^{-1}. Since ΔH represents the enthalpy of the hydrogen bond, $\Delta\Delta H$ represents the difference in H-bonding strengths between the most weakly H-bonded carbonyl in the active site (the C=O groups from the *cis* acyl–enzymes) and the most strongly bonded (the C=O groups in 5MeTA-subtilisins).

Insights into Enzyme Mechanism

As was detailed in the previous section, there is an increase in H-bonding strength between the acyl carbonyl oxygen and the oxyanion hole of −27 kJ mol^{-1} upon going from the most unreactive acyl–enzyme to the most reactive species. The acyl–enzyme is, of course, the ground state on the reaction profile for the deacylation reaction. It is remarkable that the $\Delta\Delta H$ value is very similar to the change in activation energy ($\Delta\Delta G$) throughout the series, where $\Delta\Delta G$ represents changes in the differences between the ground and transition state energy levels. $\Delta\Delta G$, calculated from k_3 using $\Delta G = -RT \ln k_3$ and using $G = 0$ when $k_3 = 1$ s^{-1} for obtaining $\Delta\Delta G$, is plotted on the right-hand ordinate of Figure 3. $\Delta\Delta G$ changes by −24 kJ mol^{-1} throughout the series of acyl–enzymes, close to the value of $\Delta\Delta H$ of −27 kJ mol^{-1}. Thus, the experimental finding is as follows:

A change in ground state property (ΔH's in the acyl–enzymes) is matched by an equal change in energy difference between the ground and transition states. It should also be emphasized that the measured ΔH involves a localized interaction in the active site, whereas ΔG is a property of the entire macromolecular complex.

The underlying significance of the relationship $\Delta\Delta G \approx \Delta\Delta H$ is not completely understood at this time. One possibility involves the concept of ground state strain, as promulgated by Jencks.[20] In this case the acyl–enzyme (the ground state) is energetically strained, due to unfavorable enzyme–substrate interactions, by an amount which exactly offsets the favorable ΔH from the oxyanion hole. The strain is released in the transition state, and the overall reduction in ΔG gives rise to rate acceleration. If we assume that the differences we observe in ΔG's are governed simply by differences in the hydrogen-bonding strengths in the acyl–enzymes and their transition states, and that, for any two acyl–enzymes, the difference in

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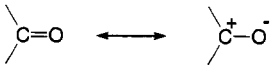
bonding in the ground states is equaled in the transition states, then the equality $\Delta\Delta G \approx \Delta\Delta H$ can be shown to hold. At this stage of our knowledge, however, we cannot reach a definitive conclusion as to the cause of the observed equality. Further experiments, for example using k_{cat}/K_M to probe changes in the transition state, are needed.

Additional insight into reactivity can be gained by considering the bond length–reactivity data set out in Figure 3. Those data clearly show that, throughout the series, as the rate constant for deacylation increases, the C=O bond of the acyl group becomes longer. In valence terms this can be depicted as the more reactive intermediates having increasing contributions from canonical form 2.



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A consequence of this is that the more reactive acyl–enzymes will have less negative charge on the carbonyl carbon atom. In other words, that atom will be more electrophilic and, in the deacylation step, more reactive to nucleophilic attack by the incoming water molecule. Thus, we can see how interactions in the acyl–enzyme complex are causing the C=O linkage to become more polarized and thus more reactive. In a sense this is a strained C=O linkage since it is being distorted away from its equilibrium conformation and electron distribution by forces in the active site. The energy for this distortion is "paid for" by favorable enzyme–substrate contacts. Quantitation of the change in C=O bond length and electron distribution should be accessible via quantum chemical calculations, and these could provide a perspective on the relationship between changes in charge density at the carbon atom and reactivity.

An alternate way to consider the data in Figure 3 is to convert log k_3 to the relative value for the 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.[4] The energy is 950 kJ mol^{-1} Å^{-1}, which is consonant with the value derived by Jones and Kirby[4] for the extension of a single C–O bond in a unimolecular heterolysis of a series of axial 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.

Future Studies

During the past 20 years the application of Raman spectroscopy to the study of enzyme–substrate complexes has been essentially limited to the investigation of chromophoric intermediates using the RR effect, and the above discussion is a case in point. Resonance conditions were necessary in order to work at concentrations of 100 μM or less and to focus selectively on active-site events. New technology is, however, greatly reducing the need for RR experiments. Recently it has become possible to obtain the Raman spectrum of a protein-bound ligand at concentrations of millimoles/liter or less under "normal" (nonresonance) Raman

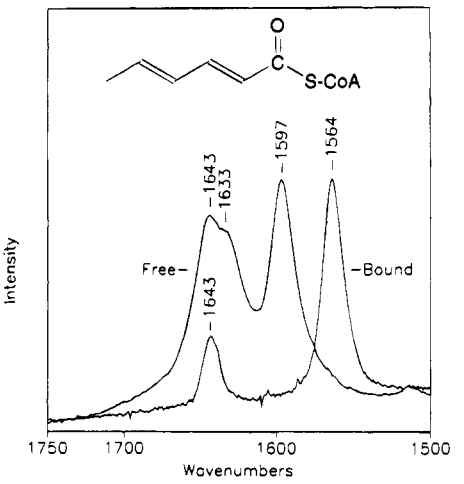

Figure 5. Raman difference spectra of hexadienoyl-CoA in solution and bound to crotonase. Spectra were obtained using 0.2 mL of 1 mM solutions and 200 mW, 647.1 nm krypton laser excitation, and the data took 100 s to collect.

conditions. The approach relies on a computer-based subtraction of the Raman spectrum of the protein from the Raman spectrum of the complex. Two advances permit the obtention of very high quality Raman data.[9] The use of charge-coupled devices as photon detectors has the twin advantages of multiplex detection and sensitivity in the red part of the spectrum. The latter is important because red-excited Raman spectra are much less prone to interference from overwhelming fluorescence. The second advance is the use of supernotch optical filters which block elastically scattered Rayleigh photons from the sample but transmit the inelastically scattered Raman photons with high efficiency. The supernotch filters replace single or double monochromators and increase greatly (by orders of magnitude) the Raman signals.

The outcome of the use of the new "Raman difference spectrometers" is that Raman is changing from being a rather esoteric method in mechanistic enzymology (and in other branches of structural biology) to a technique with widespread applicability and growing importance. Thus, "impossible" samples for resonance Raman investigation such as flavins bound to proteins[21] and highly photolabile ligands readily yield to Raman difference spectroscopy. The latter case is illustrated in Figure 5, the red-excited Raman spectra of unbound and crotonase-bound hexadienoyl-CoA are obtained with little effort using the new technology.[22] Interpretation of the Raman difference spectra in Figure 5 indicates that more than one conformation, involving rotational isomerism about the hexadiene C–C bonds, is present for the unbound ligand. However, upon binding, conformational selection occurs giving rise to a single form on the enzyme. Additionally, the spectra show that the enzyme causes strong electron polarization to occur in the –C=C–C(=O)– bonds, providing important clues on mechanism. In contrast, the data obtained by the resonance Raman approach (with excitation in the near-UV region) were very difficult to interpret due to photoisomerism and photodegradation occurring in the laser beam used to generate the RR data. The one drawback that the Raman difference approach has is that

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(22) Unpublished work, P.R.C. and P.J.T. with Dr. Vernon Anderson.

it is difficult to look at unstable complexes, given the requirement for stable spectroscopic conditions in order to carry out high-quality spectral subtractions; thus the RR technique still holds an advantage for characterizing intermediates "on the fly".

Selectivity, "focusing" on the part of the macromolecular complex of interest, in the Raman difference approach can be achieved by judicious isotopic labeling:[23] subtracting the spectrum of labeled from that of unlabeled complex results in vibrational features from only the labeled part of the system. Another factor that provides a degree of specificity is that many ligands have delocalized π electron systems and are therefore strong Raman scatterers, even under nonresonance conditions. With many of the barriers removed for obtaining good quality Raman data from mechanistically important enzyme complexes, it remains for the investigator to have an appreciation of the answers that the technique can provide and to select those systems for study for which critical information is lacking.

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