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Chemistry of Enzyme–Substrate Complexes Revealed by Resonance Raman Spectroscopy †

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1 Introduction

Chemists' fascination with enzymes stems partly from envy. Enzymes usually bring about rate enhancements of 10^8 or more while at the same time performing feats of specificity, for example at chiral centres, which we can only begin to emulate today. Thus, chemists have set out to define the properties of enzymes, using techniques of ever increasing sophistication, in the hope that they will find out how these proteins work. Armed with this knowledge and the possibility of redesigning enzymes using the methods of protein engineering[1] there are significant opportunities for creating novel, useful catalysts. Unfortunately, our understanding of the fine chemical details of enzyme function is often primitive and insufficient for knowledge-based redesign.

This being the case the question arises as to what constitutes sufficient understanding – how much do we really need to know? Many scientists would be satisfied with the coordinates of all the atoms in an enzyme–substrate complex at each point along the reaction pathway. Although this level of description remains a distant dream there is still another factor to consider. It has been realised in recent years that dynamical fluctuations of the enzyme–substrate intermediate must be included in the total description.[2] These dynamical motions, some of which may be unproductive and carry the complex momentarily off the reation pathway, provide the needed link between the static structural picture of a protein complex and the kinetic functioning enzyme. This review will attempt to show how resonance Raman (RR) spectroscopy can provide some structural and dynamical information on active site groups in functioning enzyme–substrate complexes. Of course, no one technique can provide a complete description of enzyme action and, as well as demonstrating some advantages unique to the RR method, the interplay between RR spectroscopy, *X*-ray crystallography, and enzyme kinetics will be emphasized.

RR spectroscopy can be regarded as a high information counterpart of electronic absorption spectroscopy.[3] The experimental data from the latter

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[1] W. V. Shaw, *Biochem. J.*, 1987, **246**, 1.
[2] (*a*) G. Careri, P. Fasello, and E. Gratton, *Annu. Rev. Biophys. Bioeng.*, 1979, **8**, 69; (*b*) G. R. Welch, B. Somogy, and S. Damianovich, *Progr. Biophys. Mol. Biol.*, 1982, **39**, 109.
[3] P. R. Carey, 'Biochemical Applications of Raman and Resonance Raman Spectroscopies', Academic Press, 1982.

consist of absorption bands associated with that part of the molecule giving rise to an electronic transition – the chromophore A RR spectrum is a vibrational spectrum of a chromophoric group, which, because it is sensitive to the group s conformation, environment, and electronic properties, can provide a great deal of chemical information on the chromophore RR data has the advantage that peak positions are a property solely of the electronic ground state while information on electronic excited states can be gleaned from RR peak intensities Although the RR spectrum 'reports on' the vibrational motions of the chromophore, the data can be related to molecular structure by a number of means Thus, the RR experiment occupies a bridgehead between protein structure and vibrational dynamics The key to using RR spectroscopy to study enzyme–substrate complexes is to generate a chromophore in the active site of the enzyme and thus to be able to monitor the vibrational spectrum of those bonds undergoing catalytic transformation At this time two major means of chromophore generation have been used, one involves using substrates which are intrinsically chromophoric due to the presence of an extended π-electron chain, the other creates a dithio ester chromophore RC(=S)S–Enz when a thiono ester substrate RC(=S)OR becomes transiently linked to a cysteine protease (Enz–SH) [4]

The approach taken in this review will be to outline how RR spectra can be obtained from the two classes of substrate Then two important chemical issues will be addressed, namely how the RR data provide insight into chemical forces in the active site and, secondly, how the data can begin to answer some of the questions regarding reactivity and the underlying causes of rate acceleration Finally, since the application of RR spectroscopy to the study of enzyme substrate complexes opens several novel avenues of research, a number of emerging trends will be outlined

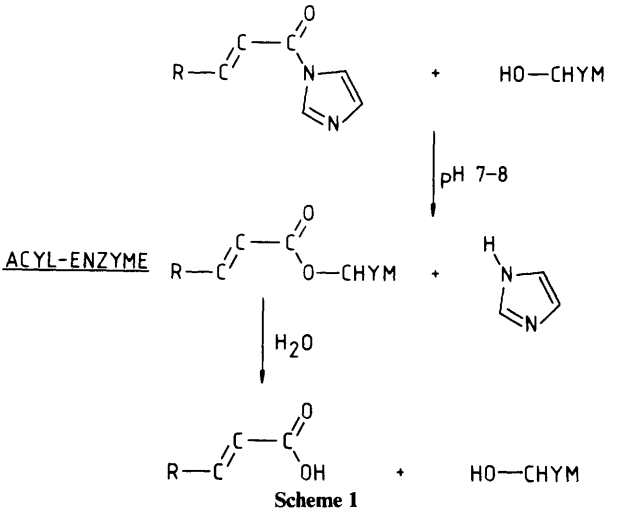
2 Using Chromophoric Substrates or Dithio Ester Intermediates to Generate Resonance Raman Spectra

A. Chromophoric Substrates.—The principles involved in obtaining RR data from the active site of an enzyme substrate complex can be illustrated by intermediates of the type R–C=C–C(=O)–O–chymotrypsin, wherein the substrate is covalently attached to the enzyme *via* the active site Ser-195 residue [5] Since the R group is usually based on a phenyl, furyl, or thienyl ring the extended π-electron chain of the bound acyl group constitutes a suitable chromophore for RR study A typical reaction scheme is shown below (Scheme 1)

The covalent intermediate, the acyl-enzyme, can be trapped during the reaction by changing pH and purified by standard methods of protein purification [6] The absorption spectrum of the acyl-enzyme is shown in Figure 1

The intense band near 340 nm is due to the substrate's acyl group bound to

[4] (*a*) P R Carey and A C Storer *Annu Rev Biophys Bioeng* 1984 **13** 25 (*b*) P R Carey and A C Storer *Pure Appl Chem* 1985 **57** 225
[5] P R Carey and H Schneider *Biochem Biophys Res Commun* 1974 **57** 831
[6] P R Carey and H Schneider *J Mol Biol* 1976 **102** 679



Scheme 1

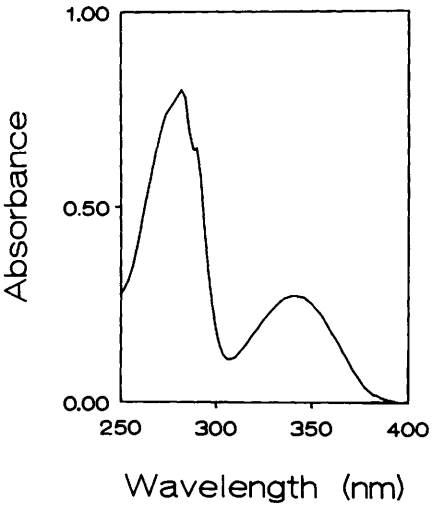


Figure 1 *Absorption spectrum of* 5-*methylthienylacryloyl-chymotrypsin at* pH 3.0

Ser-195 in the chymotrypsin active site. By exciting under this absorbance, using for example 337.5 nm Kr^+ laser radiation, the RR spectrum seen in Figure 2 can be recorded.

Because of the resonance Raman effect, which gives rise to a very intense spectrum of the chromophore, only peaks due to the acyl group are seen in Figure 2. Thus the RR spectrum allows us to obtain selectively the vibrational spectrum of the substrate without interference from the protein or solvent.

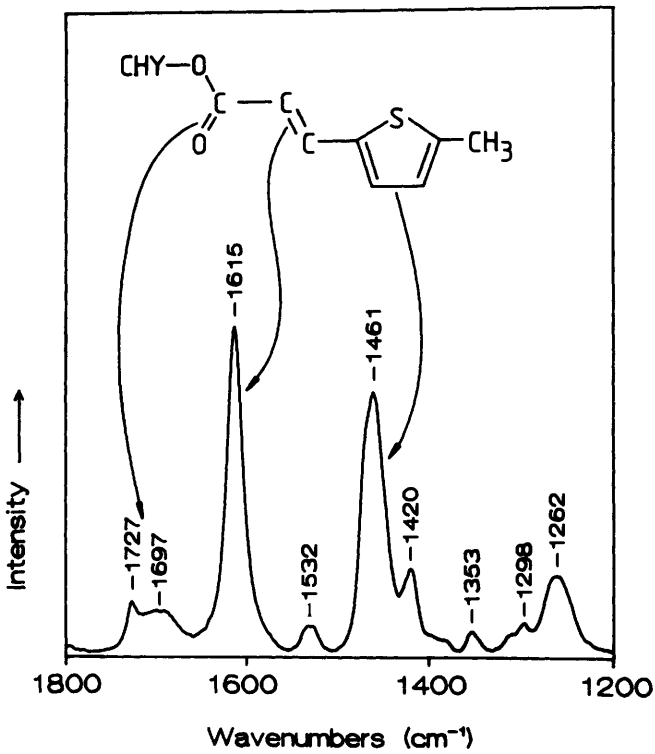


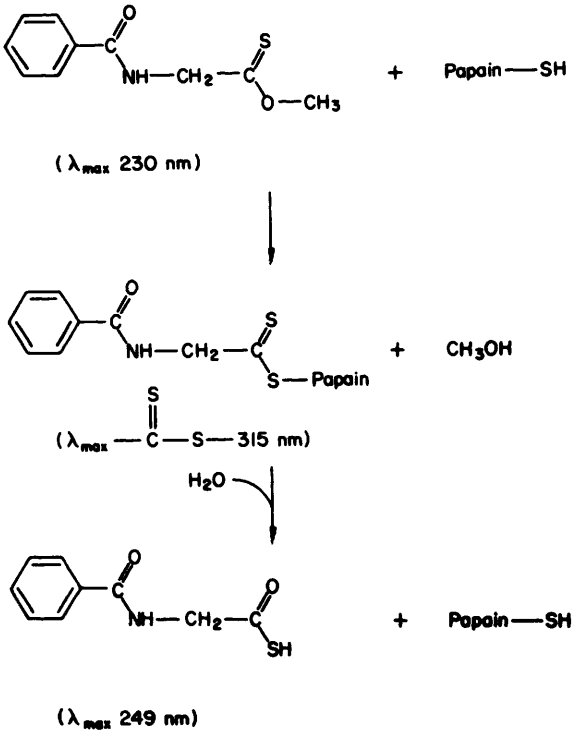
Figure 2 *Resonance Raman spectrum of 5-methylthienylacryloyl-chymotrypsin at* pH 3.0. 337.5-nm *excitation,* 100 mW, *exposure time* 10 × 10 s, 7 cm^{-1} *experimental resolution*

Features in the RR spectrum can be assigned to molecular groups, *e.g.* the peaks near 1700 cm^{-1} are due to the C=O group, the 1615 cm^{-1} band is due to the ethylenic stretching mode, $\nu_{C=C}$, and the peak at 1461 cm^{-1} is due to a thienyl ring mode.[7] Some of these groups, *e.g.* the C=O, are of mechanistic importance and the RR spectrum can be used to follow key events during the reaction. This class of substrates is equally applicable to the study of cysteine proteases.

B. Dithio Ester Intermediates.—The second strategy for using RR spectroscopy as a probe of active site events involves creating dithio ester chromophores in cysteine proteases at the time and place of catalytic transformation.[8] The reaction scheme for *N*-benzoylglycine methyl thiono ester and papain is shown below (Scheme 2).

Here the acyl-enzyme intermediate involves the creation of a transient dithio

[7] B. A. E. MacClement, R. G. Carriere, D. J. Phelps, and P. R. Carey, *Biochemistry*, 1981, **20**, 3438.
[8] (*a*) G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 189; (*b*) A. C. Storer, W. F. Murphy, and P. R. Carey, *J. Biol. Chem.*, 1979, **254**, 3163.



Scheme 2

ester group with a λ_{max} near 315 nm; it is the only species in the reaction mixture with an absorbance to the red of 300 nm. Since the acylation step is more rapid than deacylation, by using an excess of substrate it is possible to form a quasi-steady state population of acyl-enzyme for seconds or even minutes. The presence of the dithio ester chromophore for a finite time enables the RR spectrum of the acyl-enzyme to be recorded from the reaction mixture under turnover conditions. The RR spectrum contains many features in the 400–1200 cm^{-1} region which provide detailed information on conformational events involving bonds in the $-C(=O)NHCH_2C(=S)SCH_2C$(Cys-25) moiety. Owing to the requirement for an active site cysteine residue, there is at present no analogous group of substrates for serine proteases.

It is generally accepted that the acyl-enzymes formed by serine and cysteine proteases are preceded and followed by tetrahedral intermediates (THI's) for acylation and deacylation, respectively (Figure 3).[9]

It is unlikely that THI's will be identified under turnover conditions by

[9] L. Polgár and P. Halász, *Biochem. J.*, 1982, **207**, 1.

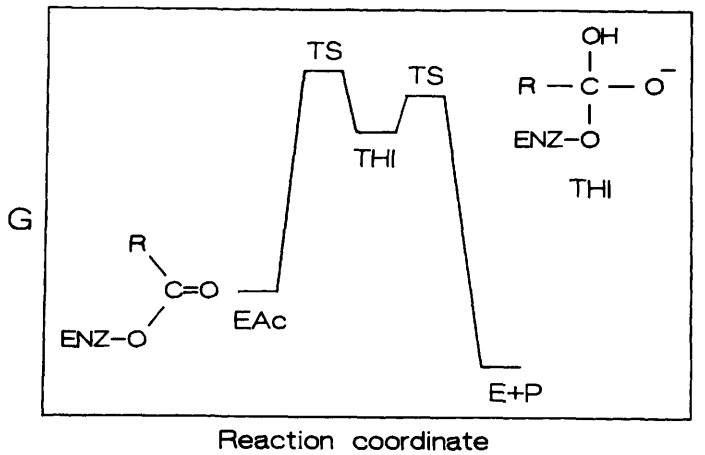


Figure 3 *Schematic representation of the free energy profile for deacylation of the acyl-enzyme* R–C(=O)–O–Enz. *The reaction proceeds via the formation of a tetrahedral intermediate (THI) which is expected to be close in structure to the transition states (TS) for deacylation. This free energy plot is analogous to that for the preceding acylation reaction in which the acyl-enzyme is generated from free enzyme and substrate*

spectroscopic or crystallographic means since it is extremely difficult to create a significant population given the THI's position on the potential energy curve, and their short lifetimes. However, their resemblance to transition states and their positions preceding and following an acyl-enzyme make THI's important species in discussions of mechanism.

3 Chemical Forces in the Active Site

A. Hydrogen-bonding to the Acyl Carbonyl Group.—The interpretation of the vibrational spectrum of an enzyme–substrate complex is greatly facilitated by the identification of a spectroscopic marker which can be directly correlated with an individual enzyme–substrate contact. In this regard the acyl-enzyme indoleacryloyl-chymotrypsin is of particular interest because the *X*-ray crystallographic structure of this complex is available. The structure determined at pH 4.0 by Henderson[10] shows the acyl carbonyl oxygen atom is hydrogen-bonded to two water molecules (Figure 4).

One of these water molecules (Figure 4) forms a strong hydrogen bond with the carbonyl oxygen and is in turn hydrogen-bonded to the protonated imidazole side chain of His-57. The other water molecule (not shown) forms only a weak hydrogen bond with the carbonyl oxygen and is in turn hydrogen-bonded to the backbone carbonyl group of Phe-41. This structure is non-productive because the acyl carbonyl oxygen is not hydrogen-bonded in the 'oxyanion hole'. In a

[10] R Henderson, *J Mol Biol*, 1970, **54**, 341

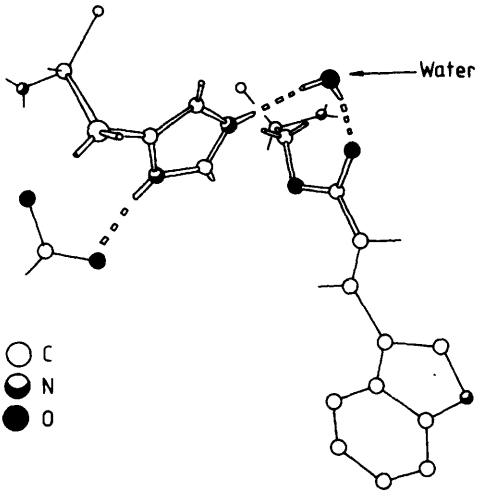


Figure 4 *The crystal structure of indoleacryloyl-chymotrypsin. The diagram shows the hydrogen-bonding interaction between the indoleacryloyl carbonyl group and the protonated imidazole side chain of* His-57 via *a bridging water molecule. The side chain of* Asp-102 *is also shown*
(Adapted from reference 10)

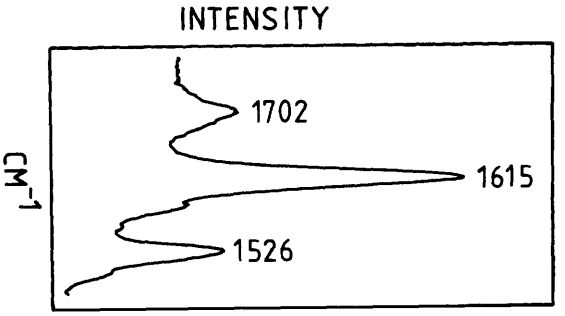


Figure 5 *Resonance Raman spectrum of indoleacryloyl-chymotrypsin at* pH 3.0. 337.5-nm *excitation,* 100 mW, *exposure time* 10 × 10 s, 7 cm^{-1} *experimental resolution*

productive complex the acyl carbonyl group is expected to be hydrogen-bonded to two enzyme NH groups (in chymotrypsin these are the backbone amide groups of Gly-193 and Ser-195). These hydrogen bonds are thought to promote catalysis by stabilizing the build up of negative charge that occurs on the carbonyl oxygen atom during substrate hydrolysis, hence the term 'oxyanion hole'.[11]

The RR spectrum of indoleacryloyl-chymotrypsin at pH 4.0 in the region 1600—1800 cm^{-1} is shown in Figure 5.

[11] A. R. Fersht, 'Enzyme Structure and Mechanism', 2nd Edn., Freeman, New York, 1985.

The RR band corresponding to the acyl carbonyl group appears as a single broad feature centred at 1702 cm^{-1}. This compares to $\nu_{C=O}$ 1685 cm^{-1} for the model compound indoleacryloyl methyl ester, in H_2O, suggesting that the carbonyl in the active site is not as strongly hydrogen-bonded as the carbonyl group of the model in H_2O wherein there are two hydrogen bonds to the carbonyl oxygen atom [12]. Thus, using the RR data, we are able to gauge the effect of protein hydrogen bond donors on the acyl carbonyl group.

B. Probing Electrical Forces in the Active Site—There is a general consensus that electrical forces are an important factor in enzymatic catalysis. For example, active site charges or dipoles can play a major role in stabilizing charge build up in the transition state. However, methods for characterizing these forces are not well developed and there is a need for experimental data to test the models for protein electrostatics[13] which are being developed by theoreticians. The 'extended π-electron chain' class of substrates in fact provides a good probe of active site electrical forces since the π electrons are polarized by nearby charges, and this effect can be followed by changes in the RR spectrum. The acyl-papain 4-dimethylamino-3-nitrocinnamoyl-papain provides a striking example of active site induced π-electron polarization [14]. Figure 6 shows that there is a complete change in the RR spectrum of the acyl group upon binding to the active site.

The spectrum of the acyl-papain has an intense band at 1570 cm^{-1} which is not seen in the RR spectrum of the product (or the substrate). In fact there is a complete lack of correspondence between RR bands in the two spectra seen in Figure 6. The clue to understanding this dramatic change was furnished by cinnamoyl model compounds which have strong electron donating groups on the phenyl ring and a strong electron withdrawing group attached to the carbonyl. These compounds have RR and electonic absorption spectra closely resembling those of the acyl-papain. The compound 4-dimethylaminocinnamoyl-imidazole mimics the spectral properties of the acyl-papain particularly well and its structure, derived by crystallographic analysis, is shown in Figure 7.

One striking feature of the structure is the difference in bond lengths in the ethylenic bonds compared to cinnamoyl compounds which do not have strong electron donors and acceptors attached to them. These 'normal' bond lengths are shown in parentheses. The changes in the ethylenic bonds, where in 4-dimethylaminocinnamoyl-imidazole the C–C and C=C linkages are shortened and lengthened respectively, are consonant with valence bond structures of the kind seen in Figure 8 making an increased contribution to the 4-dimethylaminocinnamoyl-imidazole structure [15]. The importance of structures such as that depicted in Figure 8 is due to the 'push-me pull-you' nature of the dimethylamino and imidazole substituents setting up strong polarization within the π electrons.

[12] P. J. Tonge and P. R. Carey, *Biochemistry*, 1989, **28**, 6701.
[13] (*a*) M. K. Gilson and B. H. Honig, *Nature*, 1987, **330**, 84; (*b*) A. Warshel, G. Narey-Szabo, F. Sussman, and J.-K. Hwang, *Biochemistry*, 1989, **28**, 3629.
[14] P. R. Carey, R. G. Carriere, D. J. Phelps, and H. Schneider, *Biochemistry*, 1978, **17**, 1081.
[15] P. R. Carey and V. R. Salares, *Adv. Infrared Raman Spectrosc.*, 1980, **7**, 1.

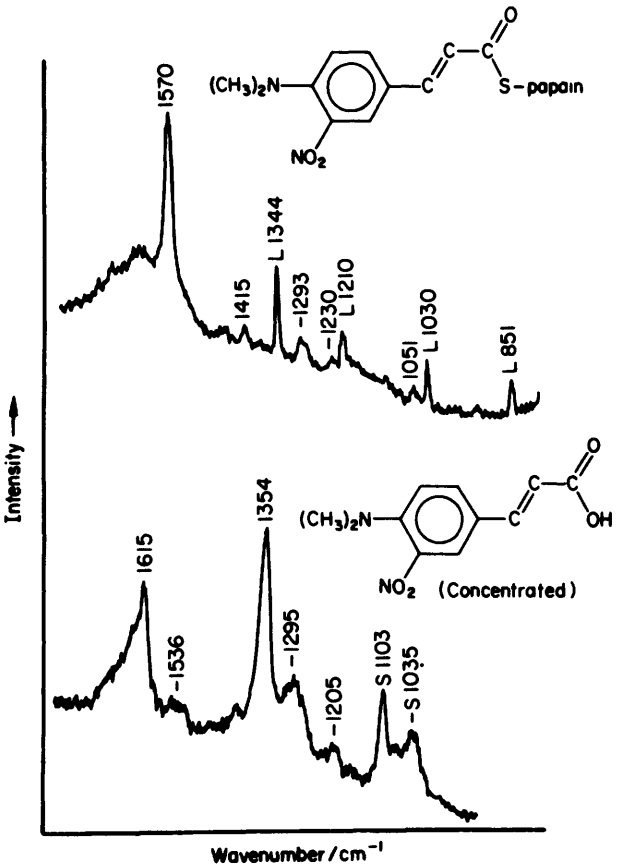


Figure 6 *Comparison of the resonance Raman spectra of* 4-*dimethylamino*-3-*nitrocinnamoyl-papain* (*top*) *and* 4-*dimethylamino*-3-*nitrocinnamic acid* (*bottom*). *L and S denote laser lines and solvent peaks respectively*
(Adapted from reference 14)

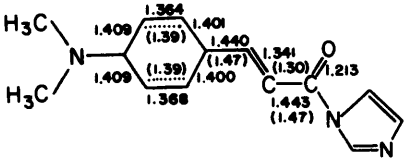


Figure 7 X-*Ray crystallographic structure of* 4-*dimethylaminocinnamoyl-imidazole*
(Unpublished work C. P. Huber and P. R. Carey)

Returning to the acyl-enzyme, it is the π-electron polarization brought about by electrical forces in the papain active site which give rise to the dramatic change in the RR spectrum of the bound acyl group. In the acyl-enzyme the

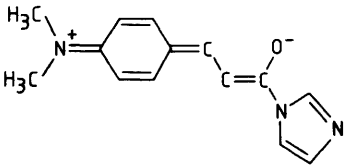


Figure 8 *Valence bond structure of* 4-*dimethylaminocinnamoyl-imidazole which contributes to the true structure*

polarization is occurring 'intermolecularly', rather than intramolecularly as in the case of 4-dimethylaminocinnamoyl-imidazole. A prime active-site candidate for bringing about the π-electron reorganization is the dipole moment from an α-helix involving a number of peptide residues and ending at cysteine-25[16] – the very residue which binds the acyl group.

This system also demonstrates the advantage of combining RR studies of acyl-enzymes with RR and crystallographic analysis of suitable model compounds. The RR data can be used as a 'vector' to carry precise structural information from the model to the enzyme-bound acyl group and to define some structural aspects of the acyl moiety in the active site very accurately. The same stategy has also been used to define small bond length changes in the geometry of the dithio ester bonds in dithioacyl papains.[17]

Some degree of π-electron polarization is very common when 'extended π chain' substrates bind to cysteine or serine proteases. However, it is usually much less marked than in the above example and is accompanied by small shifts in $\nu_{C=C}$ in the RR spectrum and a red shift in λ_{max} of the chromophore's absorption band. These phenomena are discussed in detail elsewhere.[3,4a]

C. Conformer Selection.—When there is the possibility of rotational isomerism about one or more of the substrate bonds the question arises as to which, or how many, of the various conformers is bound in the active-site. Additionally, we can ask if the bound conformer is distorted away from its relaxed low energy minimum by twisting about torsional angles. Such questions have an important place in the theory of enzyme action since geometric strain, along the reaction coordinate from the ground to the transition state, has long been proposed as a source of rate acceleration.[18]

Conformer selection and possible strain about torsional angles have been studied extensively using the RR spectra of *N*-acylglycine dithioacyl papains. The conformers discussed involve the Ramachandran-like torsional angles φ′ and ψ′ (Figure 9).

Again, the conformational properties of the acyl-enzymes are derived by reference to suitable model compounds such as $RC(=O)NHCH_2C(=S)SC_2H_5$. The models have been analysed in depth by Raman and FTIR spectroscopies

[16] W G J Hol, P T van Duijnen, and H C Berendsen, *Nature*, 1978, **273**, 443
[17] C P Huber, Y Ozaki, D H Phura, A C Storer, and P R Carey, *Biochemistry*, 1982, **21**, 3109
[18] W P Jencks, *Adv Enzymol*, 1975, **43**, 219

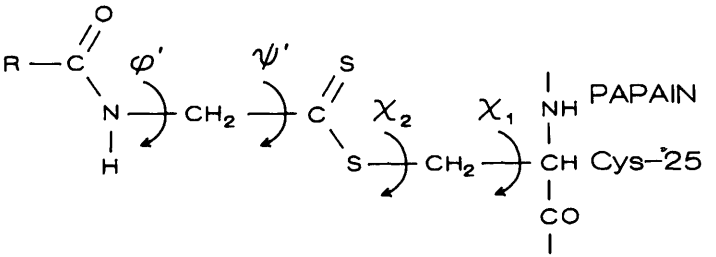
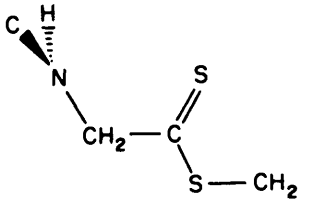
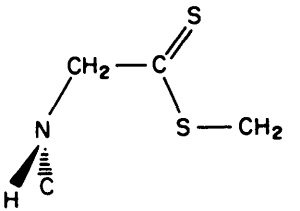


Figure 9 *The torsional angles* φ′, ψ′, χ_1, *and* χ_2 *for* N-*acylamino acid dithioacyl papains*



A



B

Figure 10 N-*acylamino acid dithio ester conformers* A *and* B

and by *X*-ray crystallography.[17,19] In aqueous solution there are two conformational states involving the φ′ and ψ′ angles. These are shown in Figure 10.

For glycine-based dithio esters the usual form in the crystal state is conformer B, which is characterized by a small ψ′ angle and close approach of the N atom to the thiol S atom.[20] Figure 11 compares the RR spectra of *N*-benzoylglycine ethyl dithio ester in aqueous solution and in its crystalline form with the spectrum of the corresponding dithioacyl papain. The close resemblance of the

[19] (*a*) H. Lee, A. C. Storer, and P. R. Carey, *Biochemistry*, 1983, **22**, 4781; (*b*) P. R. Carey, H. Lee, Y. Ozaki, and A. C. Storer, *J. Am. Chem. Soc.*, 1984, **106**, 8258; (*c*) C. P. Huber, P. R. Carey, S.-C. Hsi, H. Lee, and A. C. Storer, *J. Am. Chem. Soc.*, 1984, **106**, 8263; (*d*) V. M. Jardim-Barreto, J. J. C. Teixeira-Dias, P. R. Carey, and A. C. Storer, *Rev. Port. Quim.*, 1984, **26**, 131.

[20] K. I. Varughese, A. C. Storer, and P. R. Carey, *J. Am. Chem. Soc.*, 1984, **106**, 8252.

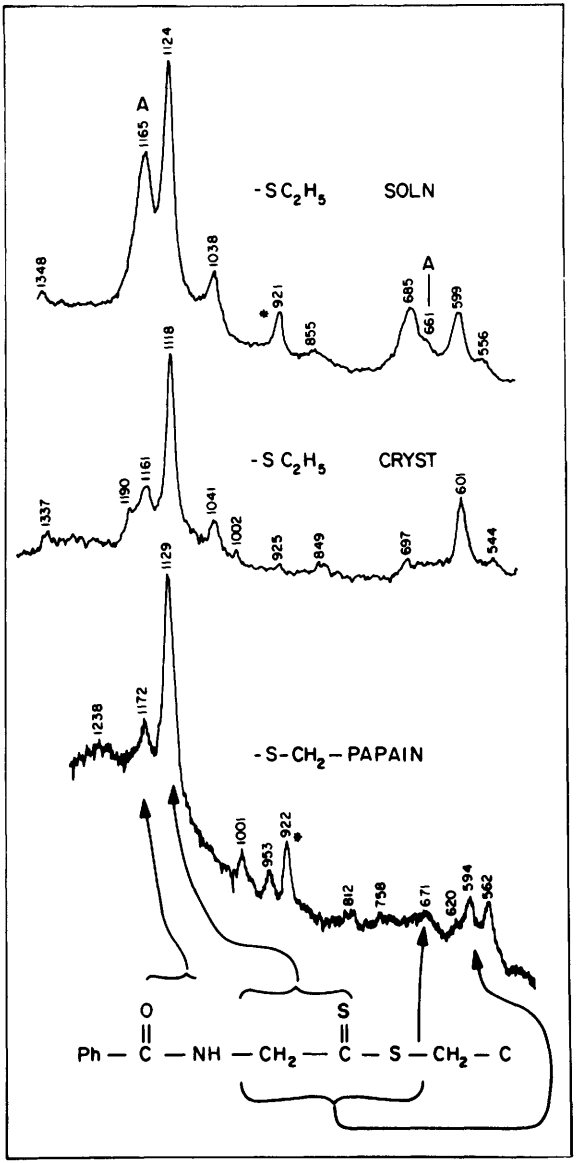


Figure 11 *Comparison of the* 324-nm *excited resonance Raman spectra of* N-*benzoylglycine dithio esters: the ethyl ester in aqueous solution (top); the ethyl ester in its polycrystalline form containing only conformer* B (*middle*); *the dithioacyl papain* (*bottom*)
(Taken from reference 4a)

solid and acyl-enzyme spectra is an immediate indication that the bound acyl conformation is the same as in the solid model, namely a B-conformer.

This conclusion is supported by analysis using, for example, isotopic substitution. To date 16 *N*-acylglycine dithioacyl papains have been analysed and all have the acyl group binding as the B conformer. No evidence has been found for the presence of any other conformer; thus for this class of substrate the active site of papain exerts strong conformer selection in favour of conformer B. The same conclusion is reached for *N*-acylglycine dithioacyl-enzymes involving the other plant cysteine proteases chymopapain, bromelain, ficin,[21] papaya peptidase II, and actinidin,[22] as well as the mammalian enzyme cathepsin B.[23]

In addition, there is evidence for a modest amount of distortion. This takes the form of differences in peak position for the intense band in the region 1130—1140 cm^{-1} for the acyl-papain compared to the corresponding B-marker band for the model in solution. The acyl-enzyme band is always several wavenumbers higher and this difference, Δ, is larger for good compared to poor substrates, for example *N*-methyloxycarbonyl-L-phenylalanylglycine dithioacyl papain[24] compared to *N*-benzoylglycine dithioacyl papain[19a,25] where Δ is 8 and 5 cm^{-1}, respectively and the k_3's, the rate constants for deacylation,[24,19b] are 0.53 and 0.082 s^{-1}. The upshift in the intense acyl-enzyme band is interpreted as being due to an increase in ψ' such that this torsional angle is distorted towards the value found in the next intermediate on the reaction pathway, the tetrahedral intermediate for deacylation. However, it should be emphasized that this distortion is small, of the order of 10—25°, and is probably energetically inexpensive.

4 Reactivity

Our understanding of the forces and effects which contribute to the specificity and catalytic efficiency of an enzyme continues to improve. It is clear that no single 'magical' effect is responsible for the remarkable performance of enzymes as catalysts. In fact it is likely that most of the factors proposed in the theory of enzyme catalytic action are used in various combinations by different classes of enzymes. For example, charge stabilization, the use of favourable binding energies to bring reactive groups in close juxtaposition, and transition state stabilization are all well documented effects in the explanation of enzyme competence.[26] RR spectroscopy can make a valuable contribution to this fund of knowledge since it can characterize the molecular properties of *functioning* intermediates on the reaction pathway. In this section the modulation of the deacylation rate constant by an intramolecular electronic effect will be illustrated by RR studies on a series of *N*-benzoylglycine dithioacyl papains and their corresponding model compounds. Results from other dithioacyl papains will lead to the surprising possibility that acyl groups binding in the active site in two

[21] P. R. Carey, Y. Ozaki, and A. C. Storer, *Biochem. Biophys. Res. Commun.*, 1983, **117**, 725.
[22] K. Brocklehurst, P. R. Carey, H. Lee, E. Salih, and A. C. Storer, *Biochem. J.*, 1984, **223**, 649.
[23] P. R. Carey, R. H. Angus, H. Lee, and A. C. Storer, *J. Biol. Chem.*, 1984, **259**, 14357.
[24] R. H. Angus, P. R. Carey, H. Lee, and A. C. Storer, *Biochemistry*, 1986, **25**, 3304.
[25] A. C. Storer, H. Lee, and P. R. Carey, *Biochemistry*, 1983, **22**, 4789.
[26] W. P. Jencks, *Cold Spring Harbor Symposia on Quantitative Biology*, 1987, **52**, 65.

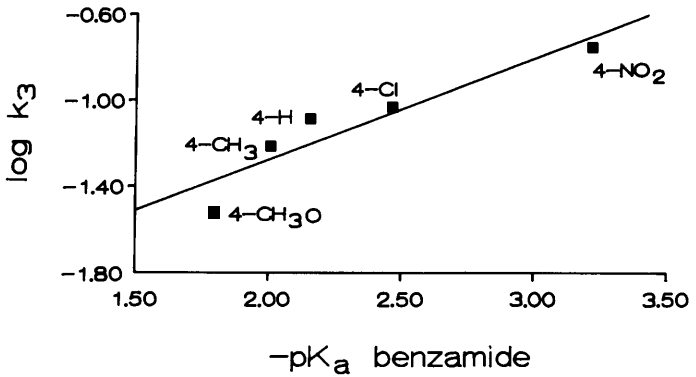


Figure 12 *Plot of* log k_3 *for the series of* para-*substituted* N-*benzoylglycine dithioacyl papains against* $-pK_a$ *for the corresponding benzamide fragments*
(Data taken from reference 19b)

disparate ways can still deacylate with very similar rates. Finally, it will be shown that RR analysis of the carbonyl profile of some acyl-chymotrypsins and acyl-subtilisins can cast light on the question of the contribution of ground state destabilization *versus* transition state stabilization to rate enhancement.

A. Control of Rate of Deacylation by an Intramolecular Electronic Effect.—For a series of *N*-benzoylglycine dithioacyl papains, RR and kinetic studies have shown that small variations in the strength of a single enzyme substrate contact caused by an intramolecular electronic effect can be correlated with changes in k_3, the rate constant for deacylation.[19b] The series is derived by placing the *para*-substituents $-OCH_3$, $-CH_3$, –H, –Cl, and $-NO_2$ on the benzoyl ring. RR spectra for these intermediates show that each has an identical B-type conformer for the acyl group in the active site, in particular the φ' and ψ' torsional angles of the $NH-CH_2-C(=S)$ bonds are invariant throughout the series. The B-conformer has a characteristic N···S contact involving the glycine N atom and the thiol S from cysteine-25. This is a HOMO–LUMO interaction between the nitrogen atom's lone pair electrons and the empty sulphur *d* orbitals.[20] The electron withdrawing or attracting nature of the *para*-substituent changes the electron density at the nitrogen lone pair which in turn modulates the strength of the N···S interaction. The latter conclusion was reached by RR studies on *para*-substituted *N*-benzoylglycine ethyl dithio esters in solution.

Returning to the acyl-enzymes, a plot of log k_3, where k_3 is the rate constant for deacylation, *versus* $-pK_a$ for the corresponding benzamide fragment,[19b] is given in Figure 12.

The straight line plot seen in Figure 12 indicates a strong correlation between k_3 and the electronic nature of the substituent, with an increase in the electron-withdrawing ability of the substituent leading to an increase in k_3.

Taking the acyl-enzyme and model data together, it can be concluded that, in

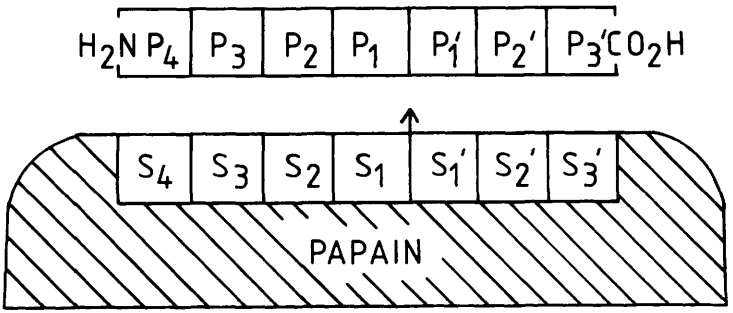


Figure 13 *Schematic representation of the seven binding subsites in the active site of papain* (S_4—S_3') *and the corresponding seven substrate residues* (P_4—P_3') *as determined by Schecter and Berger.*[28] *The arrow indicates the substrate bond* (P_1–P_1') *cleaved in the hydrolysis reaction*

the active site, the *para*-substituent modulates the electron density at the glycinic nitrogen and thereby modulates the strength of the N · · · S(thiol) interaction. The increase in strength is expressed in a reduction in k_3, suggesting that the rate limiting step in deacylation of the dithioacyl papain involves breaking the N · · · S contact. This is consonant with the view that the acyl-enzyme is followed on the reaction pathway by a tetrahedral intermediate, since in the latter case, steric considerations show that the N · · · S interaction is absent.[27]

B. Acyl Group Binding at Different Sites.—A model for the binding of a protein substrate to the active site of papain has been proposed by Schecter and Berger.[28] It envisages that for the polypeptide chain the enzyme binds up to four amino acid residues on the acyl side and three residues on the leaving group side, as shown in Figure 13.

X-Ray crystallographic analysis of a number of chloromethyl ketone–papain complexes by Drenth and co-workers[29] have shown binding in the S_1 and S_2 subsites with the acyl group in a B-type conformation. This is fully substantiated by the RR results for 16 glycine-based dithioacyl papains, $RC(=O)NHCH_2$-$C(=S)S$–papain. The RR data demonstrate clearly that in every case the acyl group is bound to Cys-25 as a B-conformer (Figure 14).

However, recent results for a series of substrates which have a side chain on the C_α atom of the first amino acid, $RC(=O)NHCHR'C(=S)S$–papain with R′ = $-C_2H_5$, $-C_3H_7$, and $-C_4H_9$, have shown that the acyl chain of the substrate is binding as an A-conformer.[30] One way of accommodating the A-conformer in the active site and still meeting the criterion for effective general base catalysis by the His-159 imidazole residue of papain is shown in Figure 15.

It is interesting that two such radically different modes of binding might occur

[27] A. C. Storer and P. R. Carey, *Biochemistry*, 1985, **24**, 6808.
[28] I. Schecter and A. Berger, *Biochem. Biophys. Res. Commun.*, 1968, **32**, 898.
[29] J. Drenth, K. H. Kalk, and H. M. Swen, *Biochemistry*, 1976, **15**, 3731.
[30] P. J. Tonge, R. Menard, A. C. Storer, B. P. Ruzsicska, and P. R. Carey, to be published.

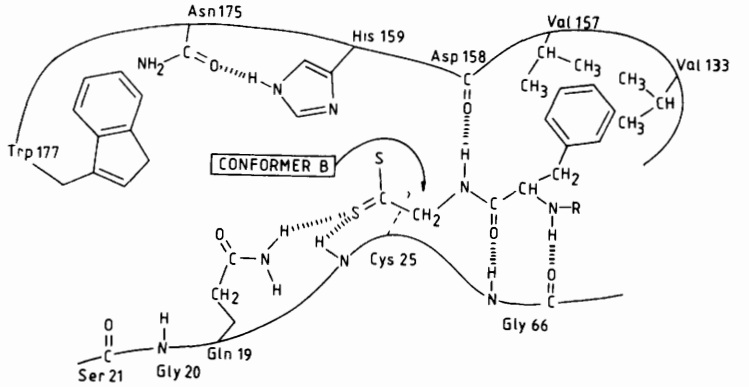


Figure 14 *Schematic representation of the binding of the* L-*Phe-Gly acyl group in the active site of papain as a* B *conformer Note that the Phe side chain is bound in the* S_2 *binding site (comprised in part by the enzyme side chains of* Val-133 *and* Val-157*) and that the dithio ester* C=S *group is hydrogen-bonded in the oxyanion hole (back-bone* NH *of* Cys-25 *and side chain* NH *of* Gln-19*)*

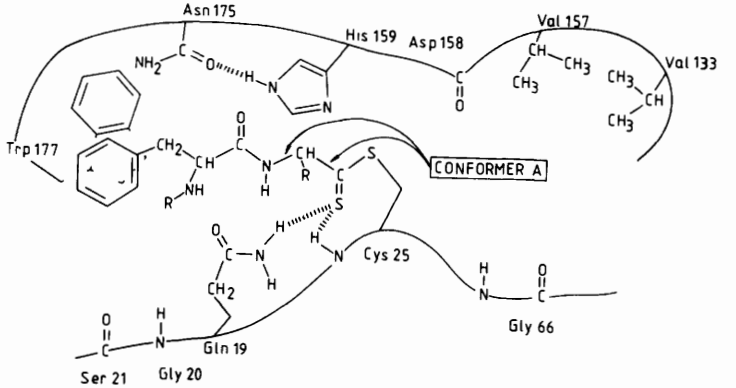


Figure 15 *Schematic representation of the binding of a* L-*Phe-amino acid acyl group in the active site of papain as an 'A-type' conformer. Note that whilst it is proposed that the dithio ester* C=S *group is bound in the oxyanion hole, the acyl group is bound 'backwards' in the active site and occupies the* S_1' *and* S_2' *enzyme subsites*

and the interest is heightened by the similar kinetics for the two classes of intermediates. The A-type conformers deacylate with k_3 values of about 2 s^{-1}, which is three times faster than the best B-conformer acyl-enzyme. This is a very small difference which can easily be explained by the retarding effect of the N • • • S contact in the B-conformers.[30]

C. Probing Reactivity *via* the Acyl Carbonyl Group.—In serine proteases deacylation is brought about by nucleophilic attack at the acyl carbonyl group by a water molecule. The nucleophilic attack is assisted by two of the three

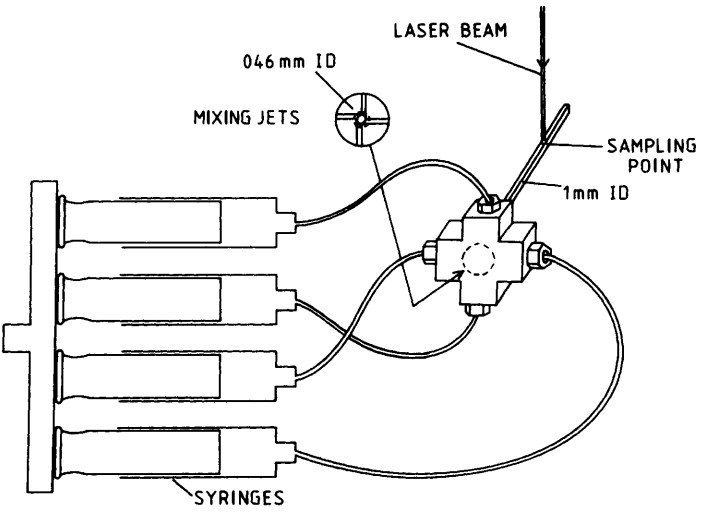


Figure 16 *Flow system for acquiring resonance Raman data of unstable acyl-enzyme intermediates at alkaline* pH

members of the so-called charge relay system, namely Asp-32 and His-57.[31] Near neutral pH the side chains of these two residues are in the correct ionization state to assist in general base catalysis, and deacylation occurs. However, at acid pH, for example at pH 3, the pair of side chains acquires a proton, and deacylation is shut down. This means it is possible to prepare stable acyl-chymotrypsins at pH 3 and to study the effect of activating the deacylation mechanism by jumping the pH to above neutral values. This is achieved in a rapid-mixing rapid-flow system[32] (Figure 16) where buffer is mixed with the stable acyl-enzyme and the RR spectrum is recorded of the unstable intermediate before significant deacylation has occurred.[12]

The above methodology will be illustrated using 5-methylthienylacryloyl-chymotrypsin. RR spectra of this acyl-enzyme in the carbonyl region are shown in Figure 17.

At pH 3.0 $\nu_{C=O}$ is characterized by at least two carbonyl bands; one at 1727 cm^{-1}, ascribed to a carbonyl population in a nonbonding environment, and one at 1697 cm^{-1} assigned to a hydrogen-bonded population of carbonyl groups.[7] As can be seen in Figure 17 the effect of fully activating the deacylation mechanism by going to pH 10 is to cause an apparent shift in $\nu_{C=O}$ centred at 1697 cm^{-1} to lower frequency. Specifically for 5-methylthienylacryloyl-chymotrypsin $\nu_{C=O}$ moves from 1697 to 1685 cm^{-1}. The pK for this change can be measured from the RR data and it is found that the pK is identical to that derived from the deacylation kinetics. This shows that the RR C=O features are indeed reflecting

[31] J. Kraut, *Annu. Rev. Biochem.*, 1977, **46**, 331.
[32] L. R. Sans Cartier, A. C. Storer, and P. R. Carey, *J. Raman Spectrosc.*, 1988, **19**, 117.

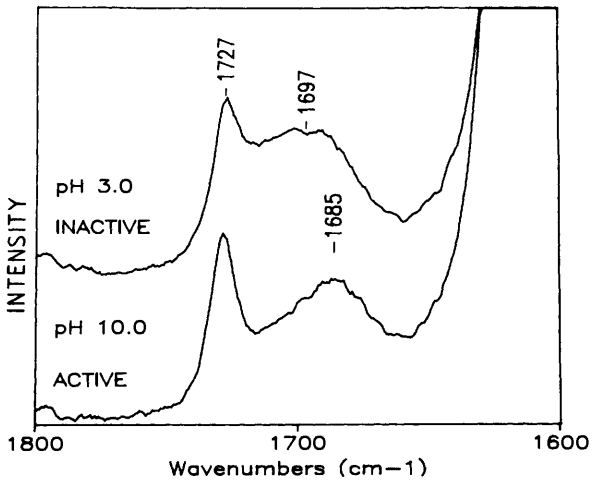


Figure 17 *Resonance Raman spectra of 5-methylthienylacryloyl-chymotrypsin at* pH 3 0 *and* 10 0 *in the* 1600—1800 cm ^{1} *region* 337 5-nm *excitation* 100 mW *exposure time* 10 × 10 s, 7 cm ^{1} *experimental resolution*

the ionization properties of His-57 and that the effect of titrating the charge relay system is being characterized [12]

The second notable finding is that for a series of acyl-chymotrypsins and the position of the hydrogen-bonded $\nu_{C=O}$ observed at high pH correlates with reactivity The lower the carbonyl frequency, the faster deacylation occurs as is seen in Figure 18

The relative decrease in $\nu_{C=O}$ observed for a more reactive acyl-enzyme is ascribed to polarization of the carbonyl group, that is canonical forms such as II (Figure 19) make an increased contribution to the structure [33]

Increasing the contribution of II increases the single bond character of the carbonyl bond and shifts the stretching mode to lower frequency In all likelihood carbonyl polarization is brought about by hydrogen-bonding to the two protein hydrogen-bond donors which are designed to stabilize charge build-up in the transition state, *viz* the 'oxyanion hole' (see above) One salient feature of the RR data is that it provides evidence for polarization in the ground state The distortion of the carbonyl electrons takes the carbonyl away from an unperturbed acyl-enzyme structure towards the next intermediate on the reaction pathway, namely the tetrahedral intermediate There has been controversy for some time regarding the relative contributions of ground state distortion and transition state stabilization, both of which can decrease the activation energy, to rate acceleration Now the RR experiments can approach this question by probing ground state destabilization of the carbonyl moiety

[33] P J Tonge and P R Carey to be published

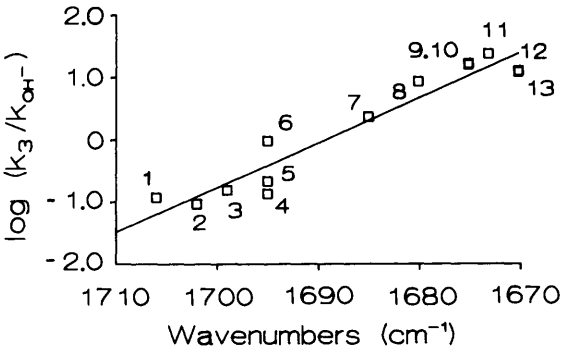


Figure 18 *Plot of* log ($k_3/k_{(OH^-)}$) *against* $\nu_{C=O}$ *for a series of acyl-serine proteases.* k_3 *is the limiting deacylation rate constant,* $k_{(OH^-)}$ *is the base-catalysed hydrolysis rate of the corresponding acyl-imidazole at* pH 10.5 *and* $\nu_{C=O}$ *is the position of the low frequency* C=O *feature observed in the resonance Raman spectrum at the* pH *of the maximum deacylation rate. 5-Methylthienylacryloyl-subtilisin:* (1) *Asn155G1n,* (2) *Asn155Arg,* (8) *BPN′ wild-type,* (11) *Carlesberg wild-type;* (3) *furylacryloyl-chymotrypsin;* (4) *4-methoxycinnamoyl-chymotrypsin;* (5) *thienylacryloyl-chymotrypsin;* (6) *indoleacryloyl-chymotrypsin* (7) *5-methylthienylacryloyl-chymotrypsin;* (9) *4-methoxycinnamoyl-subtilisin Carlsberg;* (10) *furylacryloyl-subtilisin Carlsberg:* (12) *indoleacryloyl-subtilisin Carlsberg;* (13) *5-ethylthienylacryloyl-subtilisin Carlsberg*

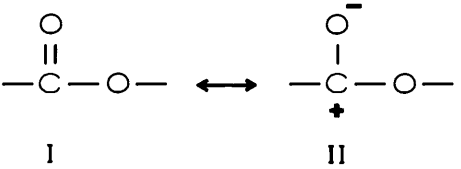


Figure 19 *Canonical structures of the* –C(=O)–O– *group*

5 Emerging Trends

A. Protein Engineering.—RR spectroscopy can contribute to defining the properties of enzymes which have had individual amino acid residues changed by site-selected mutagenesis. One example involves the serine protease subtilisin which has been the subject of pioneering protein engineering studies by a number of groups.[34] The oxyanion hole in subtilisin consists of hydrogen-bonding donors from the backbone –NH of Ser-221 and the $-NH_2$ group from the side chain of Asn-155. Replacing Asn-155 by any of a number of other residues results in a large reduction (10^2—10^3-fold) in the deacylation rate.[35] For example, substituting Asn-155 with Leu reduces the deacylation rate of the 5-methylthienylacryloyl-acyl-enzyme by *ca.* 190-fold. The effect of this substitution on the behaviour of the acyl carbonyl can be examined in the RR spectrum.

[34] J. A. Wells and D. A. Estell, *Trends in Biochemical Sciences*, 1988, **13**, 291.

[35] (*a*) J. A. Wells, B. C. Cunningham, T. P. Graycar, and D. A. Estell, *Phil. Trans. R. Soc. Lond. A*, 1986, **317**, 415; (*b*) P. Bryan, M. W. Pantoliano, S. G. Quill, H. Y Hsiao, and T. Poulos, *Proc. Natl. Acad Sci. USA*, 1986, **83**, 3743.

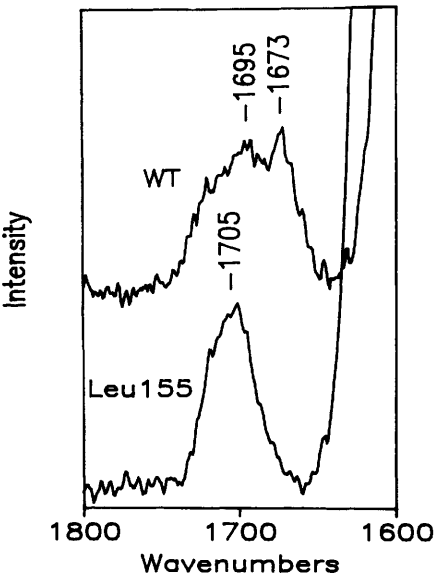


Figure 20 *The* 324-nm *excited resonance Raman spectra of 5-methylthienylacryloyl-subtilisin BPN′ wild-type (WT) and Asn155 Leu (Leu155) in the* 1600—1800 cm^{-1} *region*

Figure 20 compares the RR spectra of 5-methylthienylacryloyl-subtilisin at active pH for both the wild-type and the re-engineered (Asn155Leu) enzymes. Whilst the carbonyl region for the wild-type intermediate indicates the presence of more than one acyl carbonyl population in the active site a very significant observation is the appearance of a carbonyl band at 1673 cm^{-1}. Notably, however, this band is absent in the RR spectrum of the mutant acyl-enzyme. Thus, the RR results demonstrate vividly that the Asn to Leu change in the oxyanion hole ligand changes the nature of the hydrogen-bonding about the carbonyl oxygen such that the ability to form strong hydrogen bonds is removed or is greatly attenuated. The resulting inability to perturb the ground-state structure and, we infer, to stabilize the build-up of negative charge in the transition state explains the greatly reduced catalytic effectiveness of the 'mutated' enzyme.

B. Protein Dynamics and Cryoenzymology.—During the past decade it has been increasingly emphasized that the static 'ball and stick' depiction of proteins is inadequate.[36] Many proteins, such as enzymes, have to move in order to perform their function and a complete understanding (and manipulation) of function requires a description of protein movement or dynamics. In particular, the role and contribution of protein dynamics in catalytic efficacy is of high interest.[2]

One way to approach this problem is to recognize that many important protein motions are driven by heat, and that by cooling an enzyme–substrate

[36] H. Frauenfelder, F. Parak, and R. D. Young, *Annu. Rev. Biophys. Biophys. Chem.*, 1988, **17**, 451.

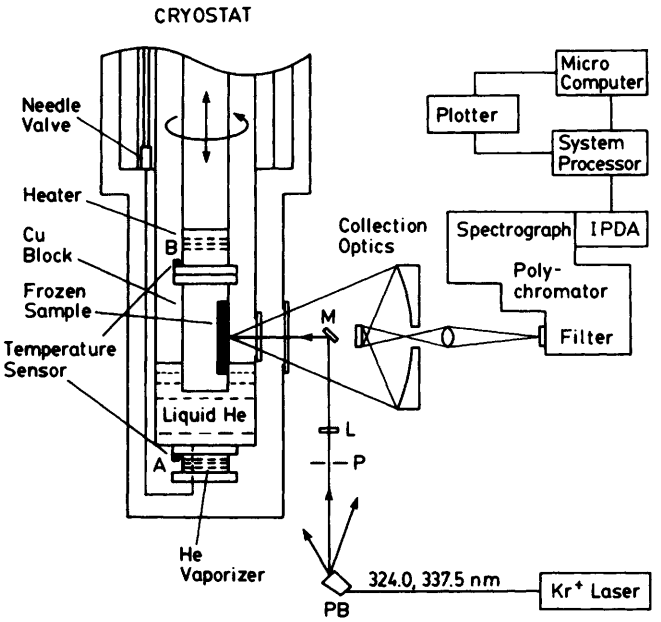


Figure 21 *Cryostat-based data collection apparatus for obtaining resonance Raman spectra of enzyme–substrate intermediates in ice matrices near* 4 K

complex it should be possible to freeze out successive orders of motion. Then, by probing the bound substrate *via* its RR spectrum as a function of temperature, it should be possible to characterize the effect of the protein dynamics on the properties of the acyl group in the active site.

Recently this strategy has been used to study the dynamics of key groups in the active sites of some dithioacyl papains.[37] The experiments involve rapidly freezing reaction mixtures containing the chromophoric acyl-papain. At low temperatures this results in the formation of a permanent population of intermediate; however the change is reversible and the reaction proceeds normally upon warming to above 273 K. The RR spectrum of the frozen reaction mixture is examined in a cryostat as shown in Figure 21.

The RR spectrum is collected by scattering the laser beam at 180° off the surface of the ice containing the acyl-enzyme. With this apparatus it is possible to obtain high quality RR data down to 4 K.[38] The spectroscopic results are startling.[39] For example, for the intermediate *N*-methyloxycarbonyl-glycine-

[37] P. J. Tonge, H. Lee, L. R. Sans Cartier, B. P. Ruzsicska, and P. R. Carey, *J. Am. Chem. Soc.*, 1989, **111**, 1496.

[38] L. R. Sans Cartier, P. J. Tonge, and P. R. Carey, *Indian J. Phys.*, 1989, **63**, 5170.

[39] M. Kim, P. J. Tonge, and P. R. Carey, 'Spectroscopy of Biological Molecules – State of the Art', ed. A. Bertoluzza, C. Fagnano, and P. Monti, Società Editrice Esculapio, Bologna, Italy, 1989, p. 77 (Proceedings of the Third European Conference on the Spectroscopy of Biological Molecules, Bologna, Italy, 1989).

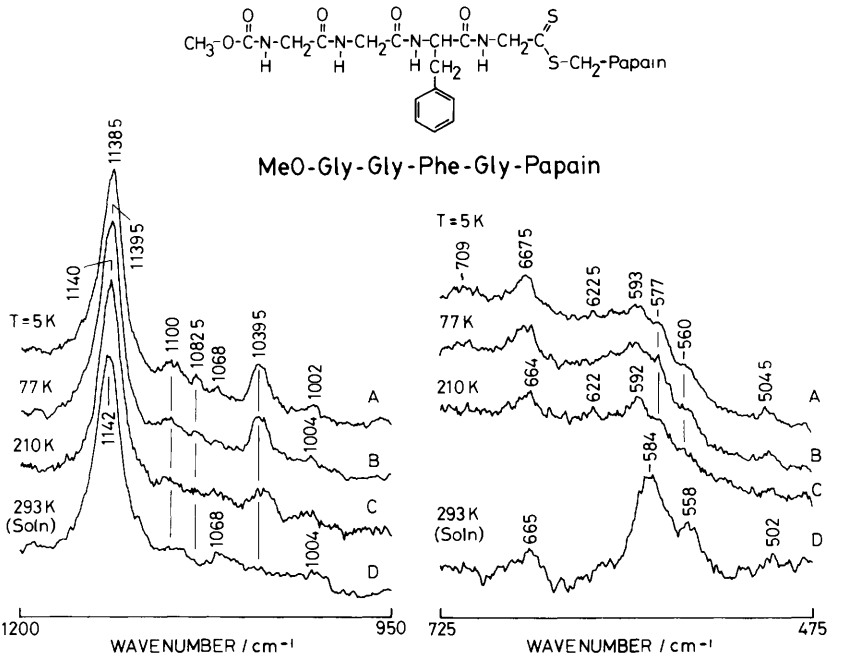


Figure 22 *The* 324-nm *excited resonance Raman spectra of* N-*(methyloxycarbonyl)-Gly-Gly-*L*-Phe-Gly dithioacyl papain from room temperature to* ca. 4 K

glycine-L-phenylalanyl-glycine dithioacyl papain there are many changes in the RR spectrum upon going to very low temperatures; principally, these involve the appearance of a 'new' band near 1035 cm^{-1}, a 3-fold increase in the intensity of the feature near 665 cm^{-1}, complex changes in the band shape of the intense feature near 1140 cm^{-1}, and numerous shifts in peak maxima.[40] These changes can be seen in Figure 22.

The key to interpreting the RR data is that many of the changes involve variations in the torsional angles φ', ψ', χ_1, and χ_2 (Figure 9).

For example, features in the 650—720 cm^{-1} region of the spectrum are from ν_{S-C} and are dispersed over this range by rotational isomerism about χ_1 and χ_2. Changes in the 1140 cm^{-1} band profile are due principally to variations in φ' and ψ'. The low temperature data demonstrate convincingly that fluctuations about the torsional angles are important at room temperature and provide an estimate of the magnitude of the angle changes and the energies involved. These fluctuations are potentially important because some may be driven by the protein matrix and may be used to explore conformational space along the reaction coordinate. Essentially this is saying that the fluctuations induced or allowed in

[40] M. Kim and P. R. Carey, to be published.

the active site are in the direction necessary to promote the progress of the acyl-enzyme along the reaction pathway to the next catalytic intermediate.

C. Fourier Transform Infrared Spectroscopy (FTIR).—FTIR can provide vibrational data for proteins at a high signal-to-noise level. The spectra can be used to elicit information on secondary structure[41] and to address problems such as the bonding of CO or O_2 to haem proteins.[42] One limitation of FTIR is that there is no equivalent to the RR effect making it difficult to obtain selective vibrational data on, for example, an active site. However, there have been attempts to probe substrate carbonyl features in enzyme-substrate complexes using FTIR since the spectral region near 1700 cm^{-1} is relatively unobscured by protein modes.[43]

For 5-methylthienylacryloyl-chymotrypsin at pH 3.0—6.0 it is possible to compare directly data obtained for the carbonyl stretching region by FTIR and RR. These two data sets are shown in Figure 23 and appear to be remarkably dissimilar.[44]

The RR spectrum is obtained directly without significant data manipulation. However, in order to remove amide I contributions the FTIR spectrum of chymotrypsin has been subtracted from the spectrum of the acyl-enzyme. The hazardous nature of this latter operation is demonstrated by the FTIR difference spectrum of PMS(F)-chymotrypsin minus chymotrypsin (Figure 23). PMSF is a covalent active site binding inhibitor, phenylmethylsulphonyl fluoride, which contains no C=O groups. Yet 'C=O' features appear in the FTIR spectrum. The apparent C=O peaks are probably due to the fact that binding PMSF perturbs the protein secondary structure and therefore PMS(F)-chymotrypsin minus chymotrypsin FTIR spectra contain 'new' peptide bands. This effect probably accounts for *ca.* 50% of the 'C=O intensity' in the FTIR spectrum of the acyl-enzyme in Figure 23. Thus, the FTIR data cannot easily be used to monitor the carbonyl of the bound acyl group.[44] However, it is possible to obtain an artefact-free FTIR spectrum by subtracting acyl-enzyme labelled with ^{13}C in the acyl moieties carbonyl group from unlabelled acyl-enzyme. This procedure gives a profile strongly resembling that centred near 1695 cm^{-1} in the RR spectrum.[44]

One additional observation stemming from the FTIR studies is that there is no feature in the FTIR spectrum of the acyl enzyme which corresponds to the RR band at 1727 cm^{-1}. In all likelihood the 1727 cm^{-1} RR feature originates from a population of carbonyl groups which have been 'photo-induced' by the laser beam. This band is present in the RR spectra of only some intermediates but does offer the possibility of performing some interesting experiments in protein dynamics. These could take the form of monitoring the decay of the 1727 cm^{-1} species in the FTIR after creating the population with a laser pulse.

[41] W. K. Surewicz and H. H. Mantsch, *Biochim. Biophys. Acta*, 1988, **952**, 115.

[42] (*a*) S. Yoshikawa and W. S. Caughey, *J. Biol. Chem.*, 1982, **257**, 412; (*b*) W. T. Potter, M. P. Tucker, R. A. Houtchens, and W. S. Caughey, *Biochemistry*, 1987, **26**, 4699.

[43] (*a*) P. J. Tonge and C. W. Wharton, *Biochem. Soc. Trans.*, 1985, **13**, 929; (*b*) C. W. Wharton, S. Ward, and A. J. White, 'Spectroscopy of Biological Molecules – State of the Art', ed. A. Bertoluzza, C. Fagnano, and P. Monti, Società Editrice Esculapio, Bologna, Italy, 1989, p. 49 (Proceedings of the Third European Conference on the Spectroscopy of Biological Molecules, Bologna, Italy, 1989).

[44] P. J. Tonge, A. J. White, C. W. Wharton, and P. R. Carey, to be published.

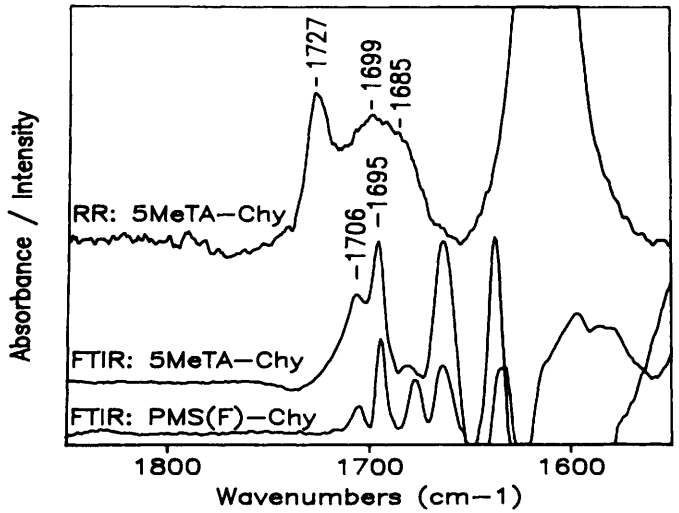


Figure 23 *Resonance Raman and Fourier transform infrared (FTIR) spectra of 5-methylthienylacryloyl-chymotrypsin (5MeTA-Chy) and phenylmethylsulphonyl-chymotrypsin (PMS(F)-Chy). Top:* 324-nm *excited RR spectrum of 5MeTA-Chy in* ^2H_2O *at* pM 6.0. *Centre: FTIR spectrum of 5MeTA-Chy (spectrum of acyl-enzyme minus spectrum of free enzyme) in* ^2H_2O *at* pM 6.0. *Bottom: FTIR spectrum of PMS(F)-Chy (spectrum of enzyme–inhibitor complex minus spectrum of free enzyme) in* ^2H_2O *at* pM 6.0

D. Other Enzymes.—The majority of RR studies of enzyme–substrate complexes have involved chymotrypsin or papain. However, RR analysis involving the dithio chromophore has been extended to include *S*-acetyl dithio coenzyme A binding to citrate synthase[45] and has provided information on conformational selection about the dithio linkages in the bound ligand.

Another study involved the search for a putative anhydride intermediate in the active site of carboxypeptidase A. RR data was collected for *O*-(*trans-p*-dimethylaminocinnamoyl)-L-β-phenyllactate reacting with the enzyme in a cryosolvent in the range 243 K to 258 K.[46] The putative intermediate would be a transitory mixed anhydride involving the carbonyl of the substrate and the γ-carboxylate of Glu-270. Although good quality spectra were obtained for the reaction mixture in the absence and presence of a powerful inhibitor, no evidence was found for an anhydride complex. While this was disappointing from the point of view of confirming the existence of a novel enzyme intermediate it clearly showed the utility of the RR spectrum in assisting the characterization.

These examples point the way to a number of future studies. Some applications, for example in cryoenzymology, require considerable experimental skill and effort but this is easily offset by the unique nature of the information which is obtained.

[45] V. E. Anderson, P. R. Carey, and P. J. Tonge, to be published.
[46] S. J. Hoffman, S. S.-T. Chu, H. Lee, E. T. Kaiser, and P. R. Carey, *J. Am. Chem. Soc.*, 1983, **105**, 6971.