Biochem. J. (1986) 233, 25–36 (Printed in Great Britain)

REVIEW ARTICLE

Infra-red and Raman spectroscopic studies of enzyme structure and function

Christopher W. WHARTON
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham, B15 2TT, U.K.

Introduction

Any physical technique must be judged in terms of its utility on the basis of several criteria. The nature, quality and amenability to precise interpretation of the information which it provides must be considered in addition to considerations of experimental complexity and cost of the equipment required. X-ray diffraction and n.m.r. may be regarded as techniques which have reached an advanced state of maturity in terms of applicability to the study of biological systems despite stringent technical requirements and high cost. It is thus apparent that these latter factors do not, in reality, deter the development and effective application of a particular technology provided the net result is deemed sufficiently desirable.

The vibrational spectroscopic techniques to be described in this Review which are currently being applied to the study of biological materials fall into a similar category in that the equipment used is presently rather expensive. They must be judged in terms of specificity and selectivity as well as complementarity (see, e.g., Theophanides, 1979; Carey, 1982; Tu, 1982; Parker, 1983; Carey & Storer, 1984).

Both i.r. and Raman spectroscopies give data which describe vibrational frequencies together with information concerning the absorption intensity and scattering cross section respectively. The means by which this information is obtained is greatly different in each case, as are the methods used to ensure that they yield accurately assignable data (Carey & Storer, 1984; Wharton, 1984).

I.r. and Raman spectroscopies rely upon fundamentally different physical processes, namely absorption and scattering. Most biochemists are familiar with the concepts of resonant absorption. This, in the case of i.r. spectroscopy, relies upon a change in the dipole moment of the absorbing species during a vibrational cycle. Since asymmetric species have larger dipole moments than more symmetric species, strong i.r. spectral features arise from polarized groups and antisymmetric vibrations of symmetric groups.

Raman scattering intensity depends upon the degree of modulation of the polarizability of the scattering species during a vibrational cycle. Thus symmetric vibrational modes of symmetric species and groups which contain polarizable atoms such as sulphur tend to scatter strongly. Raman scattering is inherently a very weak process, the intensity of scattering being approx. 10^{-6} that of the Rayleigh (laser frequency) scattering. Scattered light is emitted at lower (Stokes) and higher (anti-Stokes) frequency than the laser frequency and the Stokes scattering is stronger than the anti-Stokes.

The vibrational frequency of a particular mode is given by the difference between the scattered frequency and the laser frequency.

Resonant enhancement of Raman scattering may be realized if the laser frequency is coincident with that of an *electronic* transition of the target molecule or group. This effect can give up to a 10^5-fold enhancement of scattering and has been extensively exploited in studies of biological materials which often can only be obtained in solution at low concentration.

It is important to be aware that the vibrational frequencies that are obtained relate to atomic groupings within molecules, not simply in most instances, to pure diatomic modes. Thus while heavy atom (O, N, S) hydrogen stretch frequencies approximate closely to the frequency expected for a diatomic oscillator (i.e. are largely independent of the remaining portions of the molecule), most atomic groupings give rise to frequencies which arise from more complex vibrational modes. Thus the carbonyl group, which has a more equal mass distribution between the carbon and oxygen atoms, gives a vibrational frequency which is affected by the heavy atom framework that is bonded to the carbonyl carbon atom.

Provided a vibrational feature can be identified with a vibrational mode (often by means of isotope subsitution), the information that can be deduced from observation of the frequency is of fundamental importance. Thus it is possible to calculate bond strengths and some aspects of bonding geometry. Since the energy of the vibrational photons is relatively low, easily measurable frequency shifts are expected to result from relatively weak interactions with the group of atoms responsible for the vibrational mode. Such interactions are of particular interest in the study of the mechanism of enzyme-catalysed reactions, since in many instances the formal covalent bond exchange processes have now been established (Wharton & Eisenthal, 1981; Fersht, 1984).

Hydrogen bonding, which in view of the prevalance of general catalysis plays a crucial role in enzymic catalysis, is an example of such a process that will produce significant spectral shifts. If useful deductions are to be made regarding the intimate details of enzymic catalysis it is essential that any effects which may result from hydrogen bonding may be clearly distinguished from other factors such as geometric distortion and medium (i.e. including dielectric constant) effects.

In principle much additional information may be deduced from the measurement of the intensities and line-widths of specifically assigned spectral bands. For instance, line widths in vibrational spectroscopy are related to collisional and flexural mobility, but have yet

Abbreviations used: F.t., Fourier transform; r.R., resonance Raman.

to be utilized in any but the most qualitative sense in the interpretation of spectra of proteins and enzymes. Overall protein mobility can be assessed by using measurements of ^1H–^2H exchange in which the change in i.r. spectrum is used to monitor the exchange, but this is a global technique in which specific sites cannot be resolved (Parker, 1983). N.m.r. by contrast can be used to follow exchange at precisely defined sites and so has a decisive advantage in this type of study.

In this Introduction I have attempted to delineate the criteria by which emergent techniques should be evaluated; it is clearly the case that it is the degree of specificity that is crucial, given that rich interpretative pickings are there for the taking if this is sufficient.

In this short Review I include a brief description of the application of i.r. and Raman spectroscopies to the study of some proteins that cannot be described as enzymes. This has been done in order to give an impression of the types of information that may be obtained from such studies which are often at the forefront of the application of these spectroscopic techniques. Some of these techniques will usefully be applied to the study of enzymes in the future.

Modern instrumentation

Infrared. Although cell materials suitable for use with aqueous samples have been available for many years, the strong background absorbance of water and ^2H_2O (see Fig. 1) has discouraged studies using these solvents. Because of this strong absorption it is necessary to work in the 'windows' of the aqueous solvent spectra, sometimes making use of a change from water to ^2H_2O in order to shift the 'window' so as to coincide with the band or bands of interest. For this reason, much of the early work on proteins was done using anhydrous films (Gordon *et al.*, 1974; Ruegg & Hanni, 1975).

A further problem in such studies has been the nature of the instrumentation available. Dispersive i.r. spectrometers traditionally scan rather slowly, have a relatively low light throughput and have only a modest noise performance.

The advent of Fourier transformation (F.t.) has revolutionized i.r. spectroscopy of biological aqueous materials and, in particular, of metastable biological systems. The scanning rate of F.t.-spectrometers is measured in seconds or less as compared with 10–20 min for a dispersive instrument. This and the high light throughput (there are no slits) combine to allow very low noise spectra to be obtained by coaddition in a period of a minute or less (Martin, 1980). The digital computerized nature of the instruments allows extensive data manipulation; without a doubt subtraction procedures (i.e. sample minus reference) are the most important of these. The vast majority of F.t-i.r. spectrometers are single beam instruments, are widely commercially available, and cost in the region of £30000–£70000 for an instrument suitable for many of the studies described in this Review. High resolution is not needed for biological studies, 2 cm^{-1} being generally sufficient.

Raman. Raman spectroscopy is, in the reviewer's opinion, an experimentally more demanding technique than i.r. spectroscopy. As is now well-known, the use of lasers has revolutionized this technique by providing extremely intense, highly monochromatic, light. An enormous advantage of Raman spectroscopy in the study of biological materials is the weak scattering of water. Indeed, rather than being a difficult solvent to work with it may often be regarded as the solvent of choice. Until recently Raman spectrometers which use scanning monochromators were the only type available, but the advent of multi-channel technology has allowed an enormous increase in the data acquisition rate. Thus developments in instrumental technology have played a crucial role in the realization of vibrational spectroscopy

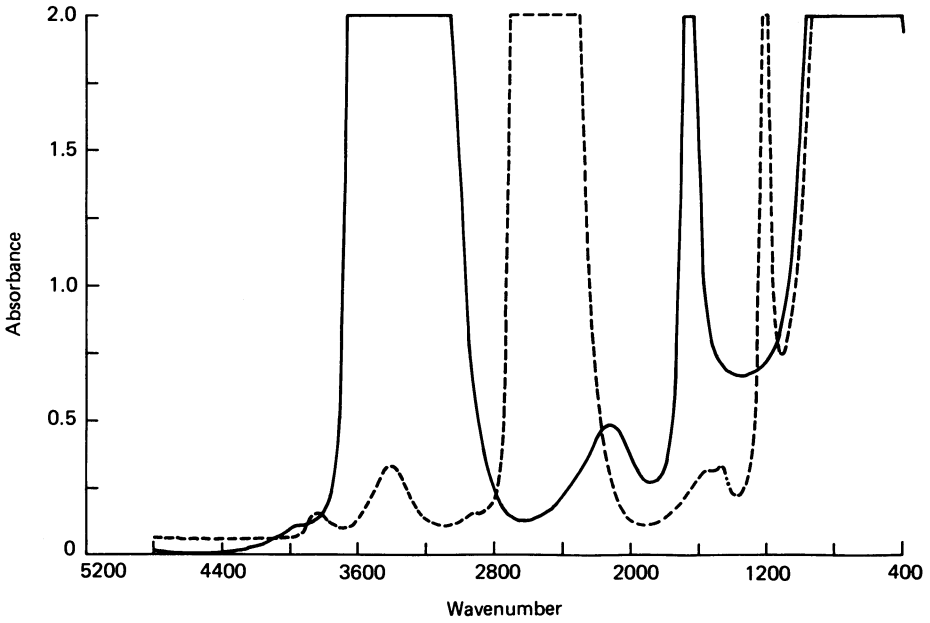


Fig. 1. I.r. spectra of ^1H_2O (——) and ^2H_2O (– – –)

An 0.073 mm pathlength CaF_2 cell was used. The peak at 3400 cm^{-1} in the ^2H_2O spectrum shows some contamination with ^1H_2O.

of aqueous biological materials, as has been the case in the application of n.m.r. to such studies.

The apparatus required for Raman spectroscopy has not yet been developed to the 'push button' stage achieved in the case of F.t-i.r. instruments (but see Asher *et al.*, 1983). Also a modern apparatus will cost two or three times as much as an F.t.-i.r. instrument and will be more difficult to use. Without a doubt it is the multi-channel detection system that has done most to enhance the usefulness of Raman spectroscopy in the study of transient species. The entire spectrum is collected simultaneously using the intensified diode array and many spectra may be coadded, depending upon the time available, to achieve the desired degree of noise reduction. The availability of lasers that emit, using doubling crystals at the lower end, over the range 190 nm upwards allows the phenomenon of resonance enhancement to be exploited to 'focus' upon small ligands bound to macromolecules by using the specific electronic absorption characteristics of the ligand to give enhancement of this portion of the macromolecular complex. Pulsed lasers can be used to study very short-lived phenomena, since the extreme rapidity of the Raman scattering process essentially freezes molecular motion on the nuclear vibrational time scale.

Despite these spectacular technological advances Raman spectrometers remain rather difficult to use in terms of optical alignment and sample presentation, and it is to be hoped that further attention will be given to this aspect in the near future (see, e.g., Asher *et al.*, 1983).

The cost and complexity of the modern Raman spectrometer is such that relatively few research groups will either be able or indeed desire to have such an instrument on their home site. The author's group make use of an instrument at the Central Laser Facility, Rutherford–Appleton Laboratory of the Science and Engineering Research Council.

Studies of protein and enzyme structure by using vibrational spectroscopy

Polypeptide chain structure. The amide groups of proteins can be shown, by normal mode analysis, to have three in-plane vibrational modes (see, e.g., Miyazawa *et al.*, 1958; Miyazawa, 1960). These are by far the most important in the study of the structure of proteins and are known as amide I, II and III respectively. Amide I, which is primarily characterized by carbonyl stretch, occurs in both i.r. and Raman spectra, being particularly intense in the i.r. It has a frequency in the range of 1630–1670 cm^{-1} dependent upon the secondary structure. Model polyamino acids have been used to assign amide I frequencies to particular structures. Thus α-helix is characterized by a range of 1645–1660 cm^{-1}, β-sheet 1665–1680 cm^{-1} and disordered structure or random coil 1660–1670 cm^{-1}.

Amide II is i.r.-active but Raman-inactive and occurs near to 1550 cm^{-1}, having an intensity some one-tenth of that of amide I. It is a mixed bending and stretching mode which has a significant component of N–H bend. When the amide proton is exchanged for ^2H a large frequency shift to approx. 1475 cm^{-1} occurs. This shift in amide II has frequently been used in the form of a loss of absorbance at 1550 cm^{-1} to monitor $^1H \rightarrow ^2H$ exchange in proteins and hence as a measure of global protein mobility; it has little utility in backbone structural analysis (Pershina & Hvidt, 1974; Sørup *et al.*, 1977; Parker, 1983).

Amide III is i.r.-inactive, Raman-active and occurs at 1230–1300 cm^{-1}. It has been used in conjunction with amide I for estimating the relative contributions of α-helix, β-sheet and random coil in a number of proteins (Chen & Lord, 1976; Lippert *et al.*, 1976; Vincent *et al.*, 1984). In some instances the results have been shown to coincide with those from X-ray crystallography (Lippert *et al.*, 1976; Eckert *et al.*, 1977). Spectral differentiation between the various structural elements of a protein is not easy, since small band-shifts have to be interpreted; amino acid homopolymers given cleaner spectra since the internal structure diversity is much reduced.

It has been proposed that the dihedral conformation of disulphide bonds in proteins may be determined from their Raman scattering frequencies (Van Wart *et al.*, 1973; Van Wart & Scheraga, 1976; Van Wart *et al.*, 1976). However, a number of conflicting claims have been made with respect to the details of the analysis and the results should be accepted with caution. Disulphide bond formation can readily be monitored by the observation of Raman scattering at 510 cm^{-1}. Groups containing sulphur generally scatter strongly in Raman spectra as a result of the high polarizability of sulphur; conversely i.r. absorption is weak.

I.r. spectra of proteins in solution are intrinsically restricted by H_2O and ^2H_2O absorption and so are rather limited in range. Such spectra, even in the range of observation that is possible (e.g. in ^2H_2O from 1900 to 1200 cm^{-1}), are relatively featureless compared with Raman spectra. In addition to amides I and II there are some weak aromatic features. Solid state spectra show more structure and are not range-limited by the solvent, but they have doubtful relevance in view of the ability of the Raman technique to give highly detailed solution spectra over a very wide range of frequencies, e.g. 3500 cm^{-1} to 10 cm^{-1}. Most of the bands in these spectra have been assigned to side chain structures. Rather small changes occur on denaturation, primarily in the Phe and Tyr regions (Lord & Yu, 1970; Chen & Lord, 1976; Porubcan *et al.*, 1978).

In conclusion, the reviewer considers that vibrational spectroscopy has yet to be proved to be more than peripherally useful in the determination of protein or enzyme secondary structure.

Proteins with prosthetic groups. Although proteins do not absorb in the visible region, three general categories may be distinguished that carry prosthetic groups which confer colour on the complete molecule. These are the haem proteins, the visual pigments and proteins which carry bound metal ions, in particular Fe^{3+} and Cu^{2+}.

The property of visible light absorption renders these proteins susceptible to study by resonance Raman spectroscopy. By using laser light which electronically excites either the haem group or the liganded metal atom much information has been obtained regarding the crystal field structure of the chelated metal atoms. The effect of ligand binding (e.g. O_2, CO and CN^-) to such haem groups has provided insight into the geometric reorganization that occurs upon ligand interaction and the time dependence of these processes (Spiro & Burke, 1976; Felton & Yu, 1978; Kitagawa *et al.*, 1979; Desbois *et al.*, 1979; Kincaid *et al.*, 1979; Remba *et al.*, 1979; Friedman *et al.*, 1982; Argade *et al.*, 1984*b*; Bangcharo-

enpaurpong *et al.*, 1984; Desbois *et al.*, 1984; El Naggar *et al.*, 1984; Schweitzer-Stenner *et al.*, 1984; Johnson *et al.*, 1985).

Recently some delightful studies have been reported concerning the mode of action of the bacterial purple membrane protein bacteriorhodopsin (Oseroff & Callender, 1974; Marcus & Lewis, 1977; Ehrenberg & Lewis, 1978; Campion *et al.*, 1977*a*,*b*; Terner *et al.*, 1979; Smith *et al.*, 1984) and rhodopsin (see below). Bacteriorhodpsin, which is a transmembrane protein, pumps protons consequent upon light absorption in order to produce a gradient for ATP synthesis and possesses a retinal prosthetic group which is attached to the protein by a Schiff's base linkage with a lysine residue. A wide range of very advanced techniques such as rapid-flow, pump and probe and dual beam spectroscopy have been employed in studies of *cis–trans* isomerization of the retinal group and Schiff base deprotonation which are the two earliest light-induced processes. Isotope substitution, for example replacement of ^1H_2O by ^2H_2O, has been used to study the protonation states of the Schiff base intermediates in the photochemical cycle. Bacteriorhodopsin has considerable resemblance to rhodopsin, the mammalian visual protein, in that both carry retinal which is linked to protein via a Schiff's base. The latter protein has been intensively studied, in particular the *cis–trans* isomerization at position 11 of the polyene chain which occurs on photoexcitation. This process, the state protonation of the C=N linkage, and the cleavage of the Schiff's base by which the all-*trans* retinal is released from opsin have been partially characterized and are topics to which state-of-the-art Raman technology is being applied (Lewis, 1976, 1978; Doukas *et al.*, 1978; Cookingham & Lewis, 1978; Eyring & Mathies, 1979; Honig *et al.*, 1979; Aton *et al.*, 1980; Birge, 1981; Rudzki & Peters, 1984).

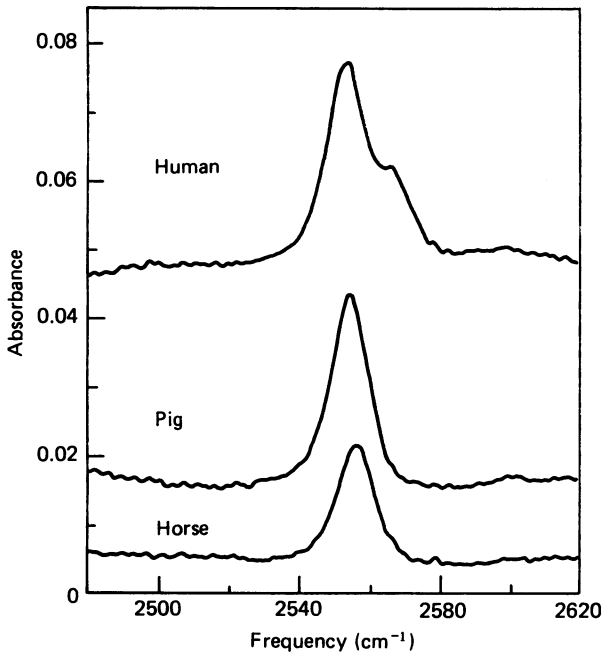


Fig. 2. I.r. difference spectra of human (17 mM-haem), pig (16 mM) and horse (11 mM) carboxyhaemoglobins each with bovine carboxyhaemoglobin subtracted as reference

Before this water was subtracted from each of the samples, the amount to be subtracted being determined from the near i.r. absorption at 1.92 μm. Four sets of 256 interferograms were collected using a CaF_2 cell with a pathlength of 0.2 mm. Redrawn from Bare *et al.* (1975).

Some examples of the application of F.t-i.r. in the study of enzyme structure and function

Thiol groups. It is perhaps surprising that the thiol group has attracted attention from F.t.-i.r. spectroscopists. As a result of the high polarizability of sulphur the i.r. absorption is weak, being approx. 1% of that of a typical carbonyl group. The thiol stretch frequency of approx. 2550 cm^{-1} lies in a 'window' in the water spectrum (see Fig. 1) so subtraction procedures can be used with long path-length cells (e.g. 0.2 mm).

Haemoglobin, as has so often been done before, will be treated for the purposes of this Review as an 'honorary' enzyme. Haemoglobin is a very soluble protein and F.t.-i.r. spectra have been taken at haem concentrations of 10–17 mM. Haemoglobins from several species have been studied with a view to characterizing the thiol groups of the proteins (Alben *et al.*, 1974; Bare *et al.*, 1975; Alben & Bare, 1980). Bovine haemoglobin has two cysteine residues per tetramer at β-93. The i.r. spectrum shows no observable peak in the region of 2550 cm^{-1}, so the thiol group must absorb weakly. Human haemoglobin has six cysteine residues per tetramer at β-93, β-112 and α-104, and shows a difference spectrum when bovine haemoglobin is subtracted (see Fig. 2) that has two components. Horse and pig haemoglobin have β-93 and β-104 and show a single peak characteristic of the stronger component in human haemoglobin. By means of further subtraction or deconvolution the α-104 and β-112 peaks were resolved. The α-104 absorption has the lower frequency and higher intensity, which on the basis of model studies indicates strong hydrogen bonding; β-112 is moderately hydrogen bonded while β-93 is exposed to solvent. The frequencies move to lower values in the presence of ^2H_2O, the shift being almost exactly that found for methanethiol. These thiol spectra which have been ingeniously resolved by the use of natural structural variation have been interpreted using the crystallographic structure of haemoglobin in terms of intramolecular interactions at the α_1–β_1 interface. It is proposed that strong intrachain hydrogen bonding occurs between the α-104 thiol group and the carbonyl oxygen of leucine α-100. This interaction replaces the normal amide–*N*-carbonyl interaction seen in the horse α-subunit G-helix. β-112 is involved in a somewhat similar, but weaker, interaction within the human β-G helix.

Perturbation of the thiol spectra has been used to monitor ligand binding to human haemoglobin (Alben & Bare, 1980). The frequency of α-104 thiol stretch has been shown to increase in the order of $HbCO < HbN_3 < HbCN \sim HbO_2 < Hb$, where $\Delta\nu_{SH}$ $(Hb - HbCO) = 4.0\ cm^{-1}$. The spectral perturbations seen on ligand binding have been interpreted in terms of the R and T structural states of haemoglobin and have been related to interactions in and between the chains of the tetramer.

Myosin. The thol groups of heavy meromyosin have been shown to absorb with a centre frequency of approx. 2565 cm^{-1} (Nakanishi *et al.*, 1981). This material has an M_r of 240000 and 26 cysteine residues/molecule. Ingeniously lysozyme, which has no free thiol groups, was used for subtraction. Addition of ATP and ADP increased the intensity of the absorption; this was

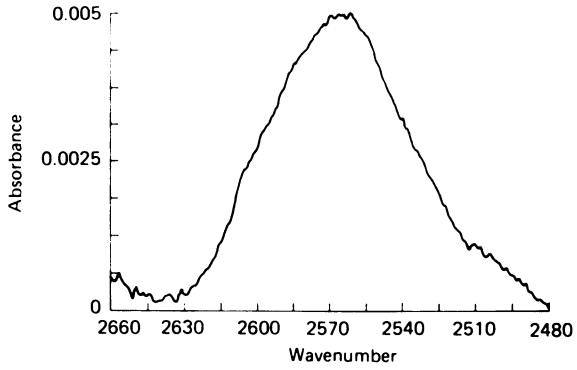


Fig. 3. I.r. difference spectrum of chymopapain in water

The enzyme was concentrated at 260 mg·ml^{-1} by using vacuum dialysis and 29000 scans were obtained using an 0.2 mm pathlength CaF_2 cell. The pH was 3.8 and enzyme that had been treated with 1 molar equivalent of iodoacetate, which caused complete loss of enyzmic activity, was used for subtraction purposes. Redrawn from Wharton (1984).

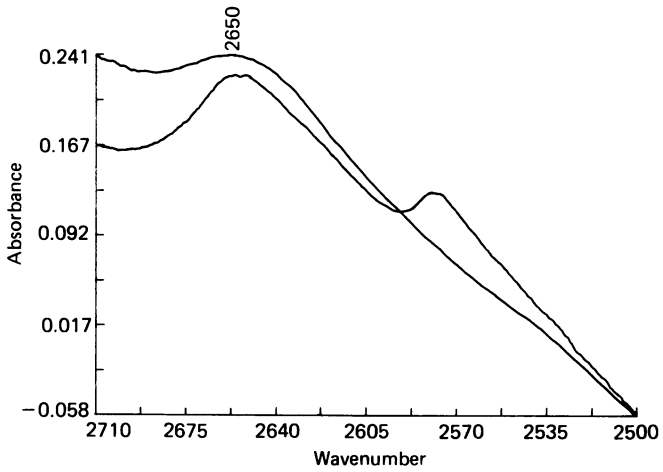


Fig. 4. I.r. difference spectra of papain (upper trace) and 2-thioethylbenzimidazole

Papain (5.4 mM, pH 3.5 in 0.1 M-KCl/1 mM-EDTA) had 0.8 reactive thiol groups/mol and was scanned 960 times in an 0.2 mm pathlength CaF_2 cell. Enzyme treated with 1 molar equivalent of iodoacetate, which caused complete loss of enzymic activity, was used as a reference for subtraction. 2-Thiomethylbenzimidazole was scanned 320 times as an 85 mM solution in water in an 0.2 mm pathlength CaF_2 cell and the solvent was subtracted (Tonge, 1985).

proposed to result from enhanced hydrogen bonding of the thiol groups.

Thiol proteinases. The S–H stretching vibration of the active centre cysteine residues of chymopapain (see Fig. 3) has been observed by using a 10 mM solution of the enzyme at pH 3.8 (Wharton, 1984; C. W. Wharton, unpublished work). When the pH was raised to 6 the intensity of the peak was much reduced, which suggests formation of a ImH^+–$S^{(-)}$ zwitterion at neutral pH (see also Polgár & Halász, 1982). Enzyme that had been inhibited with iodoacetate was used for subtraction purposes. Attempts to observe the equivalent peak in papain solution at pH 3.5 have proved unsuccessful.

The compound 2-mercaptomethylbenzimidazole may be regarded as a model of the papain active centre His-Cys pair (Stuchbury *et al.*, 1975). In Fig. 4 is shown the i.r. spectrum of this compound in water. A peak that has been assigned using the ^2H-shift to the N^+–H stretch of imidazolium ion occurs at 2647 cm^{-1}, the other peak at 2578 cm^{-1} represents the thiol stretch. Fig. 4 also shows the spectrum of papain minus *S*-carboxymethylated papain at pH 3.5. The peak at 2650 cm^{-1} has been tentatively assigned to the imidazolium ion, but the thiol stretch, as mentioned above, is not seen at this pH (Tonge, 1985). The reason for the appearance of this peak in these conditions is presently unclear. Our failure to observe the thiol stretch may indicate that the His-Cys pair is in the zwitterionic form even at this pH (Lewis *et al.*, 1976) or that the intensity of the absorption is low, as is that of β-93 of haemoglobin (Bare *et al.*, 1975). It remains a possibility that the band at 2650 cm^{-1} represents the thiol absorption but this would involve an unprecedented shift in the thiol absorption frequency. A lowered frequency relative to a simple model compound would be predicted in aqueous solution, since hydrogen-bonding both lowers the frequency and raises the intensity of such bands (Bare *et al.*, 1975).

Carbonyl groups. *Triosephosphate isomerase and aldolase.* Knowles, in a pair of excellent papers, has shown how F.t-i.r. can be used to measure vibrational frequencies of substrates bound in enzyme–substrate complexes (Belasco & Knowles, 1980, 1983).

Triosephosphate isomerase. The equilibrium of the triosephosphate isomerase-catalysed reaction, namely the interconversion of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, is heavily in favour of dihydroxyacetone phosphate. Further, dihydroxyacetone phosphate comprises > 70% of enzyme-bound species at equilibrium (Albery & Knowles, 1976). Fig. 5 shows the spectrum of enzyme-bound dihydroxyacetone phosphate, obtained by a subtraction procedure, which has two bands in the carbonyl stretching region at 1732 cm^{-1} and 1713 cm^{-1}. Binding of the potent inhibitor phosphoglycolohydroxamate eliminated these bands as did alkylation of the enzyme. Measurement of the spectrum in $^2H_2{}^{18}O$ showed only weak absorptions at 1732 and 1713 cm^{-1}, since the carbonyl oxygen of the substrate rapidly exchanges with the solvent. The new C=^{18}O peaks were not observed, since they are predicted (1697 cm^{-1} for dihydroxy[2-^{18}O]acetone phosphate in free solution) to occur below 1700 cm^{-1} in a region of intense protein (amide I) absorbance where subtraction was found to be unreliable. Thus the peaks cannot be due to enzyme perturbation since ^{18}O will not exchange into enzyme groups. ^2H-labelling of dihydroxyacetone phosphate was used to show that it is this species that is responsible for the spectral features rather than D-glyceraldehyde 3-phosphate or the *cis*-enediol intermediate (Rieder & Rose, 1959; Webb & Knowles, 1974); ^{13}C-substitution at C-1 has also been used for this purpose.

Free dihydroxyacetone phosphate absorbs at 1733 cm^{-1} but with a much broader band width (29 cm^{-1}) than the enzyme-bound forms (13 cm^{-1} and 8 cm^{-1} respectively). Such band narrowing seems to be a feature

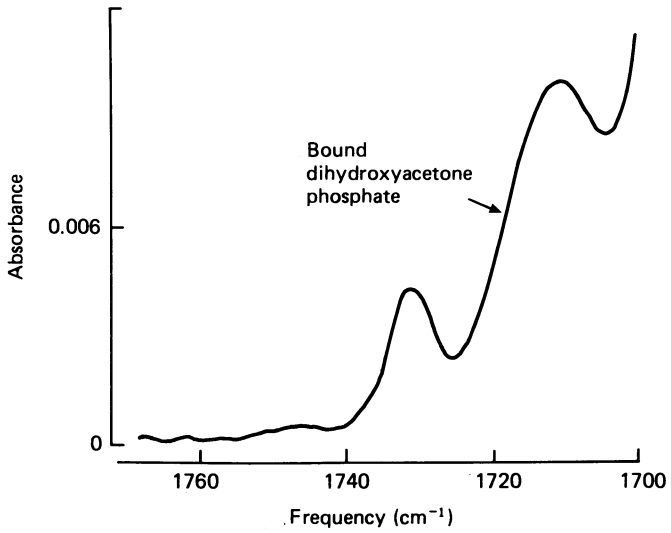


Fig. 5. I.r. spectrum of 1(*R*)-[1-^{2}H]dihydroxyacetone phosphate bound to triosephosphate isomerase

Spectral subtraction of enzyme and unbound substrate was used to give a flat baseline above 1740 cm^{-1}; 500 interferograms were averaged and an 0.108 mm CaF_2 cell was used. From Belasco & Knowles (1980).

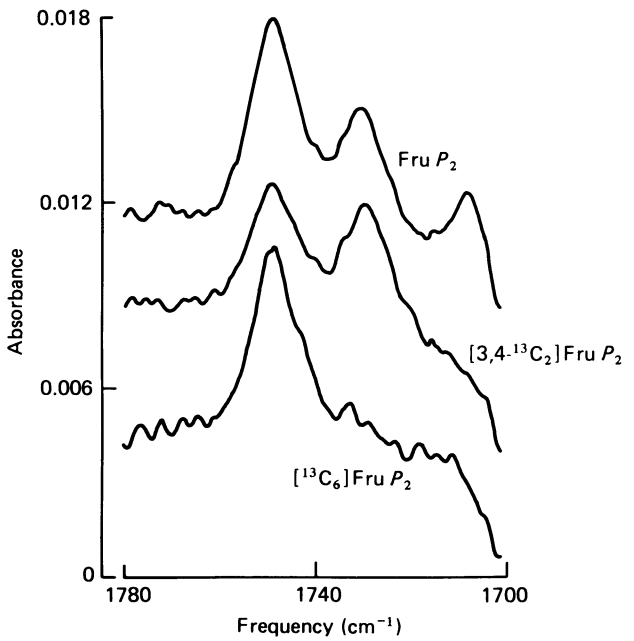


Fig. 6. I.r. spectra of fructose 1,6-bisphosphate bound to aldolase

Note that only the peak that results from enzyme perturbation remains when [$^{13}C_6$]-fructose bisphosphate is used as substrate. Aldolase was 1.93 mM and fructose bisphosphate 7.4 mM; all spectra were taken in ^2H_2O. Other conditions similar to Fig. 5 from Belasco & Knowles (1983).

of ligation to the enzyme and presumably reflects a reduced collisional mobility.

Since there are two peaks in the spectrum there must be two enzyme-bound species. The peak at 1732 cm^{-1} relates to a structure in which the carbonyl group is almost unperturbed. That at 1713 cm^{-1} is characteristic of what would be expected from an electrophilic interaction of an enzyme group with the carbonyl oxygen of the substrate; such an interaction would lengthen and weaken the carbonyl bond. The shift of 19 cm^{-1} to lower frequency is equivalent to an interaction of some 0.23 $kJ \cdot mol^{-1}$ and would represent a distortion of the substrate towards the transition state for *cis*-enediol formation (Scheme 1).

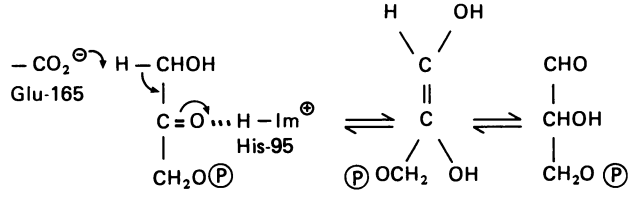
The minor carbonyl band at 1732 cm^{-1} may represent an interaction of the enzymic electrophile (here shown as imidazole of His-95) with C-1; this is an interaction that could have a role polarizing the C-1 carbonyl of glyceraldehyde 3-phosphate in the reverse reaction. Alternatively the unperturbed species may represent a non-productive mode, which since the enzyme is fully evolved (Albery & Knowles, 1976), may not have much effect on the overall reaction rate.

Aldolase. Aldolase, which catalyses the interconversion of fructose 1,6-bisphosphate and glyceraldehyde 3-phosphate plus dihydroxyacetone phosphate, has been studied in a similar way (Belasco & Knowles, 1983). Fig. 6 shows a difference spectrum which represents fructose 1,6-bisphosphate bound to aldolase. The 1730 cm^{-1} band has been assigned to the carbonyl group of bound fructose bisphosphate and/or dihydroxyacetone phosphate, and is surprisingly unperturbed relative to free substrate, while that at 1706 cm^{-1} represents bound glyceraldehyde 3-phosphate. The peak at 1748 cm^{-1} is not subject to displacement to lower frequency by ^{13}C substitution and was assigned to an *enzyme* carboxyl group perturbed in an apolar environment. ^{13}C-substitution at positions 3 and 4 was used to identify glyceraldehyde 3-phosphate as being responsible for the band at 1706 cm^{-1}, since the carbonyl bands of fructose 1,6-bisphosphate and dihydroxyacetone phosphate are almost unaffected by such a substitution.

The 24 cm^{-1} shift in the frequency of the glyceraldehyde 3-phosphate carbonyl stretch in binding to the enzyme represents a significant polarization; the enzyme electrophile presumably has a role in the enzyme mechanism. It is notable that dihydroxyacetone phosphate and/or fructose 1,6-bisphosphate are unpolarized, in contrast with the results of the experiments with triosephosphate isomerase.

The studies on triosephosphate isomerase and aldolase described above have been carefully interpreted and illustrate very well the main requirements for the success of such studies. Thus the bands to be observed must be clear of major solvent bands as well as the amide I band of the enzyme. Isotope substitution will always be required if assignment is to be made with any degree of confidence, while subtraction procedures may be used judiciously to remove background spectral features.

Chymotrypsin. Chymotrypsin-catalysed hydrolyses proceed via acyl-enzyme intermediates which are esters



Scheme 1. Reaction scheme for triosephosphate isomerase

of Ser-195 of the enzyme (see, e.g., Wharton & Eisenthal, 1981; Fersht, 1984). By using reactive acylating agents such as *trans*-cinnamoyl imidazole at approx. pH 4 it is possible to prepare stable acyl-enzymes having relatively non-specific acyl groups (Schonbaum *et al.*, 1961; Bender *et al.*, 1962; Kogan *et al.*, 1982). The F.t.-i.r. spectra of *trans*-cinnamoyl- and β-phenylpropionyl-chymotrypsins have been measured by using subtraction of the unacylated enzyme and are shown in Fig. 7 (Wharton, 1984; Tonge & Wharton, 1985). Both spectra show dual maxima in the carbonyl region, which indicates that the acyl group is capable of adopting two conformations in the enzyme active centre. The broad feature centred at 1708 cm^{-1} in the spectrum of β-phenylpropionyl-chymotrypsin is characteristic of a hydrogen-bonded or polydisperse conformation. A discrete hydrogen-bonding interaction with the enzyme would be expected not only to lower $\nu_{C=O}$ but also to give a relatively narrow band width in view of Knowle's findings with triosephosphate isomerase (Belasco & Knowles, 1980). The sharper, lower frequency band at 1693 cm^{-1} may represent a more productively bound conformer. Both bands occur at a lower frequency than that of ethyl β-phenylpropionate in acetonitrile/^2H_2O (1:9, v/v) (1711 cm^{-1}).

The two partially resolved peaks at 1700 cm^{-1} and 1707 cm^{-1} in the difference spectrum of *trans*-cinnamoyl-chymotrypsin appear to have a similar band width. The model compound ethyl cinnamate has $\nu_{C=O}$ at 1690 cm^{-1} in acetonitrile/^2H_2O (1:9, v/v) so both carbonyl bands in the acyl-enzyme spectrum are shifted to higher frequency and show no evidence of hydrogen bonding. The feature seen at 1680 cm^{-1} is not believed to be an acyl group carbonyl band since it is not apparent in a $^{12}C-{}^{13}C$ (C-1) difference spectrum. This subtraction technique in which acyl-enzyme labelled with ^{13}C at the carbonyl carbon atom is subtracted from the ^{12}C version, eliminates all features that arise from enzyme perturbation. The negative-going feature at 1737 cm^{-1} seen in Fig. 7 is eliminated, as is the feature at 1680 cm^{-1}. The former feature is the result of perturbation of one or more carboxyl groups in a hydrophobic environment which possibly also involves some proton loss.

Neither of the acyl-enzymes studied here can be regarded as being very specific; the pH-independent deacylation rate constants are 0.013 s^{-1} for *trans*-cinnamoyl- and 0.28 s^{-1} for β-phenylpropionyl-chymotrypsins (Kogan *et al.*, 1982) as compared with approx. 150 s^{-1} for the Ac-L-Phe-enzyme (Zerner *et al.*, 1964).

The dramatic features seen in Fig. 7 from 1600 to 1670 cm^{-1} have not been interpreted in any detail but are very repeatable from one difference spectrum to another; it thus seems that reliable subtraction is possible in this region using an enzyme concentration of 50 mg·ml^{-1} and 0.07 mm cells. The lineshape is characteristic of a line narrowing of amide I in the acyl-enzyme; this may be the result of a 'tightening' of the polypeptide backbone of the enzyme upon acylation giving reduced spectral dispersion (the $\nu_{C=C}$ of the cinnamoyl group is superimposed but is much less intense than $\nu_{C=O}$).

Resonance Raman spectroscopy

Early studies of enzyme mechanisms using r.R. spectroscopy involved the use of chromophoric reagents that absorb, when enzyme-bound, in the visible region of the spectrum, since argon and krypton ion lasers were available for excitation in this region (Carey, 1982; Tu, 1982).

Chymotrypsin. Carey & Schneider (1976) used a flow system and exciting light at 450 nm to study the r.R. spectrum of 4-amino-3-nitrocinnamoyl-chymotrypsin, which was prepared using the acyl-imidazole. At pH 3, where the acyl-enzyme was stable, the conformation of the acyl group was similar to that found in free solution,

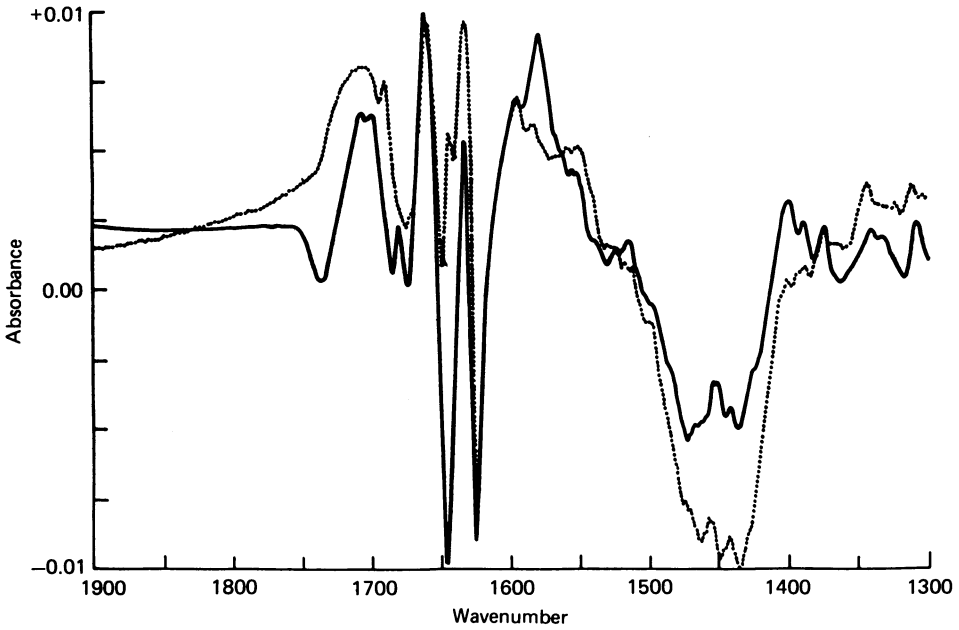


Fig. 7. I.r. difference spectra of *trans*-cinnamoyl- (——) and β-phenylpropionyl- (.....) chymotrypsins

360 scans were taken at p^{2}H values of 4.05 and 3.95 respectively. The acyl-enzymes were prepared by acylation of 1.5 mM active chymotrypsin using the appropriate acylimidazole. Equimolar imidazole was added to an aliquot of enzyme for subtraction. From Tonge & Wharton (1985).

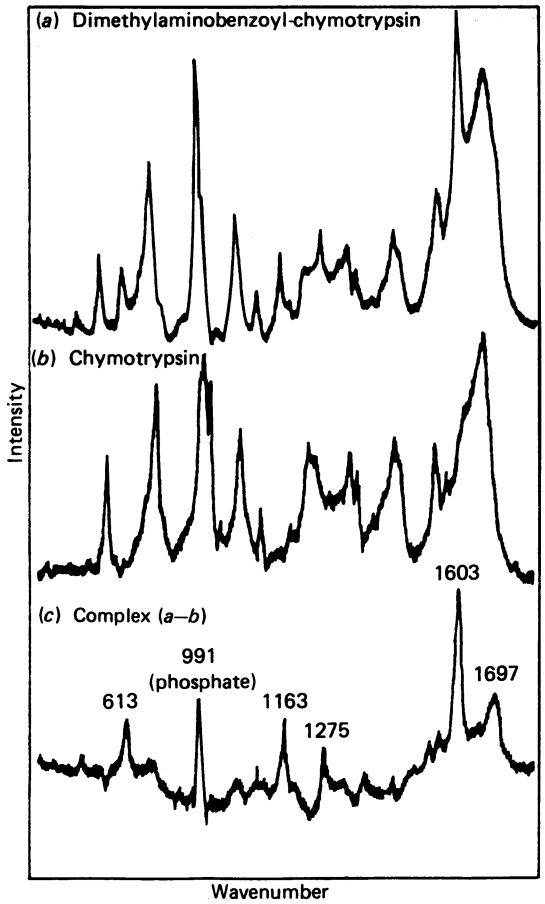


Fig. 8. Raman spectra of *p*-dimethylaminobenzoyl-chymotrypsin (*a*), chymotrypsin (*b*) and the difference between the two (*c*)

Spectra were recorded using 458 nm laser excitation, the enzyme being 2.8 mM in each case. Adapted from Argade *et al.* (1984*a*).

namely *trans* and planar. When the pH was raised to neutrality a large change occurred in the 1625 cm^{-1} band. This feature was assigned to the C=C–CO–OEnz portion of the acyl group and has a major C=C component. That the change in the 1625 cm^{-1} band titrated between pH values of 5.7 and 7 was taken to mean that an ionization in the enzyme was responsible for a change in conformation of the acyl group from an inactive to an active form. The active conformation of the acyl group was either distorted from planarity or less polarized relative to the inactive form.

MacClement *et al.* (1981) have studied the r.R. spectra of a range of β-substituted acryloyl-chymotrypsins by using laser light at 350.7 nm. Again the acyl-enzymes were studied at low pH and, by flow mixing, at neutral pH. Subsequently, ^{13}C-substitution in the acyl group has been used to achieve more accurate assignment of the various features seen in the r.R. spectra (Carey & Phelps, 1983). Of particular note was the observation of two peaks that were assigned to $\nu_{C=O}$ of the acyl carbonyl group. This indicates that two conformations of the bound acyl group coexist, but the observation that the lower frequency feature is broader than that at higher frequency contrasts sharply with the results of the F.t.-i.r. studies of β-phenylpropionyl-chymotrypsin quoted above.

Recently Argade *et al.* (1984*a*) have studied the Raman spectrum of *p*-dimethylaminobenzoyl-chymotrypsin using 457 nm excitation. In Fig. 8 are shown the Raman spectra of the enzyme, the acyl enzyme and the difference spectrum. The 1697 cm^{-1} peak is assigned to $\nu_{C=O}$ of the acyl group, which compares with 1706 cm^{-1} for this feature in the model methyl ester in acetonitrile. The u.v. absorption maxima of the acyl imidazole, the acid and the aldehyde, determined in solvents of differing dielectric constant, have been shown to be linearly correlated with the carbonyl stretching frequencies. The acyl-enzyme is found to lie on the line that correlates these factors for the aldehyde rather than the other compounds and this had led the authors to propose that the acyl group has 'aldehyde' character. This, in turn, is interpreted to mean that the spectroscopic characteristics of the acyl group cannot be interpreted in terms of any simple medium effect but must result from a much more specific interaction within the active site of the enzyme. When the acyl-enzyme was partially unfolded by heating and cooling the spectroscopic parameters changed in such a way as to move the point towards the line for the methyl ester, thus providing evidence that the specific interaction was reduced.

Papain. Papain, like chymotrypsin, forms an acyl-enzyme intermediate, albeit a thiolester, in the course of catalysis (Bender & Brubacher, 1966; Wharton & Eisenthal, 1981; Polgár & Halaśz, 1982). Carey, Storer and their colleagues have produced a long and praiseworthy series of publications which report the study of the r.R. spectra of acyl papains (see, e.g., Carey & Storer, 1983, 1984).

Early work concentrated upon use of coloured acylating agents such as 4-dimethylamino-3-nitro-(α-benzamido)cinnamoyl-imidazole (Carey *et al.*, 1976, 1978). The resulting thioacyl-enzyme could be prepared in quasi-stable form for r.R. spectroscopy using 441.6 nm excitation. A large difference was apparent between the spectra of the enzyme-bound acyl group and the substrate. The difference in the spectra was interpreted in terms of rearrangement of the α-benzamido group of the substrate upon interaction with the enzyme; an increase in amido C=N and electrophilic bonding to oxygen being postulated. It is interesting that a change in conformation of the α-benzamido group is proposed, since this group may be regarded as conferring a degree of specificity upon the substrate. Smaller changes were observed as the pH was raised, which in turn resulted in a decreased half-time for the deacylation reaction.

Clearly the acyl group described above cannot be regarded as being specific since the half-time for deacylation at pH 7 is approx. 35 s. In what has proved to be a very successful attempt to move towards the study of specific substrates Carey and coworkers have made use of *N*-acylaminoacid thionoesters as substrates for papain (see, e.g., Carey & Storer, 1983; 1984). Since papain-catalysed hydrolyses proceed via acyl-enzyme intermediates, thionesters should give rise to dithioacyl-enzymes. Simple dithioesters have a maximum in their u.v. absorption spectra close to 315 nm. Lowe & Williams (1965) were able to observe the formation and decay of absorbance at this wavelength during the course of the papain-catalysed hydrolysis of methyl thionhippurate;

this showed that the dithioacyl-enzyme was an intermediate. By using near-u.v. radiation at 324 nm from a krypton laser and a multichannel spectrometer it has proved possible to observe the r.R. spectra of a number of dithioacyl-enzymes (Ozaki *et al.*, 1983; Lee *et al.*, 1983; Storer *et al.*, 1983; Carey *et al.*, 1984*a*). The $k_{cat.}/K_m$ values for the papain-catalysed hydrolyses of thionesters are very similar to those for oxygen esters, while the $k_{cat.}$ values are some 20–30-fold less. Thus the dithioacyl-enzymes in which only the carbonyl oxygen has been changed to sulphur can be regarded as kinetically specific (Carey *et al.*, 1984*b*). A typical example of an r.R. spectrum of a dithioacylenzyme is shown in Fig. 9. In this Figure an indication is given of the structural relationships of the various spectroscopic features seen in the spectrum.

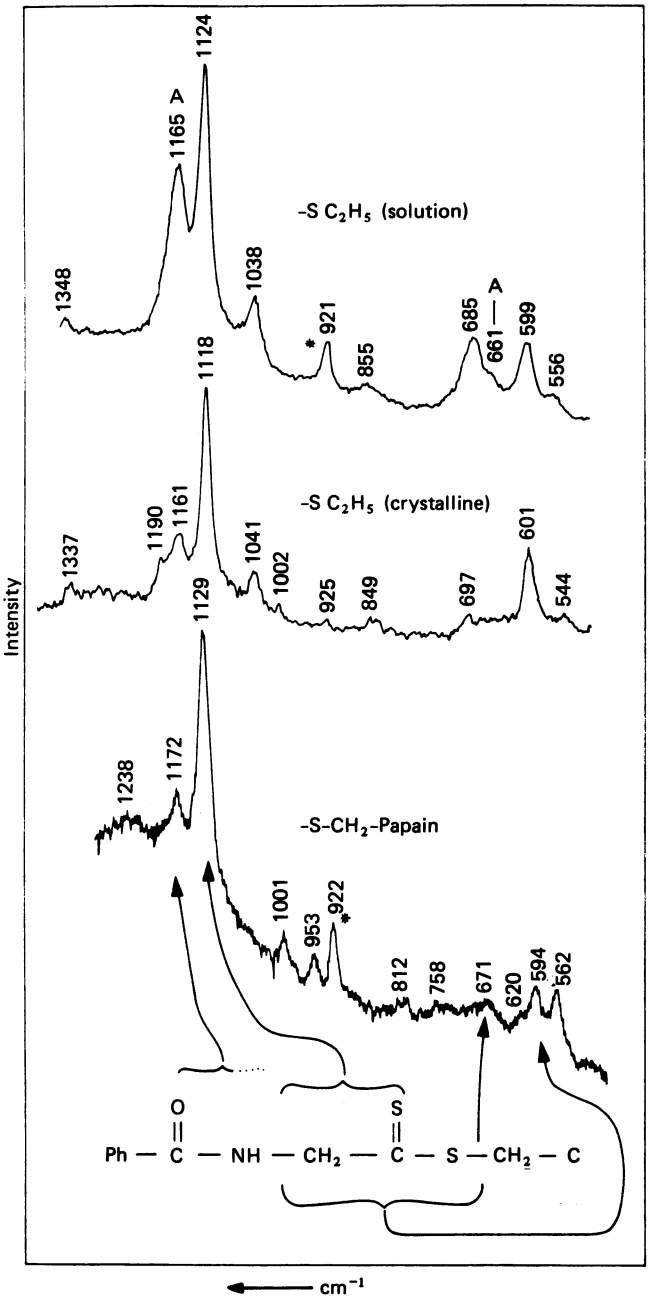


Fig. 9. Resonance Raman spectra of dithioacyl papain and *N*-benzoylglycine dithioethyl ester

The crystalline form of the model ester shows only the conformation B in which the N→S interaction is present as does the dithioacyl papain. Top and bottom spectra were obtained in water/acetonitrite (49:1, v/v) and the solvent peak is marked with an asterisk. U.v. laser excitation at 324 nm was used to obtain the spectra which were collected by using a multichannel apparatus. Data was accumulated for typically 20 s using a laser power of 40 mW. The features in the solution spectrum that correspond to conformer A are appropriately marked. From Carey & Storer (1984).

A combination of X-ray crystallographic and F.t.-i.r. studies of model compounds (Varaghese *et al.*, 1984; Huber *et al.*, 1984) has shown that dithioesters exist in two conformational forms known as A and B; these are shown in Fig. 10. The r.R. spectra show that the dithioacyl group in the dithioacyl-enzymes is in the B conformation in which there is a N→S interaction and these two atoms lie closer together than the sum of van der Waal's radii. The amide and ester planes are nearly orthogonal so that there is no intramolecular hydrogen bonding of the N–H group. The net result of the N→S interaction is an increase in double bond character of the C–S bond and a decrease in that of the C=S group with associated charge separation. The geometry of the N→S interaction in the B conformer is consistent with the nonbonded approach of N to S as proposed by Rosenfield *et al.* (1977) on the basis of nonbonding N–S contacts in crystals.

An increase in the bond strength to the enzyme would be expected to have the effect of reducing the deacylation rate and so, at first sight, would appear not to contribute to overall catalytic efficiency. However, although deacylation is rate-limiting for esters, acylation is rate-limiting for amides. Thus a showing of deacylation will probably not matter in amide hydrolysis in which case the N↔S interaction may function to trap the acyl-enzyme. This will have the effect of disfavouring return of the acylation tetrahederal intermediate to starting materials. For such a mechanism to function it is essential that the N→S interaction is significantly developed in the transition state for breakdown of the tetrahedral intermediate which gives the acyl-enzyme.

Recently a detailed comparison of the kinetics of the papain-catalysed hydrolysis of thionoesters and oxygen esters has been presented in which it had been shown that the enzyme-catalysed mechanisms are very similar (A. C. Storer, personal communication; see also Asboth *et al.*, 1985). Extensive use of ^{13}C, ^{15}N and ^{2}H isotope substitution has been made in order to allow assignment of spectroscopic features and provide a normal co-ordinate analysis of some of the dithioester model compounds (Teixeira-Dias *et al.*, 1982). Finally it is pertinent to note that the N→S interaction has been found in model thiolesters, which suggests it will also be found to occur in 'natural' thiolester acyl-enzymes.

Tonge *et al.* (1985) have made use of a line-narrowed pulsed KrF excimer laser which emits at 248 nm to make a study of a natural acylenzyme, namely *N*-acetyl-L-Phe-Gly-papain. Model thiolesters absorb in the u.v. with a maximum close to 230 nm and in aqueous solution retain some 20–40% absorbance at 248 nm. A multichannel spectrometer has been used together with a rapid flow/mixing system to obtain an r.R. spectrum of the acyl-enzyme. The spectrum of this intermediate, which has a half-life at pH 7 of approx. 100 ms, shows a small peak at 1690 cm^{-1} that has been tentatively assigned to

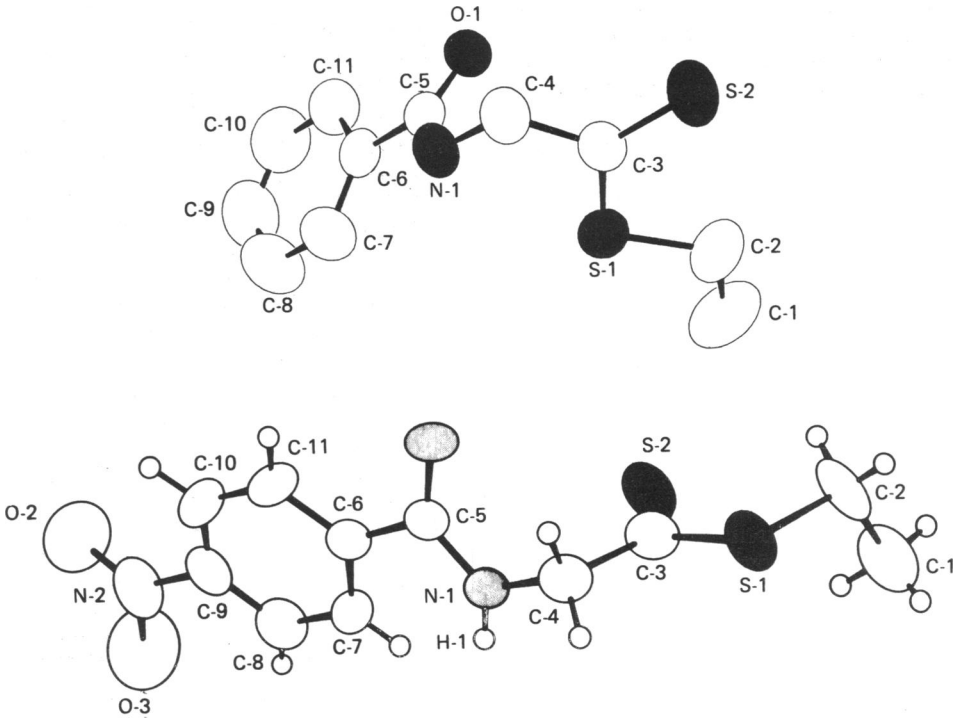


Fig. 10. Crystal structures of *N*-benzoylglycine ethyl dithioester, conformer B (top) and *p*-nitrobenzoylglycine ethyl dithioester, conformer A (bottom)

The N→S distance in conformer B is 0.29 nm as compared with 0.335 nm for the sum of their van der Waals radii. From Carey & Storer (1984).

$\nu_{C=O}$ of the thiolester link. Model thiolesters have $\nu_{C=O}$ very similar to this value, which indicates that the carbonyl group is unperturbed in this highly specific acylenzyme. Considerable pre-resonance enhancement of amide I and/or aromatic groups is seen in these spectra; this is a factor that makes studies of this type more difficult than was originally expected (Harada *et al.*, 1975; Sugawara *et al.*, 1978; Johnson *et al.*, 1984).

Other enzymes. A number of other enzyme mechanisms have been studied by using r.R. spectroscopy. These include a study of the hydrolysis of *p*-substituted cinnamoyl-β-phenyl-lactate derivatives of carboxypeptidase in cryogenic conditions (Hoffman *et al.*, 1983) and a study of β-(2-furoyl)acryloyl-glyceraldehyde-3-phosphate dehydrogenases (Storer *et al.*, 1981). Sulphonamide interaction with carbonic anhydrase has been studied in some detail (Kumar *et al.*, 1976; Petersen *et al.*, 1977; Carey & King, 1979).

Conclusions and future prospects

The application of F.t-i.r. and r.R spectroscopies to studies of enzyme mechanisms is relatively recent, so it has only been possible to describe a limited number of experiments. Those that are described above illustrate the range and incisiveness of these techniques. Apart from the rather obvious use of the techniques for the detection and characterization of covalent bonds in metastable intermediates, it is apparent that the main feature that will appeal to enzymologists is the sensitivity with respect to non-covalent interactions. Thus most of the enzyme mechanisms that have so far been studied have relatively well-established mechanisms in terms of overall covalent processes. The low energy of the photons scattered or absorbed in vibrational spectroscopy means that fairly low-energy interactions such as hydrogen bonding solvation and distortion have a marked perturbing effect on vibrational frequencies. For this reason vibrational spectroscopy is capable of being used to probe these more subtle, yet crucial, features of enzyme mechanism that are not so easily studied by other techniques.

In order that we may achieve a more complete understanding of enzyme catalysis we need to have a more accurate picture of the way in which covalent bond formation and breakdown is modulated by the enzyme structure and dynamics via the weak non-covalent forces of interaction (i.e. dipolar forces of one sort or another). The extreme rapidity of Raman scattering essentially freezes molecular structures and so may be used for the detection and characterization of extremely fast processes. Thus r.R. spectroscopy is expected, in due course, to play an important role in the study of the molecular dynamical contribution to enzyme catalysis.

Some small organic molecules (e.g. bipyridyl) have been found to give enhanced Raman scattering by up to a factor of 10^5 when bound to a metallic colloid (Cotton *et al.*, 1983; Suh *et al.*, 1983). Aminoacids and proteins have similarly been found to show Raman surface enhancement (Cotton *et al.*, 1980; Nabur *et al.*, 1983; Copeland *et al.*, 1984) while in some instances it has also been shown that proteins retain their bioactivities when so bound (Horisberger, 1983). It may prove possible to make use of this form of enhancement in studies of enzymes if the structural and mechanistic distortion that may occur on binding to the colloid is not too great.

The use of a suitable mix of techniques is quite crucial to the success of studies of enzyme mechanism. Kinetic and isotope effect studies are used to define the main features of the reaction co-ordinate in an empirical fashion. Structural techniques such as X-ray and neutron diffraction give us a framework on which to place our reaction co-ordinate, while n.m.r. and fluorescence studies relate in several respects both to structure and dynamics. Vibrational spectroscopy should allow us to decorate the reaction co-ordinate with normal mode structures at energy minima; from these it should prove possible to calculate transition state structures. It is extremely foolhardy to claim special favour for any given technique; instead, rather like a well chosen team of people, they tend to work together synergistically.

The author would like to thank P. J. Tonge for much help during the preparation of this review.

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