Molecular Details of Enzyme–Substrate Transients by Resonance Raman Spectroscopy

PAUL R. CAREY* and ANDREW C. STORER

Division of Biological Sciences, National Research Council, Ottawa, Ontario, Canada K1A 0R6

Received January 12, 1983

This Account[1] will demonstrate how we are able to observe the vibrational motions of an enzyme–substrate linkage while it is being chemically transformed in the active site. What can we hope to understand about the catalytic process by such observations? In the first instance, it is possible to use the vibrational data to obtain precise structural information on the scissile bonds. The relationship between molecular structure and vibrational motions has been extensively studied for many years[2] and in the present case is reinforced by combined X-ray crystallographic–spectroscopic studies on model compounds. The accuracy is such that changes in the length of the bond being broken can be defined to a few hundreths of an angstom—the level at which we can begin to relate structure to reactivity in terms of concepts such as the stereoelectronic[3] and frontier molecular orbital[4] theories. Moreover, the information pertains to catalytically viable complexes, and thus objections that are raised about the validity of extracting mechanistic conclusions from X-ray diffraction measurements of "static" enzyme-inhibitor or substrate analogue complexes do not apply.

In the second instance, it should be remembered that vibrational motions are a dynamical property of a molecule. Protein dynamics[5] has been an area of considerable activity recently and atomic motions in the active site take on a special significance with regard to catalysis. For example, kinetic and equilibrium isotope effects are often explained in terms of differences in the zero-point energy of certain normal modes of vibration.[6] Moreover, there are indications of how normal modes may distort a structure from one species to another along a reaction pathway.[7] These considerations remind us that by studying the vibrational properties we are close to the physical reality of the catalytic process and that it may be possible to use the vibrational data to forge a link between kinetic data, structure, and reaction pathways. Some of the theories and concepts needed to make the link are still at a nebulous stage, but the potential for development is clear.

The vibrational motions of the catalytically labile portion of enzyme–substrate intermediates are monitored by means of resonance Raman (RR) spectroscopy.[8] The RR effect, which is a special case of the Raman effect, allows us to obtain the vibrational spectrum of chromophores at concentrations of 10^{-4}–10^{-5} M in aqueous solution. Both the Raman and RR effects can be explained by reference to Figure 1. In this diagram we see the absorption spectrum of methyl dithioacetate, a relatively simple chromophore with a λ_{max} at 302 nm. Also in this diagram are shown simplified diagramatic representations of a Raman spectrum and a RR spectrum. The spectra are obtained by analyzing the light that is scattered by the sample material when is it exposed to a collimated beam of monochromatic light. The majority of the scattered light has the same wavelength as the incident light. However, a tiny portion of the scattered light occurs at discrete wavelengths differing from that of the incident light; it is this inelastically scattered light that constitutes the Raman or the RR spectrum.

The Raman effect is caused by an exchange of energy between incident photons and the sample molecules. The exchange of energy corresponds to vibrational energy level transitions within the molecules. Thus, in the present context, "peaks" in the Raman spectra correspond to vibrational transitions and the Raman spectrum measures vibrational properties of a molecule. When the excitation wavelength is far from the molecule's electronic absorption bands (see Figure 1), Raman scattering is an improbable event, so the resulting spectrum in exceedingly weak. However, under the resonance condition considerable intensity enhancement can occur. This condition is also illustrated in Figure 1; with use of an excitation wavelength (e.g., 324 nm) that lies under the absorption band of the dithio ester chromophore, a marked increase in Raman scattering is observed. Peaks occur in the same positions (usually denoted on a reciprocal centimeter scale as in infrared spectroscopy) in both the Raman and the RR spectra, but only peaks that are intimately connected with the electronic absorption process are strongly intensity enhanced under resonance conditions. For example, in the Raman spectrum shown in Figure 1 three features are evident; however, in the RR spectrum it appears that only two remain. This is because the ν_{C-S}

Paul Carey obtained his B.Sc. and D.Phil. from the University of Sussex. In 1969 he joined the NRC as a postdoctoral fellow and, at that time, shifted his research interests from applying NMR to chemical problems to applying Raman spectroscopy to biochemical problems.

Andrew Storer received his B.Sc. and Ph.D. from the University of Birmingham, England. Following this he spent two years as a postdoctoral fellow in the Chemistry Department at Cornell University before joining the staff of the NRC in 1977. His research interests include many areas of enzymology, in particular, kinetics and enzyme mechanisms. Both authors are Research Officers with the National Research Council of Canada.

(1) Issued as NRCC No. 22644.
(2) Herzberg, G. "Infrared and Raman Spectra of Polyatomic Molecules"; Van Nostrand: Princeton, NJ, 1945. Wilson, E. B.; Decius, I. C.; Cross, P. C. "Molecular Vibrations"; McGraw-Hill: New York, 1955.
(3) Dugas, H.; Penney, P. "Bioorganic Chemistry"; Springer-Verlag: West Berlin, 1981; p 232.
(4) Fleming, I. "Frontier Orbitals and Organic Chemical Reactions"; Wiley: New York, 1976.
(5) Careri, G.; Fasella, P.; Gratton, E. *Ann. Rev. Biophys. Bioeng.* 1979, *8*, 69. Karplus, M.; McCammon, J. A. *CRC Crit. Rev. Biochem.* 1981, *9*, 293.
(6) Buddenbaum, W. E.; Shiner, V. J. In "Isotope Effects on Enzyme-Catalyzed Reactions"; Cleland, W. W., O'Leary, M. H., Northrop, D. B., Eds.; University Park Press: Baltimore, MD, 1977; p 1.
(7) Bürgi, H. B. *Inorg. Chem.* 1973, *12*, 2321.
(8) Carey, P. R. "Biochemical Applications of Raman and Resonance Raman Spectroscopies"; Academic Press: New York, 1982.

0001-4842/83/0116-0455$01.50/0

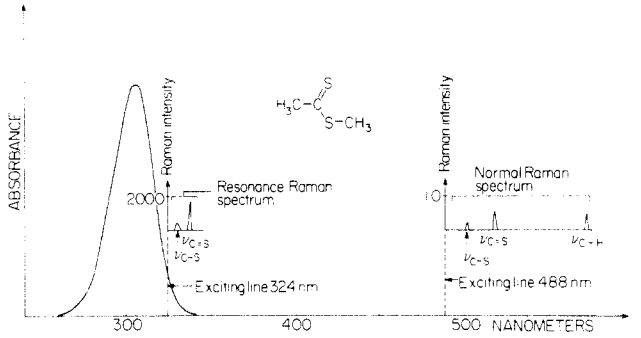


Figure 1. The main features in the absorption, resonance Raman, and normal Raman spectra of methyl dithioacetate, showing their relative positions on the wavelength scale. The choice of the 488-nm exciting line is arbitrary since any wavelength longer than 400 nm would yield a normal Raman spectrum. Reproduced from ref 8 with permission. Copyright 1982, Academic Press.

and $\nu_{C=S}$ features are associated with the electronic transition at 302 nm and so are intensity enhanced by approximately 2000-fold compared to the normal Raman case. However, the C–H bonds are not associated with the electronic transition and thus the ν_{C-H} feature is not intensity enhanced. Thus, the relatively weak ν_{C-H} is "lost" in the background noise of the RR spectrum.

It is now easy to understand how the RR effect can be used to probe selectively the vibrational spectrum of the substrate in an enzyme–substrate complex. The strategy is to form an enzyme–substrate species, wherein the substrate (or the enzyme–substrate bonds themselves) forms a chromopore which absorbs above 300 nm. Since the enzyme usually has no absorbance bands above 285 nm, it is possible to selectively excite the RR spectrum of the substrate by using irradiation lying under the latter's absorption peak. Only the intense RR signal is observed; the weak Raman peaks from the protein or solvent are normally "lost" in the background noise.

Due to the availability of laser sources, the first RR studies[9-11] of enzyme–substrate systems relied on substrates, such as substituted cinnamoyls, which have absorption bands in the visible. However, in the past few years reliable sources in the near-UV have greatly extended the range of systems that can be probed. From the spectroscopic point of view the enzyme substrate complexes fall into three classes; (1) those with spectral properties similar to those of the substrate alone; acylchymotrypsins,[12,13] or acylglyceraldehyde-3-phosphate dehydrogenase[14,15] in which the acyl group is a substituted cinnamoyl[9,10] or furylacryloyl, fall into this category, (2) those, such as acylpapains, for which the enzyme strongly perturbs the spectral properties of the substrate,[16-18] and (3) those for which the chromophore is generated in situ—at the time and place of catalysis. To eliminate confusion between substrate or product features, complexes in classes 1 and 2 usually require purification of the enzyme–substrate species prior to examination. However, those in class 3 offer the advantage that they may be studied in unstable reaction mixtures. Moreover, this group has the potential for providing answers to some of the basic questions of molecular enzymology and they form the subject of the present review.

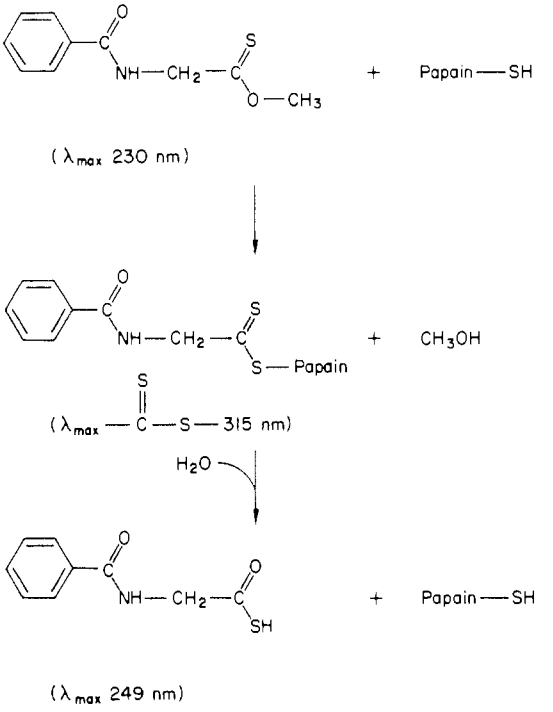


Figure 2. Reaction scheme for the formation of a dithioacyl-papain by methyl thionohippurate and papain.

Dithio Esters Used To Generate Vibrational Spectra of Scissile Bonds

The key to observing the vibrational spectrum of the scissile bonds of an enzyme–substrate complex in a reaction mixture is to ensure that the enzyme–substrate linkage itself constitutes a chromophore which is well-removed, in wavelength, from other chromophores in the mixture. Then, by exciting into the absorption band of the chromophore it is possible to obtain the RR spectrum of the transient enzyme–substrate linkage. In the present work this is achieved by generating a dithio ester RC(=S)SR′, in which the substrate and enzyme contribute the RC(=S) and SR′ moieties, respectively. The catalytic hydrolysis of ester substrates, RC(=O)-OR″, by cysteine proteases (e.g., papain) proceeds through the formation of a thiolacyl-enzyme, RC(=O)SEnz, in which a covalent linkage is formed to the S atom of the active-site cysteine residue. By a single-atom substitution and use of absorption spectroscopy, Lowe and Williams[19] were able to monitor this process during the reaction of papain with methyl thionohippurate (Figure 2). The thiono ester substrate is an analogue of methyl hippurate (a commonly used

(9) Carey, P. R.; Schneider, H. *Biochem. Biophys. Res. Commun.* **1974**, *57*, 831.
(10) Carey, P. R.; Schneider, H. *J. Mol. Biol.* **1976**, *102*, 679.
(11) Carey, P. R.; Schneider, H. *Acc. Chem. Res.* **1978**, *11*, 122.
(12) Phelps, D. J.; Schneider, H.; Carey, P. R. *Biochemistry* **1981**, *20*, 3447.
(13) MacClement, B. A. E.; Carriere, R. G.; Phelps, D. J.; Carey, P. R. *Biochemistry* **1981**, *20*, 3438.
(14) Storer, A. C.; Phelps, D. J.; Carey, P. R. *Biochemistry* **1981**, *20*, 3454.
(15) Schmidt, J.; Benecky, M.; Kafina, M.; Watters, K. L.; McFarland, J. T. *FEBS Lett.* **1978**, *96*, 263.
(16) Carey, P. R.; Carriere, R. G.; Lynn, K. R.; Schneider, H. *Biochemistry* **1976**, *15*, 2387.
(17) Carey, P. R.; Carriere, R. G.; Phelps, D. J.; Schneider, H. *Biochemistry* **1978**, *17*, 1081.
(18) Jagodzinski, P. W.; Funk, G. F.; Peticolas, W. L. *Biochemistry* **1982**, *21*, 2193.
(19) Lowe, G.; Williams, A. *Biochem. J.* **1965**, *96*, 189.

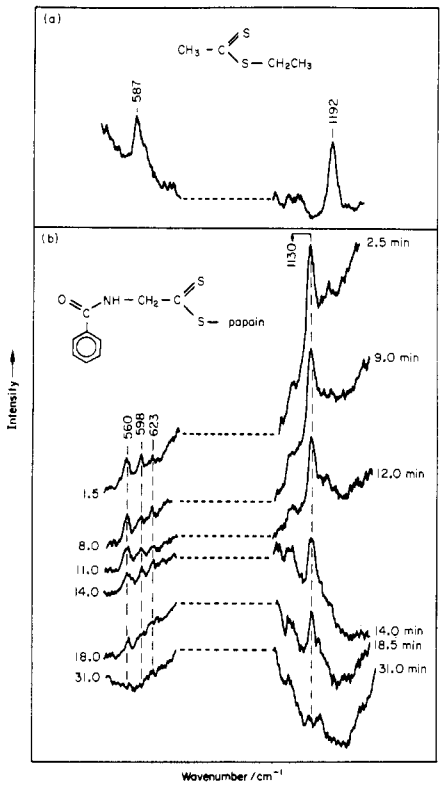


Figure 3. Resonance Raman spectra in the 600- and 1100-cm^{-1} regions of ethyl dithioacetate (a) and of a dithioacylpapain (b). For the dithio intermediate the times in minutes after mixing are denoted beside each spectral trace. The dithioacyl-enzyme was ca. 9×10^{-5} M 2 min after mixing. Excitation line 337.5 nm, 85 mW, 11 cm^{-1} spectral slit. Reproduced from ref 20 with permission. Copyright 1979, American Society of Biological Chemists, Inc.

substrate for papain) in which the carbonyl oxygen atom has been substituted by a sulfur atom. Lowe and Williams observed the transient appearance of a chromophoric intermediate with a λ_{max} near 315 nm, and using kinetic evidence and spectral comparisons with model compounds, they were able to infer that the intermediate was a dithioacyl enzyme.

The dithioacyl intermediate has a half-life of approximately 7 s, and with an excess of substrate a steady-state population is created for sufficient time for spectral observation. By employing Lowe and Williams's reaction mixture and exciting with 337.1-nm Kr^+ laser irradiation, we were able to record the RR spectra[20] shown in Figure 3. The crucial observation in Figure 3 is that for the reaction mixture the RR peaks are due to the transient C(=S)S group, i.e., we are indeed observing the vibrational spectrum of the scissile bonds. The peaks can be seen to disappear with increasing time after mixing as the excess substrate is used up. A further important point is that the spectrum of the dithioacyl-enzyme is quite different from that of a simple model compound seen at the top of Figure 3. This shows that the chemistry of the dithio ester group in the active site is markedly perturbed from that of a free dialkyl dithio ester. Efforts to achieve a detailed interpretation of RR spectra of the type seen in Figure 3 have required comprehensive multidisciplinary investigations. Before detailing these studies in the next section, several general points should be made. Firstly,

(20) Storer, A. C.; Murphy, W. F.; Carey, P. R. *J. Biol. Chem.* **1979**, *254*, 3163.

Chart I
Some of the Substrates and Model Compounds Synthesized

substrate $RC(=S)OCH_3$	dithioacylpapain $RC(=S)S$-papain	corresponding model $RC(=S)SC_2H_5$
(1) R = $PhC(=O)NHCH_2$	(4) R = $PhC(=O)OCH_2$	
(2) R = $PhCH_2C(=O)NHCH_2$	(5) R = $PhCH_2OC(=O)NHCH_2$	
(3) R = $PhCH_2CH_2C(=O)NHCH_2$	(6) R = $CH_3C(=O)NHCH_2$	

the reaction scheme of Figure 2 is capable of extension. Any cysteine protease can be used and we have had success with bromelain, chymopapain, ficin, actinidin, and papaya peptidase A, although these enzymes will not be discussed in detail here. Moreover, any thiono ester substrate is a suitable candidate, and, to date, approximately 20 thiono ester substrates have been synthesized and characterized. Most of these are *N*-acylglycine derivatives and some are discussed below. A further point is that in order to produce a suitable chromophore we have perturbed the system slightly. The natural enzyme–substrate linkage is —C(=O)S—, whereas we have —C(=S)S—. The k_{cat}/K_M values are very similar for oxygen and thiono ester substrates, although the k_{cat}'s for the latter are 20–30 times less than for the oxygen analogues.[19,21] Importantly, there is evidence that the structural properties of the dithio ester intermediates outlined below are common to thiol esters,[22] so with care we should be able to extrapolate from the dithio ester to the true thiol ester intermediates.

Detailed Information on the Scissile Bonds from the RR Spectrum

Extensive studies have been required to achieve an in depth interpretation of the RR spectra of dithioacylpapains. Even at the most basic level, vibrational and theoretical investigations had to be undertaken on simple dithio esters, such as $CH_3C(=S)SCH_3$, in order to build a reliable force field for dithio esters which could then be used as a basis for our understanding of more complex dithio ester systems. The work on simple dithio esters included Raman, RR, and infrared analysis coupled with a normal coordinate treatment of methyl and ethyl dithioacetate[23] and several ^{13}C- and D-substituted analogues. The approach was extended to molecules such as $CH_3CH_2\text{-}C(=S)SCH_3$, which with $CH_3C(=S)S\text{-}CH_2CH_3$ provided information on the sensitivity of the RR spectrum to rotation about the bonds indicated.[24] Ascending the scale of complexity the spectroscopic and physical chemical properties of dithio esters of the type $RC(=O)NHCH_2C(=S)SC_2H_5$ were studied next.[25] These *N*-acylglycine derivatives are important because they form the basis of the series of thiono ester substrates and corresponding dithioacylpapains. The series of compounds are related as shown in Chart I.

(21) Storer, A. C.; Carey, P. R., unpublished work.
(22) *N*-(β-Phenylpropionyl)- and *N*-benzoylglycine ethyl thiol esters both crystallize as B conformers having similar N···S contacts and torsional angles in the $NH\text{–}CH_2\text{–}C(=O)$ bonds compared to the dithioester analogues. Huber, C. P.; Storer, A. C.; Carey, P. R., unpublished work.
(23) Teixeira-Dias, J. J. C.; Jardim-Barreto, V. M.; Ozaki, Y.; Storer, A. C.; Carey, P. R. *Can. J. Chem.* **1982**, *60*, 174.
(24) (a) Ozaki, Y.; Storer, A. C.; Carey, P. R. *Can. J. Chem.* **1982**, *60*, 190. (b) Verma, A. L.; Ozaki, Y.; Storer, A. C.; Carey, P. R. *J. Raman Spectrosc.* **1981**, *11*, 390.
(25) Storer, A. C.; Ozaki, Y.; Carey, P. R. *Can. J. Chem.* **1982**, *60*, 199.

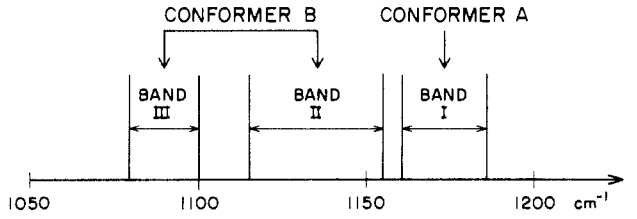


Figure 4. Relationship between the RR "signatures" and conformers in solution of *N*-acylglycine ethyl dithio esters. Reproduced from ref 28 with permission. Copyright 1982, American Chemical Society.

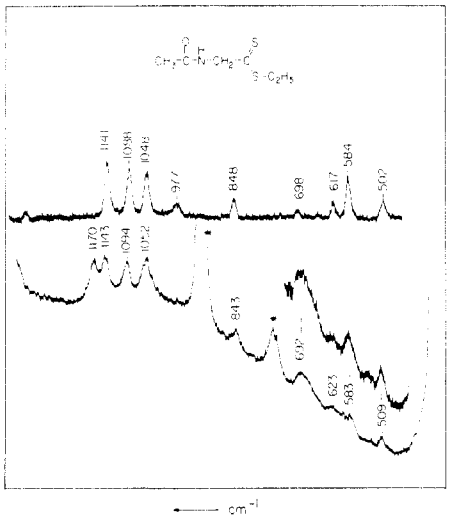


Figure 5. Raman spectra of *N*-acetylglycine ethyl dithio ester as a single crystal (top) or in an acetonitrile–water mixture (bottom). CH_3CN features are marked with an asterisk. Reproduced from ref 25 by permission.

The model *N*-acylglycine ethyl dithio esters give rise to intense RR bands in the 500–700- and 1050–1200-cm^{-1} regions of the spectrum. In solution, the relative intensities of the bands in these regions were found to be very sensitive to temperature and solvent. These facts, taken with other considerations, indicated the presence of more than one conformer. It was found that in aqueous or acetonitrile solutions there are two major conformational states designated conformers A and B. Crucially, each conformer has a characteristic and separate RR spectrum in both the 600- and 1100-cm^{-1} regions. For example, in the latter range conformer A gives rise to band I and conformer B to band II and sometimes band III as shown in Figure 4. Band I occurs at the predicted frequency of the "C=S stretching" mode for this type of molecule. However, the frequency of band II is highly perturbed, and this, taken with other evidence, such as the dependence of band III on NH to ND exchange, led to the conclusion that, in conformer B, an intramolecular interaction occurs between the amide and dithio ester groups.[25] Considerations such as these enabled us to form an approximate idea of the nature of conformers A and B. In order to form an exact description, a combined X-ray crystallographic–Raman analysis[26] was undertaken on single crystals of *N*-acylglycine ethyl dithio esters, including those shown in Chart I. Most of the *N*-acylglycine dithio esters crystallize in a form giving rise to the Raman signature of conformer B, in keeping with

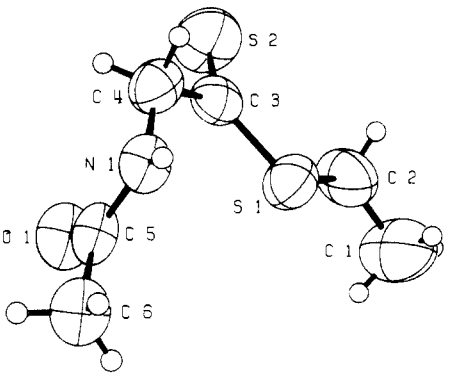


Figure 6. Conformer B. The structure of crystalline $CH_3C(=O)NHCH_2C(=S)SC_2H_5$. Reproduced from ref 26 with permission. Copyright 1982, American Chemical Society.

the higher thermodynamic stability of this form. Figure 5 compares the Raman spectra of a single crystal of $CH_3C(=O)NHCH_2C(=S)SC_2H_5$ and of this molecule in CH_3CN (allowing for the large differences in absolute intensities, the Raman and RR spectra for the dithio esters have similar appearances and conclusions from one type of measurement usually may be transferred to the other). In solution, the spectral signatures of forms A and B are present, but in the case of the crystal only the signature of conformer B is found, e.g., in the upper trace in Figure 5 the conformer A peak at 1170 cm^{-1} is missing. An X-ray diffraction analysis on the crystal now provides an accurate structure of conformer B and this is shown in Figure 6.

As predicted, the amide and dithio ester groups are in close contact; in fact, the N···S (thiol) distance is 2.9 Å, somewhat less than the sum of the van der Waals radii, which is 3.35 Å. The amide and ester planes are roughly orthogonal and thus there is no intramolecular H bonding involving the NH moiety. To date, only p-$NO_2PhC(=O)NHCH_2C(=S)SC_2H_5$ has crystallized in a form giving rise to a conformer A type RR signature[26] (a fact that may be related to the effect the p-NO_2 substituent has on the basicity of the NH group[27]). If the chemically different portions of the *N*-acetyl (Figure 5) and *N*-(*p*-nitrobenzoyl) derivatives are ignored, the major conformational differences between conformers A and B arise from a rotation of ~150° about the C(3)–C(4) bond together with a small rotation of ~10° about the C(4)–N(1) bond. Therefore, conformer A lacks the N···S (thiol) contact. The rotation about the C–C linkage changes the vibrational coupling in and about the dithio ester moiety and accounts, at least in part, for the very different Raman spectral signatures of conformers A and B.

The X-ray analyses provide the exact structures of conformers A and B and, taken with the Raman data, a set of precise structure–spectra correlations. These may now be applied to an analysis of the dithioacylpapain RR spectra. Consideration of these spectra,[28] e.g., the RR spectrum of $PhC(=O)NHCH_2C(=S)S$-substituted papain seen in Figure 7, demonstrates unequivocally that between pH 4 and 9 the great majority of acyl groups assume a B-type conformation. In Figure 7 the intense peak at 1131 cm^{-1} and the peak at 597 cm^{-1} are modes characteristic of a B conformer. The

(26) Huber, C. P.; Ozaki, Y.; Pliura, D. H.; Storer, A. C.; Carey, P. R. *Biochemistry* **1982**, *21*, 3109.

(27) Ozaki, Y.; Storer, A. C.; Carey, P. R.; unpublished work.

(28) Ozaki, Y.; Pliura, D. H.; Carey, P. R.; Storer, A. C. *Biochemistry* **1982**, *21*, 3102.

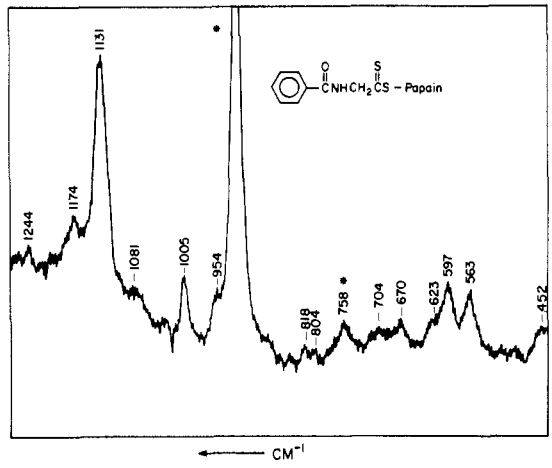


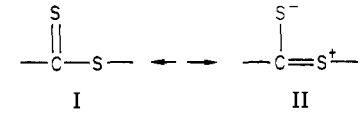
Figure 7. Complete RR spectrum of dithiohippurylpapain obtained by using diode array detection. CH_3CN peaks are marked with an asterisk. Excitation 324 nm, 50 mW, 20 s exposure (20 accumulations of 1 s).

same situation obtains for the other acylpapains listed in Chart I. There is a very close correspondence between the RR spectrum of a dithioacylpapain and that of its corresponding ethyl ester in the B form. All major peaks and most minor peaks in the acyl-enzyme spectra are due to conformer B. Although, at present, it is possible that some minor peaks are due to a small population of non-B conformers, we have no convincing evidence to support this notion. Originally, we proposed that the weak feature seen near 1174 cm^{-1} may be due to a minor non-B population, but the results of recent experiments involving ^{15}N and ^{13}C substitutions in the enzyme intermediates have rendered this possibility unlikely.[29] However, enzyme denaturation below pH 3.0 does result in conformer A peaks appearing in the spectrum,[28] and, under these conditions, the conformational population of the covalently linked acyl group reverts to that found for the corresponding ethyl ester.

Consequences for Catalysis

The RR data for every dithioacylpapain examined thus far provide strong evidence that the great majority of acyl groups assume a B-type conformation. That is, the conformation of the bound acyl group is as shown in Figure 6. What are the catalytic consequences of this finding?

The close contact between the amide nitrogen and the thiol sulfur atom in conformer B can be analyzed in terms of the criteria set out by Rosenfield et al.,[30] concerning the directional preferences of nonbonded atomic contacts with sulfur. By reexamining crystal data these authors recognized two separate preferred lines of approach, one for nucleophiles and one for electrophiles, to sulfur in a Y–S–X bonding situation. The approach of N to S in Figure 6 meets the Rosenfield criterion for the N atom acting as a nucleophile and the S atom acting as an electrophile. In fact, the nitrogen lone pair is pointing toward the sulfur, and from these considerations we might predict that confomer B will have increased contributions from canonical structures of type II. Support for this comes from comparison of the X-ray crystallographic data for conformers B and A[26] (i.e., for the *N*-acetyl and *p*-nitrobenzoyl derivatives discussed above). The C=S bond is indeed longer in conformer B (1.635 Å compared to 1.615 Å in conformer A) and the C–S single bond is shorter (1.700 Å compared to 1.727 Å in conformer A). The shorter C–S bond in conformer B leads to a remarkable conclusion, since, when we transfer this finding to the dithioacyl-enzyme, it is seen that the effect of forming conformer B in the active site is to shorten, and therefore probably strengthen, the bond being broken in the deacylation step. Why should the enzyme go to the trouble of strengthening the bond it has to cleave in the next step of the reaction? One possibility is that a conformer B type interaction is used simply to facilitate the formation of the acyl-enzyme with its accompanying entropic advantage. The N···S contact may function as part of a thermodynamic trap that favors the breakdown of the tetrahedral intermediate for acylation to the acyl-enzyme rather than allowing the tetrahedral species to revert to the Michaelis complex.



I II

It is also possible to examine the effect of the N···S (thiol) interaction in conformer B on the chemical reactivity from the molecular orbital viewpoint. According to the work of Wolfe and Kost[31] and Nguyên and Eisenstein,[32] the critical factor governing the stability of certain SN2 transition states formed during the nucleophilic attack on a carbonyl group is the effect of interactions on the π^* orbital of the C=O group. On this basis it might be worthwhile to examine the effect of the interactions in conformer B on the $\pi^*_{C=S}$ orbital. However, consideration of the geometry of conformer B and its attendant molecular orbitals shows that the lone pair of electrons on the nitrogen will have little or no overlap with $\pi^*_{C=S}$ and thus will not perturb the energy of this orbital. Rather, the lone pair will feed electron density into the σ^* orbital assocated with the S–C bond in –C(=S)SC–. Interestingly, there is experimental evidence[26] for the latter effect; S–C in conformer B is longer (1.807 Å) compared to S–C in conformer A (1.791 Å).[33]

The sensitivity of the RR spectrum to small changes in conformation allows us to detect strain, which, in the present context, is defined as geometric distortion. The conformations of the *N*-acylglycine ethyl dithio esters (Chart I) in solution are taken to be a relaxed state. Thus, we interpret any differences between the RR spectrum of a dithioacylpapain and the conformer B spectrum of the corresponding dithio ester model in solution as a measure of the strain imposed on the acyl group by the enzyme. It must be pointed out that we have found no evidence for perturbations to the RR spectrum by effects in the active site other than strain. The dithio ester spectrum is insensitive to H bonding[23]

(29) Storer, A. C.; Lee, H.; Carey, P. R., *Biochemistry*, in press.
(30) Rosenfield, R. E.; Parthasarathy, R.; Dunitz, J. D. *J. Am. Chem. Soc.* **1977**, *99*, 4860.
(31) Wolfe, S.; Kost, D. *Nouv. J. Chim.* **1978**, *2*, 441.
(32) Nguyên Trong Anh; Eisenstein, O. *Nouv. J. Chim.* **1977**, *1*, 61.
(33) Recent emphasis has been placed on the ability of protein dipoles to stabilize charges developed on catalytic intermediates (Warshel, A. *Acc. Chem. Res.* **1981**, *14*, 284). While these studies refer to intermolecular protein–substrate interactions, we should clearly consider the intramolecular effect of our substrate's amide dipole in conformer B. Model calculations for this effect are in progress.

and (in contrast to earlier studies of cinnamoylpapains[16,17]) to electrostatic effects due to protein charges or dipoles. Thus, differences between dithioacyl-enzyme and model spectra are ascribed solely to geometrical changes.

Consider the series of derivatives, *N*-(β-phenylpropionyl)glycine, *N*-(phenylacetyl)glycine, and hippuryl, denoted in Chart I as **3**, **2**, and **1**, respectively. The differences between the dithioacylpapain and corresponding model RR spectra increase along this series. There is a very close resemblance in the case of **3**, whereas for **1** marked differences are noted. For the hippuryl derivative **1**, these differences include the intensity of the 563-cm^{-1} band and the appearance of a "new" band (not seen in the model case) at 954 cm^{-1} (Figure 7). The degree of spectral perturbation for the *N*-(phenylacetyl)glycine analogue lies between those for the *N*-(β-phenylpropionyl)glycine and hippuryl compounds. These observations are supported by the results of $^{13}C{=}S$ substitutions,[29] which provide a further valuable measure of acyl-enzyme and model compound similarity. As far as specificity is concerned, the *N*-(β-phenylpropionyl)glycine derivative quite closely resembles phenylalanylglycine for which the enzyme has a high degree of specificity.[34] This resemblance diminishes upon going to **2** and decreases further upon going to **1**. From this we conclude that the acyl group with the highest degree of specificity adopts as essentially strain-free conformation, and as specifity diminishes (going to **2** and **1**, then strain increases. Thus, for a specific substrate the enzyme appears to utilize the available potential energy surface; while it does select one of the available conformational states, it does not expend energy distorting the acyl group away from the relaxed conformation found in solution. However, it must be remembered that conformer B, with its N···S (thiol) contact is a property of the acyl-enzyme alone, since it relies on the active-site cysteine sulfur. B-type conformers are not found for any of the substrates in the categories based on glycine esters or thiono esters or, indeed, the natural peptide sequences.

The present experimental evidence for a strain-free acyl group is consonant with the earlier conclusions of Lowe and Yuthavong[34] based on model building and kinetic data. They predicted that the reduction in strain in going from a distorted enzyme–substrate Michaelis complex via a tetrahedral intermediate to a strain-free acyl-enzyme could provide a driving force for the acylation process. Although a subsequent conformational change in the acyl-enzyme could produce strain, it would have to do so without using the substrate binding energy that is consumed in bringing about distortion within the Michaelis complex. For ester substrates it seems wasteful that none of the binding energy is used to the slowest step in the reaction sequence, deacylation. However, this would appear to be a sensible procedure for natural amide substrates for which acylation is the rate-determining step.

Conclusions and Prospects

The overall conclusions can be stated as follows. The great majority of the acyl groups in the active site are bound in a B-type conformation[35] (Figure 6). The enzyme is selecting one of the conformational states available to a dithio ester of *N*-acylglycine, but for a specific acyl group there is no evidence for strain distorting the geometry away from that found for a relaxed state of the corresponding dithio ester model compound in, e.g., CCl_4, solution. Consideration of the chemistry of conformer B makes it difficult to see how the dithio ester moiety is activated in the ground state toward the hydrolysis step subsequent to formation of the acyl-enzyme.

There are excellent prospects for a continued growth in our technical abilities and our understanding of the chemistry of enzyme processes. From the purely technical standpoint, developments in lasers and photon detection systems are improving our ability to study enzyme–substrate transients by RR spectroscopy by leaps and bounds. For example, the original studies of dithio ester intermediates utilized a scanning Raman spectrometer, equipped with a single photomultiplier, and due to the short lifetime of the dithio ester population, only a small spectral range could be scanned for one reaction mixture (see, e.g., Figure 3). Under these conditions, spectral reproducibility was difficult to achieve. The introduction of a UV-sensitive diode array system, which was used to record the spectrum seen in Figure 7, has radically improved the situation.[36] The diode array is akin to using 1024 minute photomultipliers simultaneously, and the consequent multiplex advantage makes it possible to produce complete and reproducible spectral traces in a few seconds. This development also makes it possible to undertake the examination of certain intermediates under cryoenzymological conditions[37] and facilitates the introduction of rapid reaction techniques.

From the enzymological viewpoint, it will be interesting to see if the conclusions so far obtained can be expanded to include substrates which have a higher degree of specificity and which possess a chiral center at the α-carbon atom, e.g., *N*-acylphenylalanyl L-alanyl thiono esters. We are also at the point of being able to relate changes in the values of an individual rate constant, k_3 for deacylation, to changes in the strength of the N···S contact in the active site. In turn, this relates to the question as to whether deacylation can occur from conformer B or whether it must be preceded by a conformational change. Another area in which our understanding is growing concerns the conformational properties of the S–C–C bonds of the cysteine residue in the dithioacyl-enzyme. When these bonds are better characterized, we will have a complete picture of the covalent linkages in and near the acyl group. Finally, studies of thiol esters are in progress in order that we may transfer the information gained on dithioacyl-enzymes to the natural thiolacyl-enzyme systems.

The pursuit of this work has involved drawing on the talents and skills of many people. The names of our collaborators appear in the list of references and it is a pleasure to record our thanks for their efforts.

(34) Lowe, G.; Yuthavong, Y. *Biochem. J.* **1971**, *124*, 107.

(35) Our present knowledge of conformer B stems from the X-ray crystallographic analysis of *N*-acetylglycine ethyl dithio ester. However, it is apparent that conformer B should be considered a conformational class rather than a single precisely defined conformation. Questions on how much variation exists within the class and its effect on spectral signatures are being actively pursued.

(36) Carey, P. R.; Sans Cartier, L. R. *J. Raman Spectrosc.*, in press.

(37) Hoffman, S. J.; Lee, H.; Kaiser, E. T.; Carey, P. R. *J. Am. Chem. Soc.*, in press.