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Relaxed and Perturbed Substrate Conformations in Enzyme Active Sites: Evidence from Multichannel Resonance Raman Spectra†

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ABSTRACT: A diode array based multichannel Raman spectrometer has made it possible to record complete, high quality, resonance Raman (RR) spectra of enzyme–substrate intermediates. The intermediates are dithioacylpapains in which the acyl group is either *N*-benzoylglycine or *N*-(β-phenylpropionyl)glycine. RR data are reported for the unlabeled dithioacylpapains as well as for the intermediates labeled separately with ND, ^{15}N, and $^{13}C{=}S$ in the glycine residue. Comparison of the results for the dithioacylpapains with that of the corresponding labeled glycine ethyl dithioesters [Lee, H., Storer, A. C., & Carey, P. R. (1983) *Biochemistry* (preceding paper in this issue)] leads to the conclusion that for both substrates in the active site the dihedral angles in the glycinic NH–C–C(=S) linkages assume an essentially relaxed type B conformation. Similarly, there is no evidence for distortion about the C(=O)–NH peptide bond which links the P_1 and P_2 sites on the substrate. However, for the *N*-benzoylglycine case there is evidence for some conformational distortion in the –S–C–C cysteine linkages. The present data favor a single homogeneous conformational population about the substrates' NH–C–C(=S) bonds in the native dithioacylpapains. However, below pH 3.0 the dithioacyl enzymes denature and the RR spectra of the $^{13}C{=}S$ substituted species confirm that the conformational population reverts to the mixture of conformers A and B found for the corresponding ethyl dithioesters in solution.

Geometric distortion of an enzyme-bound substrate is one of the factors that has been widely discussed as a contributor to rate acceleration by enzymes (Jencks, 1975; Fersht, 1977). Distortion occurs when the substrate is forced, by contacts with the active site, to assume a conformation that is strained or distorted away from the conformation found in some relaxed standard state. The unfavorable enzyme–substrate contacts will, at least to a small degree, also result in the enzyme assuming a different conformation. If the total geometric destabilization is relieved in the transition state, rate acceleration will occur. Discussion of the quantitative nature of geometric strain or distortion has been hampered by the lack of precise experimental evidence on the conformational states of substrates bound in catalytically viable complexes. However, we have shown recently that resonance Raman (RR) spectroscopy can provide such information (Huber et al., 1982; Ozaki et al., 1982a). In the present paper *N*-acylglycine ethyl dithioesters in solution are taken as standards for relaxed, unperturbed conformers with which to compare the corresponding *N*-acylglycine dithioacylpapains. In this way we are able to discuss the degree of geometric distortion in the substrates' glycinic bonds and to begin to consider perturbed conformers of the cysteine C–S–C–C bonds which are used to link the substrate to the enzyme.

The utility of the RR approach is that it enables us to monitor the vibrational spectrum of the bonds undergoing transformation in an enzyme's active site (Carey, 1981, 1982). The RR method relies on the formation of a dithioacylpapain during the enzyme-catalyzed hydrolysis of a thiono ester RC(=S)OR′. During catalysis, a transient –C–C(=S)S– linkage is formed between the substrate and enzyme which contribute the –C–C(=S)– and –S– (from cysteine-25) moieties, respectively (Lowe & Williams, 1965). The dithioester group has a λ_{max} near 315 nm, and by the use of laser irradiation at 324 nm, RR bands are observed due to the dithioester group and its immediate, covalently bound neighbors.

The RR data, taken with extensive studies on model compounds, have led to a detailed picture of the geometry of part of the enzyme-bound acyl group. It was shown that the majority of acyl groups adopt a conformation designated conformer B—which is seen in Figure 1 of Lee et al. (1983)—wherein the dihedral angles in the glycine NH–CH_2–C bonds assume a value such that the amide N atom comes into close contact with the cysteine S atom. Thus, the enzyme exerts conformational selection since conformer B is only one of a number of conformers available to a *N*-acylglycine dithioester (Lee et al., 1983). Some of the possible catalytic consequences of conformer B have been discussed (Huber et al., 1982), but now we wish to consider if any degree of geometric destabilization exists in the enzyme-bound acyl group. Of further relevance to this question is whether or not the acyl enzyme is in a single homogeneous population.

Two current advances enables us to approach these questions. First, we have synthesized several isotopically labeled substrates and model analogues of the dithioacyl enzyme intermediates. These enable us to interpret the RR spectra with greater facility and, importantly, to use the labeled model compounds as spectroscopic "benchmarks" for relaxed conformers. The second development involves the RR instrumentation. Heretofore, the RR data have been collected by using a scanning double monochromator and a single photo-

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multiplier. When this method was used, only partial RR spectra could be obtained, and it was extremely difficult to acquire reproducible, high quality spectra. This meant that only intense features could be discussed with certainty. The recent construction of a diode array based multichannel Raman spectrometer has provided a quantum jump forward in our ability to record spectra. The system, which is described in detail in Carey & Sans-Cartier (1983), enables us to acquire complete RR spectra of enzyme–substrate intermediates in 2–60 s. Moreover the spectra are highly reproducible, permitting the detection of weak features and small isotope shifts with certainty. Both these factors are potentially important to an understanding of the catalytic process. The former may unearth the presence of minor populations while the latter facilitates the comparison of isotopically substituted dithioacylpapain RR spectra with those of the corresponding model compounds.

Experimental Procedures

Materials. Papain was purchased from Sigma Chemical Co. as a suspension in sodium acetate. The enzyme was further purified by affinity chromatography (Blumberg et al., 1970). After elution from the column, the enzyme was converted to inactive papain by the addition of 1 equiv of mercuric chloride. The inactive enzyme was then concentrated to 7–10 mg/mL by using an Amicon concentrator. The concentrated enzyme was reactivated as required by adding an equal volume of 5 mM ethylenediaminetetraacetic acid (EDTA) and 4 μL of β-mercaptoethanol per 10 mg of enzyme, stirring for 30 min, and passing through a Sephadex G-15 column. The thiol content of the enzyme was determined with 5,5′-dithiobis(2-nitrobenzoic acid) as described by Ellman (1959). The enzyme prepared in this way was found to contain >70% of active cysteine/mol of protein. Efforts to raise this value to the >90% found in earlier purifications in this laboratory have thus far been unsuccessful.

The thiono ester substrates were synthesized by the procedure described in Ozaki et al. (1982a). ^{15}N- and ^{13}C-substituted thiono ester substrates were obtained from the labeled nitriles which were prepared as described in Lee et al. (1983). N deuteration of substrates was carried out by a direct H–D exchange with an acetonitrile–heavy water mixture as a solvent. All the substrates were purified by crystallization and silica gel chromatography with acetonitrile–ether (1:9) mixtures as eluant. The purity of the sample was checked by NMR and in some cases by elemental analysis.

Resonance Raman spectra were measured on a Spex triplemate equipped with a Tracor Northern DARSS (diode array rapid scan spectrometer) system. The spectrograph stage of the triplemate employed a 3600 g/mm holographic grating resulting in an approximately 900-cm^{-1} spectral range displayed across the diode array in the 335-nm region. The 324 nm line of a Coherent Radiation 2000K krypton ion laser was used as an excitation source. The near-UV laser lines were separated by using two Pellin-Broca prisms, and unnecessary laser plasma lines were eliminated by using a tunable Anaspec 300 SQ monochromator. A Cassegrain collecting optic (Anagrain, manufactured by Anaspec, U.K.) was used to collect the scattered light and focus it on the spectrometer's entrance slit (Carey & Sans-Cartier, 1983). Data acquisition was controlled by two parameters, ET (exposure time) and N (number of scans). Thus, the total acquisition was carried out in the time ET × N. Raman frequencies were calibrated by using two emission lines from an argon lamp which correspond to a Raman shift 742.5 and 1225.9 cm^{-1} with 324-nm excitation. Absolute accuracy of the calibrated wavenumbers is believed to be within ±2 cm^{-1}. For sequential spectra reproducibility is better than ±1 cm^{-1}.

The sample cell consisted of a quartz cuvette (1-cm path length), which was stirred by a magnetic pip during measurement to avoid photodegradation. The typical sample solution contains 130–190 μM papain and 3.5–4 mM substrate dissolved in 2% acetonitrile solution. The pH of the unbuffered sample solution was around 5. Solutions of enzyme containing 50 mM PO_4^{-3} pH 6.0 and 5 mM EDTA gave identical spectra. The concentration of the dithioacyl enzyme reaches ≈100 μM under these steady-state conditions. Data collection commenced approximately 30 s after mixing. For the experiments involving ND substitution in the enzyme-bound acyl group, substrate containing >95% ND was added to the enzyme in H_2O. The rate of exchange of ND with H from the solvent was such that, in the time taken to record the spectrum of the dithioacyl enzyme, the acyl group contained >90% ND. Very similar RR spectra were also recorded in D_2O, but these contained a broad feature near 1200 cm^{-1} due to the solvent's D–O–D bending mode.

Results

The 324-nm excited RR spectra of N-benzoylglycine and N-(β-phenylpropionyl)glycine dithioacylpapains are shown in Figures 1 and 2, respectively. In both figures the spectra refer to the unlabeled, RC(=O)NDCH$_2$C(=S)S–papain, RC(=O)^{15}NHCH$_2$C(=S)S–papain, and RC(=O)NHCH$_2$-^{13}C(=S)S–papain, going from top to bottom. For the N-benzoylglycine papain derivative, ND substitution leads to a large decrease in intensity of the 1172-cm^{-1} peak and a slight but reproducible increase in the frequency of the 1129-cm^{-1} band. The residual intensity observed near 1170 cm^{-1} for the ND spectrum in Figure 1 is probably due to reexchanged NH and is a consequence of the way in which the ND acyl enzyme was prepared (see Experimental Procedures; a spectrum recorded in D_2O shows no feature near 1170 cm^{-1}). The only change seen upon ^{15}N substitution is the shift of the 1172-cm^{-1} band (^{14}N) to 1163 cm^{-1} (^{15}N). In the spectrum of the ^{13}C-substituted acyl enzyme (Figure 1) the intense feature at 1129 cm^{-1} (^{12}C) moves to 1098 cm^{-1} (^{13}C), the 1172-cm^{-1} band (^{12}C) is replaced by a weaker band at 1170 cm^{-1} (^{13}C), and there is a residual peak at 1130 cm^{-1}. The latter may contain a contribution from a band shifted from under the 1170-cm^{-1} profile and/or a peak from acyl enzyme containing residual ^{12}C=S. The effects of isotopic substitution are seen only in the 1100–1175-cm^{-1} region; bands elsewhere in the spectrum do not shift by more than ±2 cm^{-1}. The recently acquired ability to record complete traces in a relatively short time span and at low CH_3CN concentrations allows us to point out several novel dithioacylpapain features of weak intensity. There are weak bands near 950 and 670 cm^{-1} that show the same time dependency as the intense 1129-cm^{-1} peak, and these features are therefore assigned to the dithioacyl enzyme species. The 1000- and 620-cm^{-1} features are due to phenyl ring modes from the substrate and product, which are present in relatively high concentrations. A further potentially interesting observation is that there is RR intensity underlying the 550–750-cm^{-1} region which is due to dithioacyl enzyme but which is only partially resolved. This is illustrated in Figure 3 where the integrated intensity in the 550–750-cm^{-1} range is seen to decrease in step with the decay of the 1129-cm^{-1} feature. The invariance of the relative intensities of the weak peaks with respect to the 1129-cm^{-1} peak means that they are not due to reaction side products or, in the pH 4.0–7.0 range, to denatured acyl enzyme. However, some of the weak features could conceivably be due to the presence of a second

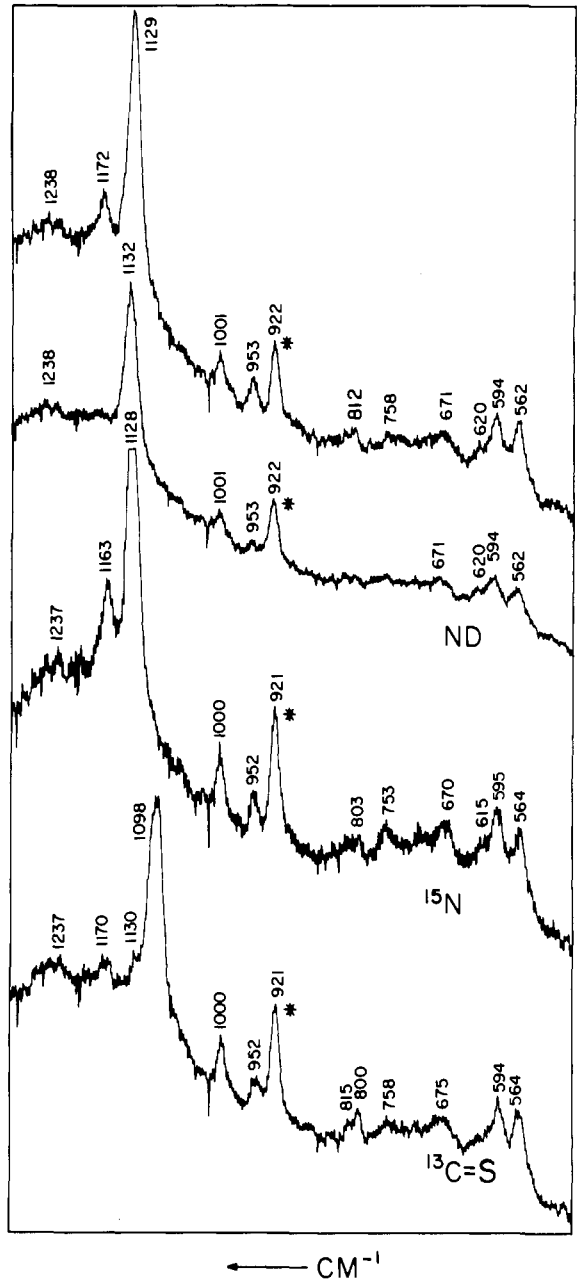


FIGURE 1: Multichannel resonance Raman spectra of unsubstituted (top) and isotopically labeled (in the glycine atoms indicated) *N*-benzoylglycine dithioacylpapain. 324-nm excitation, 100 mW, exposure time 4 × 15 s, 13-cm^{-1} spectral slit. CH_3CN feature is denoted by an asterisk.

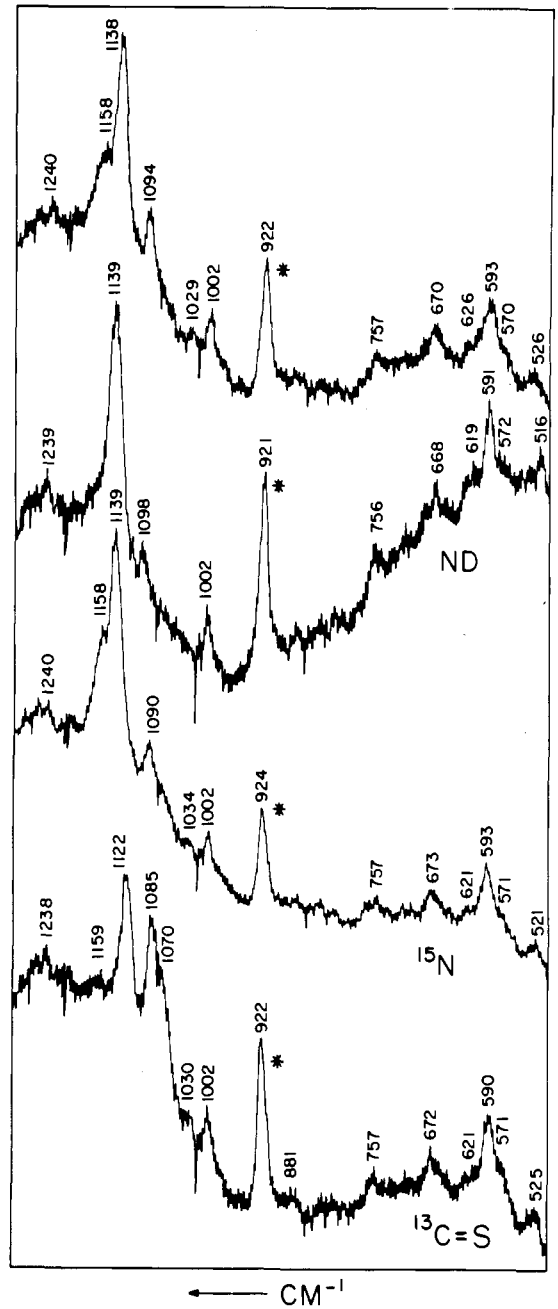


FIGURE 2: Multichannel resonance Raman spectra of unsubstituted (top) and isotopically labeled (in the glycine atoms indicated) *N*-(β-phenylpropionyl)glycine dithioacylpapain. 324-nm excitation, 100 mW, exposure time 4 × 15 s, 13-cm^{-1} spectral slit. CH_3CN feature is denoted by an asterisk.

conformational population in rapid equilibrium with the major population. RR experiments on reaction mixtures on the millisecond time scale are needed to investigate this possibility.

For *N*-(β-phenylpropionyl)glycine dithioacylpapain, ND substitution brings about two changes: the 1158-cm^{-1} shoulder (NH) is greatly decreased in intensity (as in the *N*-benzoylglycine case the residual component is probably due to a small amount of the NH compound), and the 1094-cm^{-1} band (NH) shifts to 1098 cm^{-1} and decreases in relative intensity. A further alteration concerns the band profile of the 593-cm^{-1} peak; this profile " sharpens up" in the ND derivative. The change is reproducible and is the only effect of isotopic substitution detected in the 500–750-cm^{-1} region. The outcome of ^{15}N replacement is to shift the 1094-cm^{-1} band (^{14}N) to 1090 cm^{-1} (^{15}N). The results of ^{13}C substitution are more complex. In the ^{13}C RR spectrum (Figure 2) there is a small but reproducible component near 1159 cm^{-1}, two intense peaks at 1122 and 1085 cm^{-1}, and a shoulder at 1070 cm^{-1}. Apart from the 593-cm^{-1} profile in the ND spectrum (Figure 2), the *N*-(β-phenylpropionyl) results resemble the *N*-benzoyl results in that the effects of isotopic substitution are limited to the 1050–1160-cm^{-1} range. Additionally, there is a correspondence in the appearance of the weak peaks near 1250 cm^{-1}, the phenyl ring modes near 1001 and 620 cm^{-1}, and the bands near 750 and 670 cm^{-1}. The latter bands disappear with increase in time after mixing, in synchrony with the 1138-cm^{-1} feature, and are thus ascribed to the dithioacyl enzyme species. The band at 593 cm^{-1} has been described before, and we are now confident in assigning the weak feature near 520 cm^{-1} to the RR spectrum of the intermediate (Figure 2). However, due to the broad and weak nature of the 520-cm^{-1} peak, we do not attach any significance of its apparent isotope shifts seen in Figure 2. Unlike the *N*-benzoylglycine papain spectra, a very

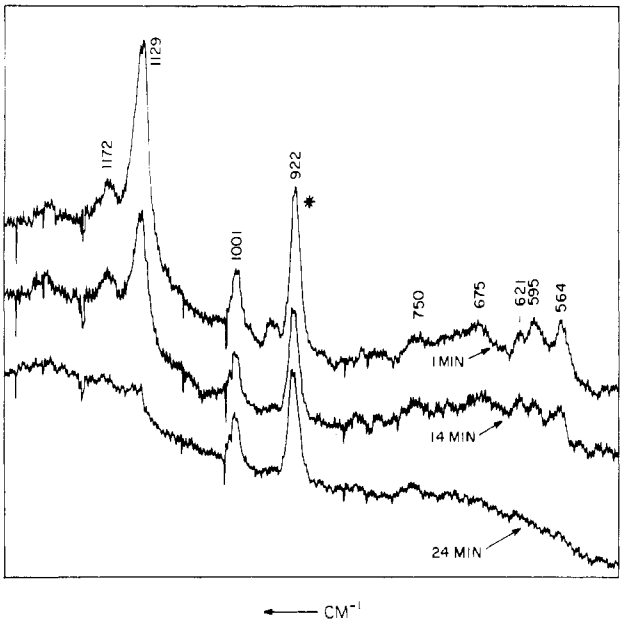


FIGURE 3: Time dependence of the resonance Raman spectrum of a reaction mixture containing *N*-benzoylglycine dithioacylpapain. Times indicated are after mixing. 324-nm excitation, 100 mW, exposure time 4 × 15 s, 13-cm^{-1} spectral slit. CH_3CN feature is denoted by an asterisk.

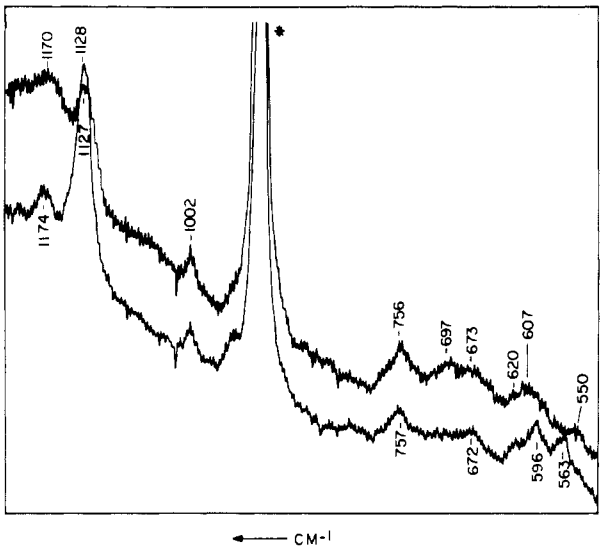


FIGURE 4: Resonance Raman spectra of native (bottom) and denatured (at pH 2.8, top) *N*-benzoylglycine dithioacylpapain. 324-nm excitation, 100 mW, exposure time 2 × 30 s, 13-cm^{-1} spectral slit. CH_3CN feature is denoted by an asterisk; CH_3CN may also contribute intensity at 750 cm^{-1}.

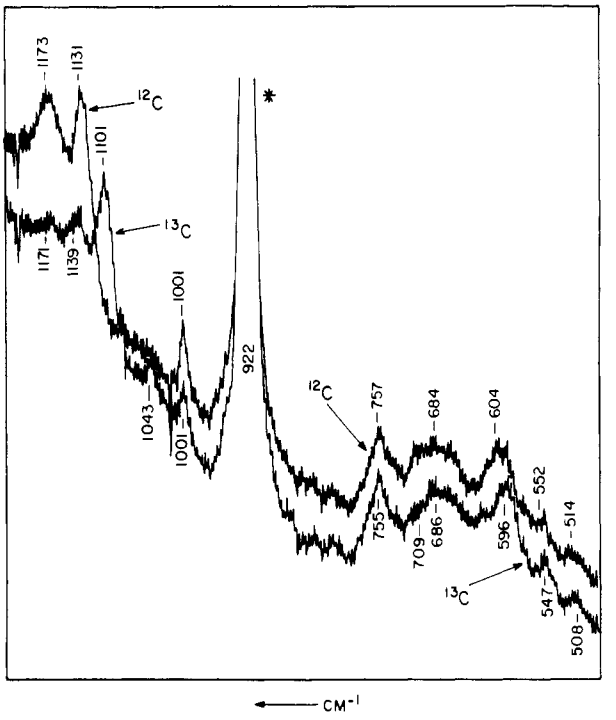


FIGURE 5: Resonance Raman spectra of denatured *N*-benzoylglycine dithioacylpapain, unlabeled and $^{13}C{=}S$ labeled. 324-nm excitation, 100 mW, exposure time 4 × 15 s, 13-cm^{-1} spectral slit. CH_3CN feature is denoted by an asterisk; CH_3CN may also contribute intensity at 750 cm^{-1}.

weak band is seen near 1030 cm^{-1} in some of the spectra in Figure 2, but there is no evidence for a peak near 950 cm^{-1}. However, both intermediates show evidence (Figures 1–3) of unresolved intensity in the 550–750-cm^{-1} region; i.e., the observed peaks in this range are either broad and only partially resolved or are "sitting" on top of one or more additional broad components.

There are several other minor features in the RR spectra that at the present level of signal-to-noise ratio are of little utility. These include the bands near 1240 and 1260 cm^{-1}, which may be due to CH_2 wagging modes (Teixeira-Dias et al., 1982), and the bands between 750 and 900 cm^{-1} (Figures 1–3). At higher levels of sensitivity some of these weak bands may provide conformational information.

The effect on the RR spectrum of taking dithio-*N*-benzoylglycine papain to low pH is seen in Figure 4. Figure 4 compares the RR spectrum of this dithioacyl enzyme at pH 6.0 with the spectrum recorded at pH 2.8 after the addition of 2 N HCl to the acyl enzyme formed near neutral pH. We have previously published RR spectra in the 1050–1200-cm^{-1} range as a function of pH; multiplex detection affords the opportunity of obtaining a complete reliable spectrum under the difficult conditions encountered at low pH. Major spectral changes are observed in the pH 2.8 spectrum. A broad intense band appears near 1170 cm^{-1}, and there is a diminution of the relative intensity of the peak at 1128 cm^{-1}. The changes observed in Figure 4 in the 500–700-cm^{-1} region are less pronounced but have been reproduced repeatedly; upon acidification there is an increase in relative intensity near 697 cm^{-1}, an apparent shift in the 598-cm^{-1} band to 607 cm^{-1}, and a replacement of the 563-cm^{-1} peak by a weaker feature at 550 cm^{-1}. Figure 5 compares the spectra recorded at pH 2.8 of the unlabeled and the ^{13}C-substituted *N*-benzoylglycine papains. The spectra are reproducible to the point of being almost superimposable in the low wavenumber region. However, a slight shift to lower frequency is seen for the peaks at 604 and 552 cm^{-1} in the ^{13}C case. The 1173- and 1131-cm^{-1} peaks (^{12}C) move to 1139 and 1101 cm^{-1} in the ^{13}C-substituted dithioacyl enzyme, and in the latter, a weak peak is revealed at 1171 cm^{-1}. A peak is observed at 1043 cm^{-1} in the derivative (Figure 5), which appears to be weak or absent in the RR spectrum of the unsubstituted compound.

The effect on the RR spectrum of taking $PhCH_2CH_2$-$(=O)NHCH_2{}^{13}C(=S)S$-papain to below pH 3.0 is shown in Figure 6. The results are discussed under Acid Denaturation.

Discussion

Presence of a Relaxed Conformation in the S_1 Active Site and in the P_1–P_2 Linkage. Schechter & Berger (1967) have shown that papain has an active site extending over about 25 Å which, on the acyl binding side, can accommodate up to four

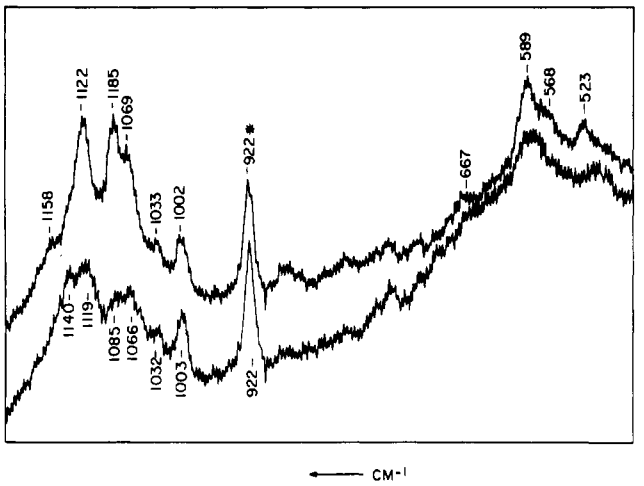


FIGURE 6: Resonance Raman spectra of ^{13}C=S-labeled *N*-(β-phenylpropionyl)glycine dithioacylpapain, native (top) and denatured (at pH 2.6; bottom). 324-nm excitation, 100 mW, exposure time 4 × 15 s, 13-cm^{-1} spectral slit. CH_3CN feature is denoted by an asterisk.

amino acid residues. In the present case the glycine is denoted as the P_1 residue and occupies the S_1 site, while the C(=O)–NH bond in the substrates links the P_1 and P_2 residues.

There is a close parallel between the RR spectra of the isotopically substituted dithioacyl enzymes and those of the B form of their corresponding ethyl dithioesters in solution. For the *N*-benzoylglycine-based derivatives the B conformer indicator, band II (near 1130 cm^{-1}), in the model and enzyme spectra is essentially unchanged by ND or ^{15}N substitution but shows a marked reduction in frequency upon ^{13}C substitution, by 31 and 24 cm^{-1}, in the dithioacyl enzyme and model compound, respectively [Figure 1; Figure 5 in Lee et al. (1983)].

A comparison of the *N*-(β-phenylpropionyl)glycine results for the dithioacylpapain and the ethyl dithioester model compound also provides a strong parallelism in the behavior of the conformer B markers. The intense band at 1138 cm^{-1} in the acyl enzyme and that near 1140 cm^{-1} in the RR spectrum of conformer B in CCl_4 or CH_3CN (Lee et al., 1983) show little change upon ND or ^{15}N substitution but markedly similar perturbations upon ^{13}C substitution. In the ^{13}C-substituted acyl enzyme there are two intense peaks at 1122 and 1085 cm^{-1} with a shoulder at 1070 cm^{-1}; the corresponding peaks for the ethyl dithioester, as conformer B, appear at 1115, 1079, and 1068 cm^{-1} [Figure 7 (Lee et al., 1983)]. Two alternative explanations were given for the appearance of the latter bands in the ^{13}C model case (Lee et al., 1983). In one instance the 1115- and 1079-cm^{-1} bands were seen as a split band II; in the other explanation the 1079- and 1068-cm^{-1} features were seen as originating from band III. In either case the splitting could be caused by a Fermi interaction with an overtone which has a fundamental frequency in the 530–550-cm^{-1} region. Whatever the explanation there is remarkable correspondence between the ^{13}C-substituted dithioacyl enzyme and model RR spectra. Since the RR signature is very sensitive to changes in the torsional angles, this demonstrates that the torsional angles in the glycinic NC–C–C(=S) bonds must be very similar in both situations. Moreover, the similarity indicates that the unusual pattern of bands seen for the ^{13}C-substituted dithioacyl enzyme originates within the glycine moiety rather than as a result of an enzyme–substrate interaction.

Band III, the conformer B mode occurring at 1094 cm^{-1} in the unsubstituted dithioacylpapain (Figure 2), also shows a very similar ND and ^{15}N dependence in the model and enzyme cases. For both dithioester and dithioacyl enzyme, upon ND substitution, band III moves slightly to higher frequency and loses intensity, while upon ^{15}N substitution, band III moves slightly to lower frequency.

The other well-established conformer B marker occurs near 595 cm^{-1} for both dithioacyl enzymes. The 595-cm^{-1} band is insensitive to isotopic substitution in both the dithioacyl enzymes and the corresponding models. This situation is akin to $CH_3C(=S)SCH_3$ and $CH_3C(=S)SC_2H_5$ where the ^{13}C=S insensitive 580-cm^{-1} RR feature is due to a mixture of $\nu_{C=S}$, ν_{C-S}, and ν_{C-C} in the C–C(=S)–S fragment. However, we believe it would be premature at this time to assign the 595-cm^{-1} dithioacyl enzyme peak to a similar normal mode.

For the *N*-(β-phenylpropionyl)glycine analogue, the weak dithioacyl enzyme peak near 525 cm^{-1} is not easily discernible in the RR spectra of the ester model compound [Figure 9 (Lee et al., 1983)]; it may, however, correspond to the band seen in the normal Raman spectrum of a single crystal of the ethyl dithioester at 532 cm^{-1} (unpublished work, this laboratory). Thus, it may be tentatively assigned to a conformer B feature of unknown normal mode origin. The weak peak seen for the *N*-benzoylglycine enzyme near 1170 cm^{-1} in Figure 1 and the shoulder near 1158 cm^{-1} for the *N*-(β-phenylpropionyl)glycine enzyme are both sensitive to ND, ^{15}N, and ^{13}C substitution. This demonstrates unequivocally that these features are *not* due to a band I like feature from a non-B conformer, since band I is insensitive to ND and ^{15}N substitution. Rather they are assigned to a mode or modes which are highly delocalized and contain contributions from the motions of the NH hydrogen and nitrogen and the C=S carbon atoms. For the *N*-benzoylglycine enzyme intermediate the 1170-cm^{-1} peak may be related to the 1165-cm^{-1} peak seen in the normal Raman spectrum of a single crystal of *N*-benzoylglycine ethyl dithioester [Figure 3 (Lee et al., 1983)]. The Raman spectra of the ^{15}N and ^{13}C=S forms of this compound as a single crystal, where it is in a B type conformation, are also seen in Figure 5 of the preceding paper (Lee et al., 1983). The pattern of peaks in the 1100–1170-cm^{-1} region for the isotopically substituted compounds is seen to correspond closely to the patterns seen in Figure 1 for the analogous dithioacyl enzymes. This suggests strongly that the 1170-cm^{-1} feature seen in the dithioacyl enzyme spectrum is a property of a B type conformation.

The results of the isotopic replacements clearly demonstrate that all major resolved features in the RR spectra may be assigned to the acyl group in a B type conformation about the NH–CH_2–C(=S) linkages. There is no evidence for a minor non-B population, although we cannot discount the possibility that such a population exists and may be found as spectral resolution and sensitivity improve. When the peak positions and isotopic shifts of the *N*-acylglycine ethyl dithioesters are taken as standards of relaxed conformers, it is apparent from the close parallel with the dithioacylpapain results that conformer B in the active site is essentially in a relaxed state. That is, there is no major geometric destabilization involving rotations in the glycinic linkages, and in the terminology of Schechter & Berger (1967) there is no distortion in the S_1 subsite, at least when it is occupied by glycine. We say that the glycine conformation is essentially relaxed, because for the *N*-benzoylglycine case there is evidence for a very modest distortion. This evidence rests on the shifts seen in band II for ^{13}C substitution. As seen in Table I there is a tendency of band II to increase in frequency upon going from the ester in a single crystal to the ester in solution and to increase further in going to the dithioacyl enzyme, from 1120 to 1124 and to

Table I: Band II Positions and ^{13}C Shifts for *N*-Benzoylglycine Derivatives

	position	$\Delta\ ^{13}C$ (cm^{-1})
ethyl ester, Raman, single crystal	1120	17
ethyl ester, in H_2O, RR	1124	24
ethyl ester, in CH_3CN, FTIR	1123	24
dithioacylpapain, pH 6.0, RR	1129	31

1129 cm^{-1}, respectively. At the same time, the shift in band II upon ^{13}C substitution increases from 17 to 24 and to 31 cm^{-1}, respectively. Band II probably contains major contributions of C=S and C–C(=S) stretching coupled to other motions of the glycine dithioester skeleton. It appears that as band II increases in frequency [and becomes more like a "normal" $\nu_{C=S}$ (Teixeira-Dias et al., 1982; Storer et al., 1982)] it becomes more localized in the C–C(=S)–S fragment. When the solution values are taken as a standard, the conformation in the single crystal appears to be slightly distorted such that the normal mode giving rise to band II becomes more delocalized, while in the enzyme a different minor perturbation has the opposite effect, making the mode appear more localized. We cannot presently quantitate the changes in torsional angles, which bring about the observed differences in band positions and isotopic shifts. Such information should become available when more *N*-acylglycine ethyl dithioesters have been analyzed by X-ray crystallography. The complex ^{13}C isotopic effects for the *N*-(β-phenylpropionyl)glycine derivatives prevent us from applying a similar analysis to these compounds.

The presence of features in the dithioacylpapain RR spectra which are sensitive to ^{15}N and ND substitution provides a probe of conformation in the C(=O)–NH peptide bond linking the P_1 and P_2 residues. The clearest example involves band III of the *N*-(β-phenylpropionyl)glycine derivatives. Band III shows the same response to ^{15}N and ND substitution in both the dithioacyl enzymes and in the corresponding ethyl dithioester in solution. This strongly suggests that the normal mode pattern involving the NH residue is similar in both environments and, therefore, that the conformation about the NH group is the same in both cases. Thus, we have no evidence for any distortion in the C(=O)–NH peptide bond in the papain-bound *N*-(β-phenylpropionyl)glycine substrate. This is contrary to the suggestions of Makinen and co-workers (Kuo et al., 1983) and Bernhard and co-workers (Bernhard & Lau, 1971), who conclude that distortion does occur in this region of peptide substrates when they are bound to carboxypeptidase and chymotrypsin, respectively.

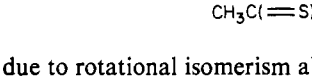
Discrepancies between Dithioacyl Enzyme and Model RR Spectra. It is apparent from the foregoing section that there is a close correspondence between most of the major features in the RR spectra of the dithioacyl enzymes and the ethyl dithioester models in their B conformation. However, there are several discrepancies, and these are of sufficient importance to warrant discussion. First, there is a moderately intense feature in the RR spectra of the model esters, which appears to have no counterpart in the dithioacyl enzyme spectra. This is the band seen near 1040 cm^{-1} in Figures 7–10 of Lee et al. (1983); in the models it is insensitive to ND and ^{15}N substitution, and it moves slightly to lower frequency and increases in relative intensity in the ^{13}C=S analogues.

The second discrepancy concerns the broad band seen in all acyl enzyme RR spectra near 670 cm^{-1} (Figures 1 and 2). This mode has hitherto been undetected in the dithioacyl enzyme spectra and has no counterpart in the model compounds. It is tentatively assigned to a mode associated with the S–C stretching motion in the cysteine moiety. In simple dithioesters the S–C mode is a fairly pure vibration occurring near 730 and 690 cm^{-1} in $CH_3C(=S)SCH_3$, and $CH_3C(=S)SC_2H_5$, respectively (Teixeira-Dias et al., 1982), and it occurs at the same positions for the *N*-acylglycine analogues $RC(=O)NHCH_2C(=S)SCH_3$ and $RC(=O)NHCH_2C(=S)SC_2H_5$ (Lee et al., 1983). Thus, we assign the 670-cm^{-1} enzyme feature to ν_{S-C} with the frequency decreasing along the series S–CH_3 (730 cm^{-1}), S–C_2H_5 (690 cm^{-1}), and S–C–C–papain (670 cm^{-1}). It is unlikely that this mode is very sensitive to changes in the glycinic N–C–C(=S) bonds. The absence of a conformer A band near 1160 cm^{-1} eliminates the possibility that the 670 cm^{-1} feature is due to conformer A.

The third and fourth discrepancies relate to dithio-*N*-benzoylglycine papain. This species has RR features at 953 and 562 cm^{-1} (Figure 1) that are insensitive to ^{15}N, ND, and ^{13}C=S substitution and that are not observed in the RR spectra of the *N*-benzoylglycine ethyl dithioester model in solution (Lee et al., 1983). Features are observed at 962 and 538 cm^{-1} in the normal Raman spectrum of the model [Figure 3 (Lee et al., 1983)] as a single crystal, but clearly, if these are related to the bands seen in the RR spectrum of the dithioacylpapain, we have to account for substantial frequency and intensity changes.

Since the 670-cm^{-1} dithioacyl enzyme peak is assigned, we have to account at the present level of spectral reproducibility for three discrepancies between the model and dithioacyl enzyme spectra: (1) the nonappearance of a conformer B mode near 1040 cm^{-1} in both the dithioacyl enzyme spectra and, (2 and 3) for *N*-benzoylglycine papain, the appearance of new bands at 953 and 562 cm^{-1}. Some clues as to the origin of the discrepancies are found in the data for the RR spectra of the acid-denatured dithioacyl enzymes discussed in the next section.

Acid Denaturation. The RR spectra of dithio-*N*-benzoylglycine papain at pH 6.0 and pH 2.8 are compared in Figure 4. The latter sample was prepared by adding 2 N HCl to the dithioacyl enzyme formed near neutral pH. Below pH 3.0 the dithioacyl enzyme rapidly and irreversibly denatures, and deacylation does not occur (unpublished work, this laboratory). The drop in relative intensity of the 1128-cm^{-1} peak and the concomitant increase in the intensity of the 1170-cm^{-1} band is readily explained in terms of denaturation, leading to a decrease in the population of acyl groups in a B type conformation. The broad nature of the 1170-cm^{-1} feature, in the spectrum at pH 2.8, suggests that the non-B population is not a single well-defined population but is heterogeneous with differing torsional angles about the NH–C–C(=S) bonds reflecting the heterogeneous protein environments encountered in the denatured acyl enzyme. The increase in intensity in the 670–690-cm^{-1} region in the pH 2.8 RR spectrum is also consistent with the increase in population of non-B conformations such as conformer A (Lee et al., 1983). The change in position of the 596-cm^{-1} peak upon acid denaturation is more difficult to explain on the basis of the formation of non-B conformers. The diminution of the B type population should lead to a decrease in intensity of the 596-cm^{-1} band and an increase in intensity in the 680-cm^{-1} region. A shift to higher frequency by 10 cm^{-1} is more likely to be a consequence of a change in conformer population about the –S–C–C *cysteine* linkages, since, for example, shifts of this magnitude are seen in the 590-cm^{-1} peak of



due to rotational isomerism about the bond indicated (Ozaki

et al., 1982b). The low pH results also show clearly that the 953- and 562-cm^{-1} peaks are not a property of B- or A-like conformers in the denatured acyl enzyme. Upon denaturation the 562-cm^{-1} feature disappears and is possibly replaced by a weaker peak near 550 cm^{-1} (Figures 4 and 5), and the 953-cm^{-1} peak is seen to diminish greatly in intensity or to be absent altogether. These findings taken together suggest a complete spectral interpretation cannot be achieved in terms of a description of the dihedral angles of the NH–C–C(=S) bonds of the bound acyl group but that, in addition, consideration must be given to the conformational properties of the cysteine heavy atom framework, –S–C–C. The failure to detect the 562-cm^{-1} band in any model compound and the present demonstration that it is associated solely with the *native* acyl enzyme show that the conformation in the –S–C–C linkages is not that found in an equilibrium state in the absence of the intact protein matrix.

Further support for a special conformation in the cysteine bonds comes from consideration of the ^{13}C data for the denatured *N*-benzoylglycine enzyme. The RR spectra of ^{13}C-substituted and unlabeled dithio-*N*-benzoylglycine papain at pH 2.8 are compared in Figure 5. The substantial shifts in the 1173- and 1131-cm^{-1} peaks upon ^{13}C substitution confirm the assignment of these features to band I and band II type modes. The residual peak in the ^{13}C RR spectrum at 1171 cm^{-1} is probably related to the feature seen at this position in the spectrum of the native acyl enzyme and discussed above. Apart from the very small shifts in the 600-cm^{-1} region, the 500–700-cm^{-1} range is insensitive to isotopic substitution. However, a difference in the ^{12}C and ^{13}C RR spectra occurs near 1040 cm^{-1} where a peak is present for the ^{13}C species but is absent or very weak in the spectrum of the ^{12}C analogue. This observation may be related to two factors: one is that a medium intensity peak is observed near 1040 cm^{-1} in the RR spectra of the ethyl dithioesters of *N*-acylglycine derivatives but not, normally, in the corresponding dithioacylpapains; the other factor is that in the model compounds the 1040-cm^{-1} mode is associated with the –S–C–C linkages of the B conformer (it has been tentatively assigned to ν_{C-C} coupled to a degree to other motions) and increases in relative intensity upon ^{13}C substitution (Lee et al., 1983). Taking these points into consideration, we suggest that the presence of a mode near 1040 cm^{-1} in the RR spectrum is contingent upon a B type conformation in the glycine bonds and a certain, presently unknown configuration in the –S–C–C bonds. The latter conformation is present in the "relaxed" conformation of *N*-acylglycine ethyl dithioesters in solution but absent in the native dithioacyl enzymes which again points to a "special" conformation in the cysteine bonds. Upon denaturation the cysteine bonds assume a more relaxed conformation (although still in contact with a heterogeneous environment associated with the denatured protein), and we expect to see an increase in intensity near 1040 cm^{-1}. This is barely apparent for the ^{12}C derivatives but is clearly seen in the ^{13}C-substituted denatured acyl enzyme, in keeping with the observation of increased relative intensity of the 1040-cm^{-1} peak for the ^{13}C model compounds.

The results for the acid denaturation of $PhCH_2CH_2$-$C(=O)NHCH_2{}^{13}C(=S)S$-papain are shown in Figure 6. Upon denaturation the spectrum in the 1050–1150-cm^{-1} region resembles that for the corresponding ethyl dithioester in CH_3CN/H_2O [Figure 7 (Lee et al., 1983)]. In the enzyme case, however, the peaks are broader, indicating that although two main classes of conformer are present, viz., A and B, there are conformational variations within each class. This variation is probably caused by the heterogeneous nature of the protein environment around the *N*-acylglycine moiety after denaturation. The broad nature of the peaks also prevents us from making any statement about a possible increase in the intensity of the 1033-cm^{-1} peak in the denatured material. It is apparent, however, that the peak near 1035 cm^{-1} is less intense than the corresponding features in the ^{13}C-substituted *N*-(β-phenylpropionyl)- or *N*-benzoylglycine models. Unlike the *N*-benzoylglycine case, there is no evidence for a shift in the peak at 589 cm^{-1} after denaturation. No reliance is put on the apparent shift of the weak 523-cm^{-1} band seen in Figure 6; in general the broad peaks between 500 and 700 cm^{-1} in the spectrum of the denatured species prevent accurate comparison.

Distortion in the Cysteine Linkages. The findings of the preceding sections are that the acyl group appears to bind to the enzyme in a relaxed conformation about its C(=O)–$NH–CH_2–C(=S)$ bonds. However, for the *N*-benzoyl analogue, there is evidence for a nonrelaxed conformation about the cysteine C–S–C–C bonds. Moreover, from the denaturation experiments it appears that the nonrelaxed state depends, in large part, on an intact active site. Before this point is discussed further, it should be noted that no evidence has been found for perturbations to the RR spectrum by effects in the active site other than changes in the torsional angles near the dithioester moiety. The dithioester spectrum is insensitive to H bonding (Teixeira-Dias et al., 1982; Storer et al., 1982) and appears to be insensitive to electrostatic effects due to protein charges or dipoles (Huber et al., 1982; Ozaki et al., 1982a). Therefore, we interpret any differences between the RR spectrum of the dithioacylpapain and the conformer B spectrum of the corresponding dithioester model in its relaxed state in solution as a measure of the strain imposed by the enzyme.

The evidence for distortion in the cysteine C–S–C–C linkages is firm for the *N*-benzoylglycine dithioacylpapain but ambiguous for the *N*-(β-phenypropionyl)glycine analogue. For the former there are three spectral differences between enzyme intermediate and the model compound, viz., the nonappearance of the 1040-cm^{-1} feature in the enzyme and the appearance of the "new" features at 953 and 562 cm^{-1} in the native dithioacyl enzyme. On the basis of extensive studies on *N*-acylglycine model compounds, on the ^{13}C and ^{15}N substitution results, and on acid denaturation results, all three anomalies can be associated with the cysteine bonds. Moreover, the results for the denatured acyl enzyme—the disappearance of the 953- and 562-cm^{-1} bands, the shift of the 595-cm^{-1} peak, and the appearance of the weak 1040-cm^{-1} peak in the ^{13}C-labeled species—suggest that, for this native dithioacylpapain, the cysteine linkages are in a nonrelaxed state. However, for the *N*-(β-phenylpropionyl)glycine case the one difference between enzyme and model spectra is the lack of a medium intensity 1040-cm^{-1} band in the enzyme RR spectrum. Since we associate this band with ν_{C-C} from $-SC_2H_5$ in the models, the nonappearance of the 1040-cm^{-1} band may be due to a nonrelaxed conformation in the cysteine bonds. However, since it does not increase markedly upon denaturation, and since no shifts were detected in the 600-cm^{-1} region, it is equally plausible that the lack of the 1040-cm^{-1} band simply represents the different chemistry of the cysteine S–C–C bonds in the enzyme compared to the ethyl S–C–C bonds in the model compounds. Cysteine-based dithioesters of *N*-acylglycine are required to test this concept and these are under consideration. Interestingly, as far as specificity is concerned the *N*-(β-phenylpropionyl)glycine compound quite closely resembles

phenylalanylglycine for which the enzyme has a high degree of specificity (Lowe & Yuthavong, 1971). This resemblance diminishes upon going to the *N*-benzoylglycine substrate, and thus, as substrate specificity decreases, there is increasing evidence for distortion which appears to be expressed mainly in the conformation of the cysteine S–C–C bonds.

The appearance of the 953- and 562-cm^{-1} peaks in the native intermediate and the shift of the 595-cm^{-1} band upon denaturation for the dithio-*N*-benzoylglycine papain may mean that there are quite large distortions of the C–S–C–C bonds in the active site. The ethyl esters $RC(=S)SC_2H_5$ in solution almost certainly take up an *S*-cis conformation (Teixeira-Dias et al., 1982) and the possibility arises for the dithio-*N*-benzoylglycine papain that, as a result of the lack of substrate and enzyme complementarity, the conformation is *S*-trans or nonplanar in the C–S–C linkages. Additionally, heterogeneity cannot be ruled out at this stage, with *S*-cis and some non *S*-cis being in equilibrium.

In summary, we have shown how it is possible to use the RR spectrum to dissect the substrate in the active site and to probe for conformational distortion. Thus, by reference to certain standard states (usually the conformations of the corresponding dithioesters in solution), we can discuss the conformation in the $NH–CH_2–C(=S)$ bonds of the P_1 residue, the conformation in the peptide group linking the P_1 and P_2 residues, and the conformation in the S–C–C bonds of cysteine-25. For the bound *N*-benzoylglycine and *N*-(β-phenylpropionyl)glycine substrates there is no evidence for distortion in the $–C(=O)NH$ bonds linking the P_1 and P_2 residues. Similarly, there is no evidence for distortion in the $NH–CH_2C(=S)$ torsional angles for the *N*-(β-phenylpropionyl) derivative, and these angles are essentially relaxed in the *N*-benzoyl case, too. However, for the latter there is evidence for a minor perturbation, in the active site, from the relaxed conformer B state in solution. Minor discrepancies between dithioacylpapain and corresponding ethyl dithioester RR spectra are explained in terms of the conformation in cysteine-25's S–C–C bonds. Acid denaturation experiments show that most of the spectral discrepancies are a property of the intact active site and lead to the hypothesis that the torsional angles about these cysteine bonds are not those of a relaxed, unperturbed state. However, the major conclusion of this work is that the substrates are not undergoing distortions which, by being relieved in the transition state would result in rate acceleration. The enzyme is using the available, minimally perturbed, conformational space, and any distortions are minor. This finding appears to be general for *N*-acylglycine-based substrates, but it should be remembered that papain has an extended active site. It is an area of immediate concern to see if the same conclusion holds for substrates extending into the S_2, S_3, ... sites and, additionally, for substrates which do not have glycine at the S_1 position.

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