Conformational Variability in an Enzyme's Active Site: Resonance Raman Evidence for Different Acyl Group Conformations in *N*-Acylglycine and *N*-Acylalanine Dithioacyl Papains†

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ABSTRACT: It is demonstrated that the vibrational modes associated with the catalytically labile region of *N*-acylalanine dithioacyl papains undergo a major reorganization compared to the normal modes of corresponding model compounds. Thus, the resonance Raman (RR) spectrum of, e.g., *N*-benzoylalanine dithioacyl papain and its response to isotopic labeling cannot be understood completely on the basis of the RR spectrum of *N*-benzoylalanine ethyl dithio ester in one of its known conformational states [detailed in Lee, H., Angus, R. H., Storer, A. C., Varughese, K. I., & Carey, P. R. (1988) *Biochemistry* (preceding paper in this issue)]. This situation contrasts sharply to that for *N*-acylglycine dithioacyl papains whose RR spectra closely resemble those of the corresponding *N*-acylglycine ethyl dithio esters in a conformational state known as conformer B. For the *N*-acylalanine intermediates two possible causes are put forward to explain the rearrangement of the normal modes. First, the acyl groups based on alanine may bind in papain's active site in a conformation whose torsional angles near the $-C(=S)S-$ group differ markedly from those of characterized model compounds. The second, and presently favored, explanation is that the *N*-acylalanine moiety is binding in the active site in an A- or C_5-like conformation and that, in addition, there is significant vibrational coupling between some of the normal modes of the bound substrate and the normal modes associated with parts of the enzyme in contact with the substrate. The finding that deacylation for *N*-acylglycine or *N*-acylalanine dithioacyl papains must proceed from structures which are different is an indication that the mechanism of deacylation may not have strict stereochemical requirements. The intermediates discussed are *N*-benzoyl-L-alanine, *N*-(β-phenylpropionyl)-L-alanine, and *N*-(methyloxycarbonyl)-L-phenylalanyl-L-alanine dithioacyl papains and their $NHCH(CD_3)C(=S)-$ and $NHCH(CH_3)^{13}C(=S)$-substituted analogues.

By using resonance Raman (RR) spectroscopy it has been possible to define quite precisely the conformation about the bonds linking substrate to enzyme in a large number of *N*-acylglycine (dithioacyl) papains, $RC(=O)NHCH_2C(=S)S$—papain (Carey & Storer, 1984, 1985). In each case, whether the R acyl group is an organic functional group or an additional amino acid residue(s) (Angus et al., 1986), the acylglycine moiety takes up a single conformation about the $NH-CH_2-CS$ bonds known as conformer B, where the $NHCH_2-CS$ (thiol) torsional angle is ≈20° and the amide N and thiol S atoms are in close contact. We now report that *N*-acylalanine (dithioacyl) papains, $RC(=O)NHCH(CH_3)C(=S)S$—papain, take up a markedly different conformation about their $-NHCH(CH_3)—CS$ bonds.

It is necessary to introduce two pieces of information to facilitate the discussion of the *N*-acylalanine papain data. First, constant reference has to be made to the spectroscopic and structural data for model compounds consisting of *N*-acylalanine ethyl dithio esters. The model compound results are discussed in the preceding and accompanying paper (Lee et al., 1988), and that publication also defines the conformers designated A, B, and C_5. The RR signatures for these conformers, including $^{13}C=S$- and CD_3-substituted forms, are summarized in Figure 1. The second item requiring introduction concerns the position of known conformers of *N*-acylglycine and *N*-acylalanine ethyl dithio esters in a Ramachandran-like plot. By analogy to the Ramachandran angles the CNH–CHXCS (where X = H or CH_3) and NHCHX–CS(thiol) torsional angles are designated ϕ' and ψ', respectively. The ϕ',ψ' regions for the A, B, and C_5 conformers are shown in Figure 2. The RR data for the *N*-acylalanine (dithioacyl) papains allow us to discuss the placement of the acyl group conformation(s) in regions of the Ramachandran map.

Experimental Procedures

Materials

Papain was prepared, activated, and assayed as described previously (Carey et al., 1984).

Methyl Thiono Ester Preparation. Methyl thiono esters were made from their respective nitriles as previously described (Ozaki et al., 1982; Carey et al., 1984) with the following modifications. *N*-Benzoyl-DL-alanine and *N*-(β-phenylpropionyl)-DL-alanine methyl thiono esters were purified on silica gel with ether:CH_3CN (9:1) as eluant, while *N*-(methyloxycarbonyl)-L-phenylalanyl-DL-alanine methyl thiono ester was purified as described in Carey et al. (1984).

The *N*-benzoyl- and *N*-(β-z-phenylpropionyl)-DL-alanine nitriles were synthesized as previously described (Angus et al., 1985). *N*-(Methyloxycarbonyl)-L-phenylalanyl-DL-alanine nitrile was synthesized as previously described (Varughese et al., 1986).

Methods

Resonance Raman spectra were measured on a Spex Triplemate equipped with a Tracor Northern DARSS (diode

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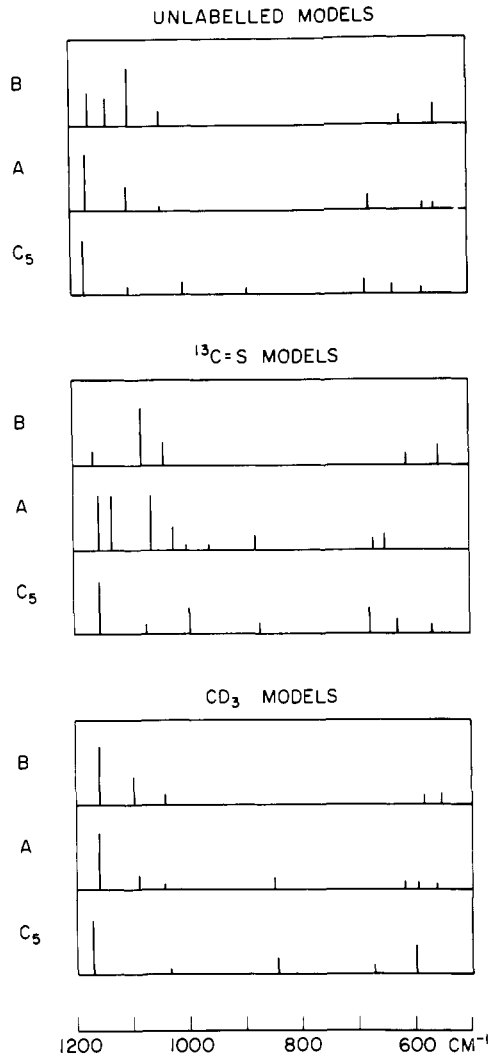

FIGURE 1: Schematic representation of the RR spectra of alanine dithio esters and their ^{13}C=S- and $CH(CD_3)C$=S-substituted analogues in conformational states A, B, and C_5. The data summarize the results of the preceding and accompanying publication (Lee et al., 1987).

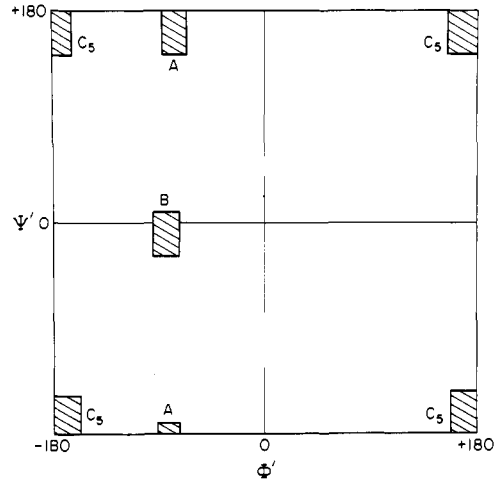

FIGURE 2: Position of A, B, and C_5 conformers in a Ramachandran-like ϕ',ψ' conformational space.

array rapid scan spectrometer system). The spectrograph stage of the Triplemate employs 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 3000K krypton ion laser was used as an excitation source. The apparatus is described

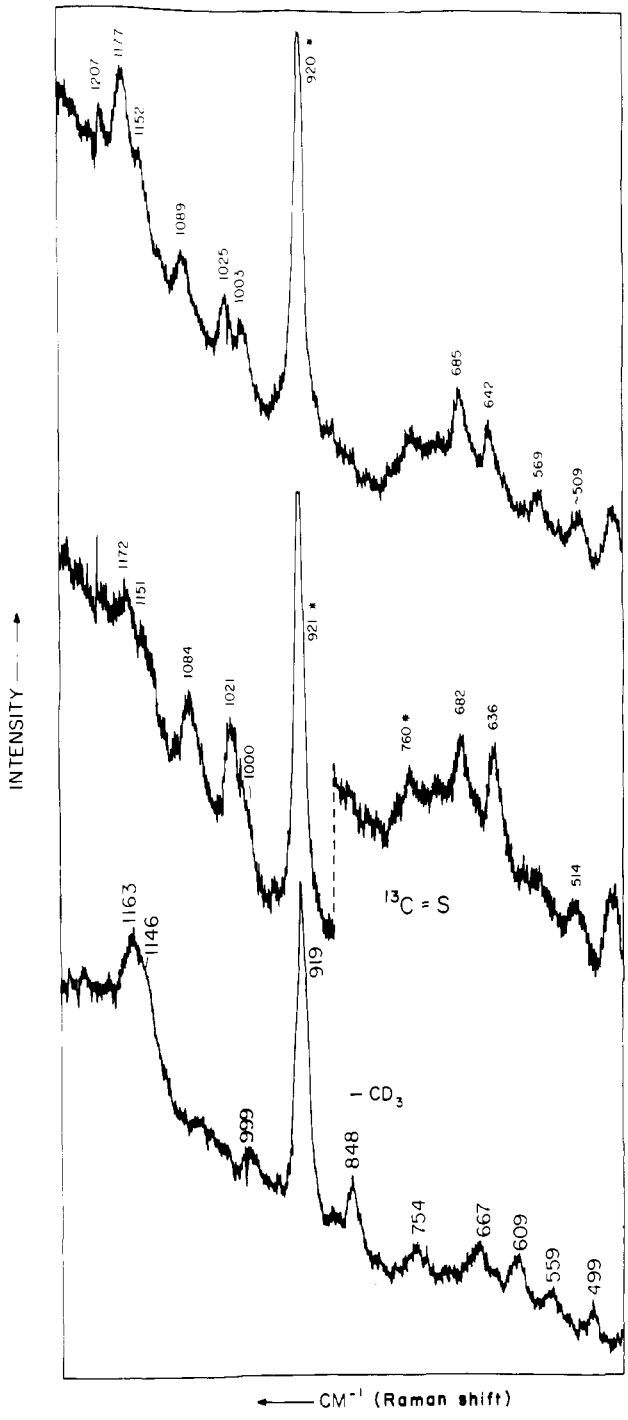

FIGURE 3: 324-nm excited RR spectra of *N*-benzoyl-L-alanine dithioacyl papain and its ^{13}C=S- (middle) or $NHCH(CD_3)C$=S- (bottom) substituted analogues.

in detail elsewhere (Carey & Sans Cartier, 1983). Raman frequencies were calibrated by using two emission lines from an argon lamp that correspond to Raman shifts of 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}. Sequential spectra reproducibility is better than ±1 cm^{-1}.

The RR sample cell for the reaction mixture consisted of a quartz cuvette (0.5-cm path length), stirred by a magnetic pip during measurement to avoid photodegradation. In a typical reaction mixture 60 μL of 25 mM substrate (in 50% CH_3CN/H_2O) was added to 240 μL of enzyme [130 μM, pH 6.0, 1 mM ethylenediaminetetraacetic acid (EDTA)]. Data collection commenced a few seconds after mixing. Total data acquisition time was 20–60 s per spectrum.

RESULTS

N-Benzoyl-L-alanine (Dithioacyl) Papain. Figure 3 compares the RR spectra of unlabeled and $^{13}C{=}S$- and CD_3-labeled *N*-benzoylalanine dithioacyl papain. The poor spectral quality is due to the low percentage of acylation (<40%) for the substrate in the active site and the presence of a weak luminescence background from an unidentified source. The determination of percentage of acylation is described in the following and accompanying publication (Storer et al., 1988). That publication also concludes that only the L form of alanine thiono ester substrates form dithioacyl papains. The unlabeled *N*-benzoylalanine intermediate is characterized by an intense peak at 1177 cm^{-1}, a series of bands of medium intensity at 1089, 1025, 685, and 642 cm^{-1}, weaker features at 569 and 509 cm^{-1}, and an unresolved shoulder at 1152 cm^{-1}. The peaks near 920 and 755 cm^{-1} are due to CH_3CN, used to carry the substrate into solution. The features near 1207 and 1003 cm^{-1} are due predominantly to excess substrate and product and have little contribution from the acyl enzyme. Substitution by $^{13}C{=}S$ in the acyl enzyme leads to only modest shifts in peak positions, of the order of 5 cm^{-1}, but a major reorganization of peak intensities. The results of CD_3 substitution at the α carbon, for the dithioacyl papain, are also seen in Figure 3. The RR spectrum of this form is characterized by a broad intense band at 1163 cm^{-1} and a series of weaker features at 848, 667, 609, 559, and 499 cm^{-1}. The weak band near 1000 cm^{-1} is due in large part to a phenyl ring mode from the relatively high concentration of substrate present in the reaction mixture.

N-(β-Phenylpropionyl)-L-alanine (Dithioacyl) Papain. Figure 4 compares the RR spectra of unlabeled and $^{13}C{=}S$- and CD_3-substituted *N*-(β-phenylpropionyl)alanine (dithioacyl) papain. The isotopically unsubstituted intermediate gives rise to intense features at 1182 and 1103 cm^{-1} and moderately intense bands at 671 and 538 cm^{-1}. Less intense peaks appear at 1031, and 645 cm^{-1}, and as for the *N*-benzoyl analogue a shoulder appears at 1152 cm^{-1}. The feature at 1003 cm^{-1} is due predominantly to excess substrate and product, and the bands at 920 and 757 cm^{-1} are from CH_3CN. Again, as in the case of the *N*-benzoylalanine intermediate, marked reorganization of band intensities occurs in the 1000–1200-cm^{-1} region upon $^{13}C{=}S$ substitution. Small downward shifts appear to occur for the 1182- and 1031-cm^{-1} bands in the $^{13}C{=}S$ compound, to 1173 and 1024 cm^{-1}, respectively. The 1103-cm^{-1} peak is replaced by an unresolved doublet at 1083 and 1072 cm^{-1}. In addition, the peak at 645 cm^{-1} shifts to 632 cm^{-1} and increases in relative intensity in the $^{13}C{=}S$ analogue. The RR spectrum of the acyl enzyme with CD_3 replacing $-CH_3$ on alanine's α carbon quite closely resembles the RR spectrum of the corresponding *N*-benzoylalanine intermediate, with a broad intense peak near 1171 cm^{-1} and weaker features at 840, 662, 603, and 529 cm^{-1}. However, in addition, for the *N*-(β-phenylpropionyl)alanine CD_3 intermediate, a weak peak is detected at 1095 cm^{-1}, which is not seen for the CD_3 *N*-benzoylalanine case.

N-(Methyloxycarbonyl)-DL-phenylalanyl-L-alanine Dithoacyl Papain. Under the conditions of the RR experiments, this substrate acylates papain to essentially 100%, in contrast to the approximate value of 40–45% for the two foregoing substrates. Thus, it is usually possible to obtain better quality data from PheAla papain reaction intermediates. The RR spectrum of the unlabeled PheAla dithioacyl papain is shown in Figure 5; it bears a striking resemblance to the RR spectrum of the *N*-(β-phenylpropionyl)alanine intermediate seen in Figure 4. The PheAla papain RR spectrum has intense features at 1176 and 1100 cm^{-1} and less intense bands at 1034, 669, 640, 594, and 529 cm^{-1}. The results of $^{13}C{=}S$ substitution (Figure 5) also bear some resemblance to the *N*-(β-phenylpropionyl)alanine case. Upon $^{13}C{=}S$ substitution the peak at 1100 cm^{-1} ($^{12}C{=}S$) appears to shift to 1067 cm^{-1} (Figure 5) and increases in relative intensity while the 1176 cm^{-1} feature ($^{12}C{=}S$) decreases in intensity and shows a modest shift to 1165 cm^{-1}. The $NHCHCD_3C(=S)$ analogue was also made for the PheAla intermediate. The results for the RR spectra are shown in Figure 5. The CD_3 intermediate has an intense mode at 1165 cm^{-1} and other major peaks of lesser intensity at 1078, 841, 659, 601, and 512 cm^{-1}.

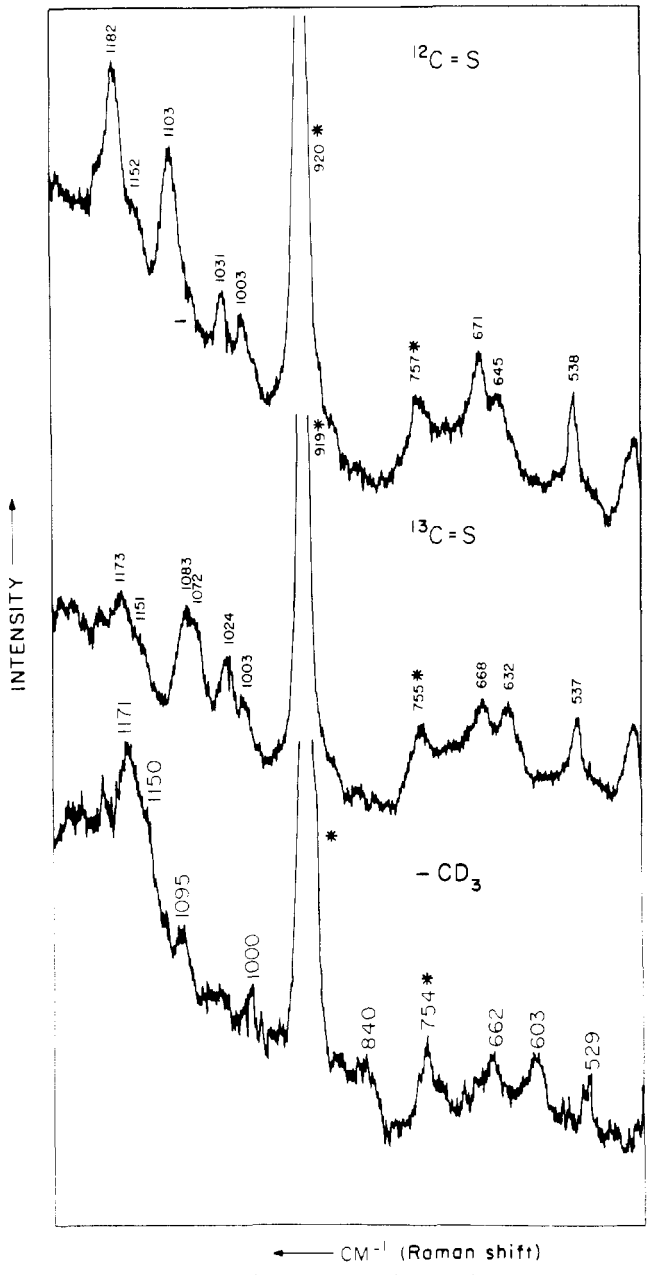

FIGURE 4: 324-nm excited RR spectra of *N*-(β-phenylpropionyl)-L-alanine dithioacyl papain and its $^{13}C{=}S$- (middle) or $NHCH(CD_3)C{=}S$- (bottom) substituted analogues.

Comparison with Model Compounds. The RR spectra of *N*-acylglycine dithioacyl papains could be interpreted in detail by reference to their corresponding model compounds, namely, *N*-acylglycine ethyl dithio esters. Joint Raman and X-ray crystallographic studies on the model glycine dithio esters were used to set up a library of spectra–structure correlations, and this library served as a basis for RR spectral interpretation

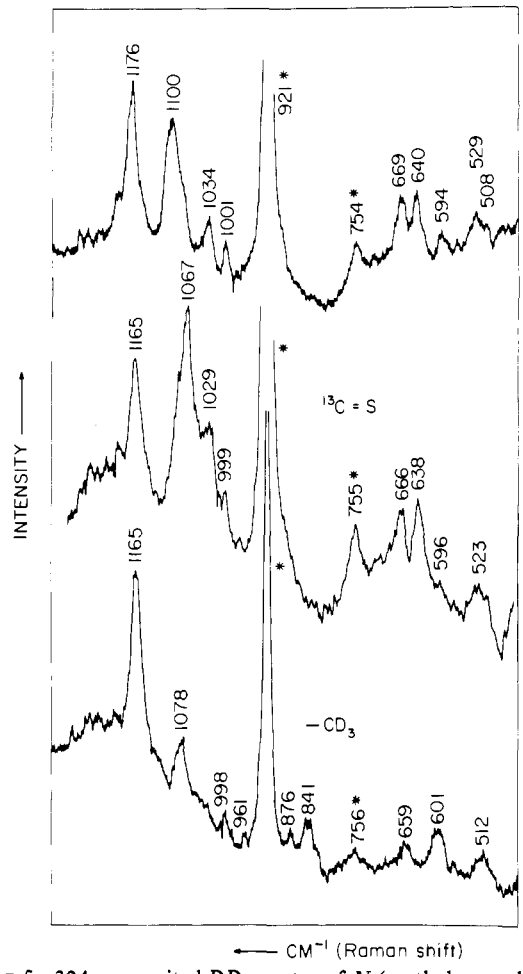

FIGURE 5: 324-nm excited RR spectra of *N*-(methyloxycarbonyl)-L-phenylalanyl-L-alanine dithioacyl papain and its $^{13}C{=}S$- or $NHCH(CD_3)C{=}S$-substituted analogues.

(Huber et al., 1982; Varughese et al., 1984). A close similarity was found for the spectra of B conformers in the library and the RR spectra of the corresponding dithioacyl papains (Ozaki et al., 1982; Storer et al., 1983; Angus et al., 1986). This similarity extended to properties such as spectral changes upon isotope substitutions, giving considerable confidence that the interpretation was sound.

In contrast, the RR spectra of the present series of *N*-acylalanine dithioacyl papains do not resemble closely the spectra of a known conformational state of *N*-acylalanine dithio esters. This is in spite of the fact that the conformational preferences in the bonds near the dithio ester group in *N*-acylalanine dithio esters have been characterized and found to be very similar to the conformational preferences of *N*-acylglycine dithio esters (Angus et al., 1985; Varughese et al., 1986; Lee et al., 1988). The RR spectra of each of the three *N*-acylalanine papain intermediates and its isotopically substituted analogues will now be compared to those of the corresponding model and to the spectral signatures determined for the A, B, and C_5 conformers of *N*-acylalanine dithio esters (Figure 1; Lee et al., 1988).

N-Benzoyl-L-alanine Dithioacyl Papain. The RR spectrum of unlabeled *N*-benzoylalanine dithioacyl papain (Figure 3) shows a fairly close similarity with an alanine dithio ester in an A- or C_5-type conformation. The similarity is seen by comparing the top spectrum in Figure 3 with the schematic spectra in Figure 1 and with the C_5 RR spectra for *N*-acylalanine dithio esters in CCl_4 in the preceding paper (Lee et al., 1988, Figures 7 and 9) and with the A-type RR spectrum for solid methyloxycarbonyl-L-phenylalanyl-DL-alanine ethyl dithio ester (Lee et al., 1988, Figure 5). The pair of bands at 642 and 685 cm^{-1} (Figure 3) are typical of a C_5-type conformer, although the RR spectrum of the intermediate lacks a medium-intensity feature near 1000 cm^{-1} seen for the C_5 conformer of *N*-acylalanine dithio esters (the 1000-cm^{-1} peak in Figure 3 is due predominantly to excess substrate). The intensity of the feature near 1000 cm^{-1} is sensitive to changes in the ϕ' angle, being present in C_5 conformers but absent in the RR spectrum of an A-like conformer (Lee et al., 1988). Overall the RR data suggest that the bound *N*-benzoylalanine dithioacyl group takes up a position in the ψ',ϕ' conformational space within the limits set by the A and C_5 forms, i.e., $180 > \psi' > 140$, $180 > \phi' > 70$.

One difference between the unlabeled *N*-acylalanine dithioacyl papain RR spectrum and that of the corresponding model in its A or C_5 conformers is that neither of the model spectra has the 509-cm^{-1} feature seen in Figure 3. Further differences concern the response of acyl-enzyme and model RR spectra to labeling the C=S group with ^{13}C. For the PheAla model dithio ester in the A-like conformation, both intense features in the 1000–1200-cm^{-1} region show large shifts upon $^{13}C{=}S$ substitution with the peaks at 1174 and 1099 cm^{-1} ($^{12}C{=}S$) being replaced by features at 1158, 1133, and 1065 cm^{-1} ($^{13}C{=}S$; Figure 5, Lee et al., 1988). In the case of the C_5 conformer the intense RR band at 1182 cm^{-1} shifts to 1156 cm^{-1} in the $^{13}C{=}S$-substituted compound. In contrast, for the *N*-benzoylalanine dithioacyl papain $^{13}C{=}S$ substitution brings about major intensity rearrangement in the 1000–1200-cm^{-1} region but only modest, ~5 cm^{-1}, downward shifts in the 1177-, 1089-, and 1025-cm^{-1} features.

On the basis of the known spectral signature of the B conformer of *N*-benzoylalanine ethyl dithio ester (Figure 1 and Angus et al., 1985) and its response to $^{13}C{=}S$ substitution (Angus et al., 1985), there is no evidence for a B-like conformer contributing to the RR spectrum of *N*-benzoylalanine dithioacyl papains shown in Figure 3. In summary, the RR spectrum of the intermediate provides some evidence for the presence of a conformer in the ϕ',ψ' space bounded by A and C_5 (Figure 2), but an explanation must be furnished for the observed non-model-like $^{13}C{=}S$ shifts. This will be taken up in a following section.

N-(β-Phenylpropionyl)-L-alanine Dithioacyl Papain. The RR spectrum of the unlabeled *N*-(β-phenylpropionyl)-L-alanine intermediate seen in Figure 4 has several peaks in the positions of conformer A or C_5 markers (Figure 1). The intense 1182-cm^{-1} band and the peaks at 671 and 645 cm^{-1} are highly suggestive of the presence of a population in the ϕ',ψ' region containing the A and C_5 conformers. An A-like population would also be expected to contribute to the peaks at 1103 and 1031 cm^{-1}, but it is hard to account for all of the intensity in these peaks on the basis of the spectra of known conformers in the A–C_5 region (Figure 1). Furthermore, the sharp peak at 538 cm^{-1} has no counterpart in the RR spectra of model compounds in the A or C_5 conformations in solution or in the RR spectrum of the solid A conformer (Lee et al., 1988).

Unlike the *N*-benzoylalanine papain case, peak shifts of more than 5 cm^{-1} are seen upon $^{13}C{=}S$ substitution, but again it is not possible to explain the $^{13}C{=}S$ RR spectra on the basis of the behavior of model compounds. In particular, the shifts seen in the 1000–1200-cm^{-1} region cannot be accounted for by the presence of a known A, C_5, or B conformer or by a blend of these conformers. If the 1182-cm^{-1} feature ($^{12}C{=}S$) was due to a mode from an unperturbed A- or C_5-like con-

former, it should shift to ≈1160 cm^{-1} upon $^{13}C{=}S$ substitution (Figure 1). On the other hand, the behavior of the peaks at 671 and 645 cm^{-1} ($^{12}C{=}S$) going to 668 and 632 cm^{-1}, with the 632-cm^{-1} peak increasing in relative intensity in the $^{13}C{=}S$ analogue, is very much like that of the A or C_5 form of the corresponding model compound (Figure 1).

The results for CD_3-substituted *N*-(β-phenylpropionyl)-L-alanine dithioacyl papain are seen in Figure 4. The intense feature at 1171 cm^{-1} and the weaker bands at 1095, 840, 662, and 603 cm^{-1} are suggestive of the C_5 conformer for the CD_3 labeled model in CCl_4 (Lee et al., 1988), but the 529-cm^{-1} band has no counterpart in the RR spectrum in CCl_4.

In toto, the RR results for this substrate indicate the presence of a major population in the A–C_5 region of the ϕ',ψ' space (Figure 2). The presence of a second population of B conformers cannot be completely ruled out and could account for some of the intensity of the 1103-cm^{-1} band seen in Figure 3 (top spectrum), but there is no B marker near 560 cm^{-1} and the existence of the B population must remain equivocal.

N-(Methyloxycarbonyl)-L-phenylalanyl-L-alanine Dithioacyl Papain. The RR spectrum of this intermediate (Figure 5), in its unlabeled form, is very similar to that of the foregoing *N*-(β-phenylpropionyl)alanine analogue. The main differences are in the 500–600-cm^{-1} range where the PheAla intermediate has at least two minor features at 529 and 594 cm^{-1}, whereas the *N*-(β-phenylpropionyl) analogue has a single sharp peak at 538 cm^{-1}. Additionally, the latter enzyme–substrate complex gives rise to a shoulder at 1152 cm^{-1} (Figure 4) that does not appear in the RR spectrum of the PheAla papain (Figure 5). Also, the 1100-cm^{-1} feature is broader in the PheAla case and is probably a composite band.

Given the overall similarities in their RR spectra, the conclusions reached for the unlabeled *N*-(β-phenylpropionyl)-L-alanine dithioacyl enzyme apply in the PheAla case, too. The 1176-, 1100-, 669-, and 640-cm^{-1} peaks (Figure 5, top) are taken as evidence for a major population state in the A–C_5 region of ϕ',ψ' space (Figure 2). This may account for all or some of the intensity of the 1034- and 1100-cm^{-1} peaks with the additional possibility that a B-conformer population may contribute to the latter feature. However, the characteristic B marker near 560 cm^{-1} (Figure 1) is not seen in Figure 5, and we cannot state with certainty that there is a second population in addition to the population found in the A–C_5 region (Figure 2).

Although there is a fair correspondence in the data for the unlabeled PheAla enzyme and model [for the solid A conformer see Figure 5, Lee et al. (1988)] RR spectra, the response to $^{13}C{=}S$ substitution in the enzyme intermediate is complex and cannot be interpreted solely on the basis of model data. The intense peaks at 1176 and 1100 cm^{-1} are replaced by bands at 1165 and 1067 cm^{-1} in the $^{13}C{=}S$ enzyme derivative, mimicking quite closely the behavior of the corresponding ethyl dithio ester model compound (Lee et al., 1988). However, the 1133-cm^{-1} peak seen in the $^{13}C{=}S$-substituted model (Figure 5, Lee et al., 1988) has no counterpart in the acyl-enzyme RR spectrum.

For the CD_3-substituted model in the solid A-like conformer (Figure 5; Lee et al., 1988) and the CD_3-substituted acyl enzyme, there are close similarities in the 800–1200-cm^{-1} range. The correspondence between the bands at 846 cm^{-1} in the model and at 841 cm^{-1} in the dithioacyl papain is particularly noteworthy, since a peak in this region appears to be a good A-conformer marker. The region from 500–700 cm^{-1} in the RR spectrum of the acyl enzyme does not resemble closely the spectrum of any alanine model compound. The enzyme spectrum has three features at 659, 601, and 512 cm^{-1}. The former peak may be due to an A conformer [compare spectra of solid A, solid B, and A and B in solution, in Lee et al. (1988)], but both the A and B conformers, in their CD_3 forms, give rise to a number of unresolved bands between 560 and 615 cm^{-1} (Figure 1 and Lee et al., 1988), whereas the acyl-enzyme RR spectrum has a single peak at 601 cm^{-1}. The enzyme feature at 512 cm^{-1} has no counterpart in model spectra.

Although, as in the *N*-benzoylalanine and *N*-(β-phenylpropionyl)alanine cases, we cannot interpret every feature in the PheAla papain spectra, the overall data provide evidence for a population in the A–C_5 region of the ϕ',ψ' conformational space (Figure 2). The evidence regarding a second B-like conformer population is inconclusive and will be considered further below.

Discussion

The key questions to be addressed concern the conformation of the *N*-acylalanine acyl groups in the active site and the possibility that there may be more than one conformational population. The method of comparing model with enzyme RR spectra works less well than the same approach with *N*-acylglycine dithioacyl papain since for the alanine compounds there is not a simple one-to-one correspondence between the RR spectrum of the enzyme species and that of known models. Not every peak in the enzyme RR spectra can be explained in terms of a model feature, and often the response to isotopic substitution in the enzyme RR spectrum finds no parallel in the model data.

There are at least three reasons why differences can occur between acyl-enzyme and model RR spectra. The first and most obvious cause is that the conformation in the active site is different from any conformations presently characterized for the model compound. We have good RR signatures for the A- and B-like and C_5 conformers of *N*-acylalanine dithio esters, and the conformation of the enzyme-bound acyl group would have to be different from any of these.

The second explanation is that the *N*-acylalanine substrate is binding as a "known" conformer but that its vibrational signature is perturbed, in at least part of the spectral range, by vibrational coupling to normal modes of the enzyme. This would give rise to "unusual" peak positions, intensities, and responses to isotopic substitution in the range wherein the coupling is most pronounced. Covalent bonding is not a prerequisite for the occurrence of vibrational coupling; the force field can be markedly perturbed by weaker noncovalent interactions. For example, vibrational coupling has been observed between noncovalently bound base pairs across a G:C polynucleotide double helix (Howard et al., 1969).

The third explanation concerns the effect of charged groups or dipoles in the active site on the RR spectrum of the bound substrate. The primary effect of charges would almost certainly be on the excited electronic states of the dithio ester group. This may lead to a perturbation in the intensity pattern of the spectrum due to the enzyme intermediates vis-à-vis the spectrum of the model compounds, but it does not offer a satisfactory explanation for differential behavior in peak positions or shifts upon isotopic substitutions.

For each of the three *N*-acylalanine dithioacyl papains and their $^{13}C{=}S$ and CD_3 forms, the bulk of the RR data can be interpreted in terms of a conformer with ϕ',ψ' angles in the region bounded by the A and C_5 conformers (Figure 2). For the unlabeled intermediates, the main lines of evidence are the intense peak near 1175 cm^{-1} and the two characteristic C_5-like conformer bands near 640 and 665 cm^{-1}. Additional data in

favor of an A-like conformer are seen in the RR spectra of the CD_3-substituted *N*-acylalanine dithioacyl papains in the form of the peak near 845 cm^{-1}, which is an A marker (Figure 1). The peaks apparent in the $^{12}C{=}S$ enzyme intermediate at 640 and 665 cm^{-1} are also seen in the $^{13}C{=}S$-substituted derivatives, which also show the small shifts to lower frequency seen in the C_5 conformer of the models (Figure 1). For the $^{13}C{=}S$-substituted compounds there are some parallels between the dithioacyl-enzyme RR spectra and those of their A-like model analogues in the 1000–1200-cm^{-1} range. However, the similarities here are not so compelling. The favored explanation for the observed discrepancies is that, in this region, vibrational coupling is occurring between substrate and enzyme motions such that the normal mode pattern of the dithioacyl group in this region is perturbed. The best evidence that this is indeed occurring is the RR spectrum of $^{13}C{=}S$-substituted *N*-benzoylalanine dithioacyl papain where unusually small peak shifts are seen. This phenomenon can be explained in terms of vibrational coupling since in this case some of the expected ^{13}C shift will be taken up by the normal modes of the enzyme, which are coupled to the normal modes of the dithioacyl group. Any normal modes of the enzyme participating in this process are not resonance Raman enhanced and therefore do not appear in the spectra.

In all the RR spectra of the *N*-acylalanine papain intermediates there are one or more peaks that have not been assigned, raising the possibility that a second conformational population may exist. This may be so, but we have not detected any consistent pattern among the additional peaks, and they do not correspond to any known conformer. For the dithioacylalanine papains the spectral region from 500–600 cm^{-1} appears quite unique to the enzyme spectra and has no counterpart in model data. In part, this may reflect the sensitivity resulting from coupling to "soft modes", e.g., bond torsions, in this region. The evidence for a major B-conformer population is equivocal—in complete contrast to *N*-acylglycine (dithioacyl) papains. The 560-cm^{-1} B markers do not appear in the alanine-based acyl-enzyme spectra. However, it is possible to invoke an additional B-like population to account for the intensity of the 1100-cm^{-1} peaks in the *N*-(β-phenylpropionyl)alanine and PheAla dithioacyl enzyme spectra, and it is interesting to note that the relative intensity of the corresponding 1089-cm^{-1} peak in the *N*-benzoyl intermediate (Figure 3) is much lower than that for the former two intermediates (Figures 4 and 5).

Summary

The RR results for alanine-based substrates bound to papain's active site are radically different from those for glycine-based substrates. The RR spectra of *N*-acylglycine dithioacyl papains can be interpreted quite precisely in terms of the RR spectra of model compounds, *N*-acylglycine ethyl dithio esters, in a well-characterized conformational state known as conformer B. In contrast, for *N*-acylalanine dithioacyl papains there is not a close correspondence between the RR spectra of the intermediates and the RR spectra of *N*-acylalanine ethyl dithio ester models in any known conformational state. Unequivocally, the *N*-acylalanine moiety is not binding in the active site in the same B-type conformation as in the *N*-acylglycine substrates. The noncorrespondence between the alanine–enzyme RR data and the alanine–model RR data, taken with the results of $^{13}C{=}S$ substitution in the two cases, demonstrates that the normal mode structure has been reorganized in the active site compared to any known state for a model alanine dithio ester. Normal mode reorganization can result from the occurrence of a new, as yet uncharacterized, conformational state, from vibrational coupling between substrate and enzyme normal modes, or from a combination of both effects. At our present level of understanding the alanine papain RR spectra are best interpreted in terms of the presence of a large population of molecules in the A or C_5 region of conformational space with, in addition, some vibrational coupling occurring between enzyme and substrate modes. There is also the possibility of the presence of a second conformational population in the active site of *N*-acylalanine dithioacyl papains but the evidence for this is equivocal.

Although the fine points of interpretation for the *N*-acylalanine papain RR data are not fully resolved, it is beyond doubt that there are major differences between the conformations (and enzyme–substrate interactions) of dithioacyl papains for *N*-acylglycine and *N*-acylalanine derivatives. Thus, we have to consider such questions as whether, for these complexes, deacylation is insensitive to intermediate stereochemistry or whether deacylation can proceed via more than one reaction pathway. These issues are taken up more fully in the following and accompanying publication (Storer et al., 1988).

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