tergent removal by dialysis or gel filtration, and it is therefore commercially economic. It can be applied to a variety of phospholipid mixtures containing PA and gives reproducible results. Preliminary experiments show that the method can be applied to large quantities of lipids. The ratio of SUV to LUV depends on a number of factors; foremost are the pH to which the phospholipid dispersion is exposed and the rate of the NaOD addition. Furthermore, the ionic strength and the nature of the ions present are important in determining this ratio and, for mixed phospholipid dispersions, the PA content. By varying these parameters, it is possible not only to control the ratio of SUV to LUV but also to vary the surface charge density of the bilayer and hence the surface potential within wide limits. By selecting suitable mixtures, it should, in principle, be possible to control another important bilayer property: lipid fluidity. In summary, the vesicles produced by pH adjustment as described here have characteristics which make them potentially useful as drug carriers. Questions of bilayer permeability and stability are important when considering such applications. Our experiments show that the unilamellar vesicles produced by this method are sufficiently stable and relatively insensitive to changes in pH and ionic strength. In their permeability to ions, these vesicles resemble sonicated PC vesicles (Gains & Hauser, 1983).

Registry No. DLPA, 55332-91-7; LLPA, 86594-49-2.

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Conformational States of *N*-Acylglycine Dithioesters in Solution: Resonance Raman Studies of Isotopically Substituted Models for Enzyme–Substrate Complexes†

H. Lee, A. C. Storer, and P. R. Carey*

ABSTRACT: Resonance Raman (RR) and FTIR spectroscopic studies, taken with X-ray crystallographic data, are used to define the three major conformational states of *N*-acylglycine dithioesters in solution and to set up spectra–structure correlations. Importantly, each conformer has a characteristic RR signature, and thus the RR spectrum can be used to follow conformational events within dithioester enzyme–substrate intermediates. The signatures are further defined in the present work by the synthesis and spectroscopic characterization of ^{13}C and ^{15}N derivatives of *N*-benzoylglycine ethyl dithioester and *N*-(β-phenylpropionyl)glycine ethyl dithioester. The observed isotope shifts offer insight into the normal mode character of the RR bands and provide standards with which to compare the shifts in the corresponding enzyme–substrate intermediates.

The impetus for the present work originates in the finding that the resonance Raman (RR) spectra of dithioacyl enzymes, of the type $RC(=O)NHCH_2C(=S)S$–papain, provide a means of monitoring the vibrational spectrum of those bonds undergoing catalytic transformation in the enzyme's active site (Storer et al., 1979). The major features in the RR spectra of the enzyme intermediates occur in the 500–1200-cm^{-1} region and, in principle, give precise information on the conformational state or states of the enzyme-bound substrate. However, to elicit this information, it has been necessary to undertake extensive investigations into the spectroscopic and conformational properties of dithioesters. These studies include de-

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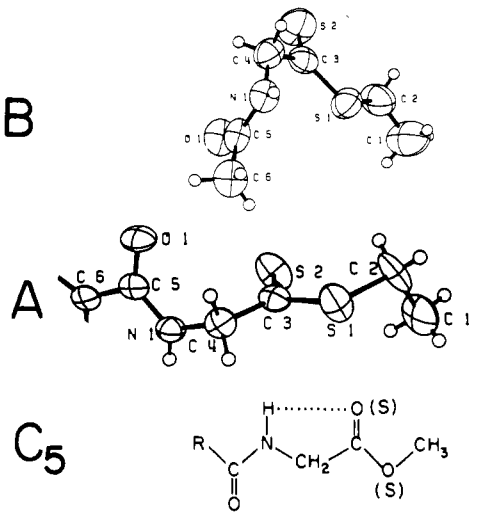

FIGURE 1: Conformers B (top), A (center), and C_5 (bottom). Structures B and A are reproduced from Huber et al. (1982) with permission.

veloping a force field for simple dialkyl dithioesters (Teixeira-Dias et al., 1982), studying the rotational isomers of dialkyl dithioesters (Ozaki et al., 1982b) and *N*-acylglycine dithioesters (Storer et al., 1982), and establishing RR spectra–structure correlations for *N*-acylglycine dithioesters by a joint Raman–X-ray crystallographic approach (Huber et al., 1982). The latter work recognized two major conformational states for *N*-acylglycine dithioesters, designated conformers A and B. Conformer B is illustrated in Figure 1 and differs from conformer A mainly by a 150° rotation about the $NHCH_2$–CS_2 bond. The outcome of these studies is that we are beginning to understand the normal mode properties of simple dithioesters, and for *N*-acylglycine dithioesters we have an initial understanding of spectra–structure correlations in the 1050–1200-cm^{-1} region.

In order to gain further insight into the enzyme-bound species, it is important to extend the correlation to the 500–700-cm^{-1} range. Thus, the objectives of the present work are to delineate a complete correlation between RR spectra and conformers A and B, to include in this correlation an additional species known as C_5 (Figure 1) which has not been discussed by us before, and by the use of isotopically substituted *N*-acylglycine dithioesters to improve our understanding of the normal mode origins of the RR bands of each conformer. The labeled compounds are also of great value for comparison with the corresponding isotopically labeled dithioacylpapains discussed in the following paper (Storer et al., 1983).

Experimental Procedures

Materials. $PhC(=O)NHCH_2{}^{13}C(=S)SC_2H_5$, $PhC(=O){}^{15}NHCH_2C(=S)SC_2H_5$, $PhCH_2CH_2C(=O)NHCH_2{}^{13}C(=S)SC_2H_5$, and $PhCH_2CH_2C(=O){}^{15}NHCH_2C(=S)SC_2H_5$ were prepared from their respective nitriles as described by Storer et al. (1982). The route to the nitriles was as follows: sodium cyanide was reacted with a mixture of formaldehyde and ammonium chloride (Gilman & Blatt, 1964a) to give the (methyleneamino)acetonitrile. The subsequent alcoholysis of this product gave the aminoacetonitrile bisulfate (Gilman & Blatt, 1964b) which was converted to the nitrile (Storer et al., 1982). The yields obtained were 56% for (methyleneamino)acetonitrile and 85% for aminoacetonitrile bisulfate. [^{15}N]Ammonium chloride (95 atom %, Merck Sharp & Dohme Canada) and sodium [^{13}C]cyanide (90 atom %, Merck Sharp & Dohme Canada) were employed as starting materials. ND analogues of the esters were obtained by dissolving the samples in an acetonitrile heavy water mixture and evaporating the solvent. Purity of the samples was checked by NMR. The unlabeled *N*-acylglycine dithioesters were prepared as described in Storer et al. (1982).

Methods. The normal Raman spectra of the single crystals, seen in Figures 3 and 4, were recorded on a Jarrell-Ash spectrometer described elsewhere (Kumar & Carey, 1975). The RR spectrum shown in Figure 11 was recorded on equipment based on a Spex 0.5-m double monochromator which is described in MacClement et al. (1981). The RR spectra shown in Figures 5–8 were recorded by using a multiplex system based on a Tracor-Northern diode array detector coupled to a Spex triplemate monochromator. The multiplex system is outlined in the following paper (Storer et al., 1983) and is described in detail in Carey & Sans-Cartier (1983). The laser power and total exposure time (=individual exposure time × number of repeats) are given in the figure captions. The FTIR data were obtained by using a Bomem DA 3.02 instrument, equipped with a medium range mercury–cadmium–telluride detector; 500 scans of maximum optical retardation 0.5 cm were coadded, apodized with a Happ-genzel function, and Fourier transformed to yield a spectrum with a resolution of 2 cm^{-1}.

Results and Discussion

FTIR Evidence for the C_5 Conformer in CCl_4 Solution. Although the C_5 conformer has not been discussed for dithioester derivatives, it has been reported widely in the literature concerning di- and tripeptides and depsipeptides (Cung et al., 1972; Avignon & Lascombe, 1972). There is a consensus that it consists of a five-membered ring that is completed by a hydrogen bond between the NH and O=C groups (Figure 1).

Although studied extensively by spectroscopic techniques, precise structural information from X-ray diffraction analysis is unavailable for the C_5 geometry. However, theoretical calculations indicate that the C_5 skeleton is essentially planar (Laurence & Thompson, 1980). The difference between C_5 and A conformers (Huber et al., 1982) consists of a ~90° rotation about the NH–CH_2 bond (Figure 1). Hydrogen bonding between the –NH (or C=O) moiety and solvent can suppress completely the C_5 interaction. Thus, a significant population of C_5 isomers occurs in solvents such as CCl_4, which possess little or no propensity for hydrogen bonding. The C_5 intramolecular interaction has a characteristic effect on the N–H stretching region in the IR spectrum; compared to isomers lacking the NH···O=C interaction the NH stretching mode in the C_5 conformer is shifted approximately 50 cm^{-1} to lower frequencies, and the band profile is broadened. The FTIR spectra of compounds **1** and **2**[1] in CCl_4 solution seen in Figure 2 each show two intense features. The sharper band near 3455 cm^{-1} is due to an isomer population containing a non-hydrogen-bonded NH group (in which conformers B and probably A are present; see below). Since intermolecular hydrogen bonding is insignificant at the concentrations used to measure the FTIR spectra, the broad feature near 3390 cm^{-1} is taken as evidence for a significant population of intramolecularly H-bonded C_5 isomers. In CH_3CN solution weak H bonding of the solvent to the –NH group brings about a similar shift in ν_{N-H} and this is also shown in Figure 2. As a result of the overwhelming concentration of the solvent, only a H-bonded N–H feature is observed in the spectrum, the sharp

[1] Abbreviations: compound **1**, *N*-(β-phenylpropionyl)glycine ethyl dithioester; compound **2**, *N*-benzoylglycine ethyl dithioester.

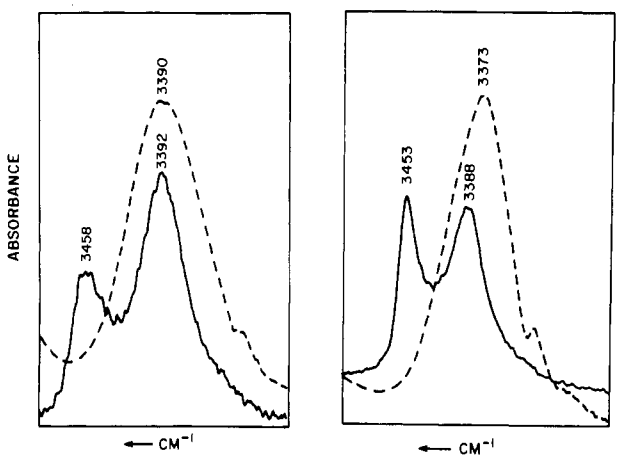

FIGURE 2: NH stretching region of the FTIR spectra of *N*-benzoylglycine ethyl dithioester (compound **2**) in CCl_4 (—) and CH_3CN (---), 10^{-3} and 2×10^{-3} M, respectively (left-hand side). NH stretching region of the FTIR spectra of *N*-(β-phenylpropionyl)glycine ethyl dithioester (compound **1**) in CCl_4 (—) and CH_3CN (---), 10^{-3} and 2×10^{-3} M, respectively (right-hand side).

N–H band seen near 3455 cm^{-1} in CCl_4 solution is absent in CH_3CN solution.

RR Signatures of Conformers A, B, and C_5. In earlier reports from this laboratory it was stated that in solution *N*-acylglycine dithioesters exist in two major conformational states termed conformers A and B (Ozaki et al., 1982a; Storer et al., 1982). In the 1050–1200-cm^{-1} region of the RR spectrum, conformer A gives rise to an intense band between 1160 and 1180 cm^{-1} (designated band I) while conformer B gives rise to an intense feature between 1115 and 1155 cm^{-1} (band II) and sometimes a second moderately intense band between 1080 and 1100 cm^{-1} (band III). The exact natures of A and B have been elucidated by joint X-ray crystallographic and Raman studies (Huber et al., 1982) which, in turn, were used to form a very precise structural model for certain dithioacyl enzyme intermediates. The analysis will be extended now to include consideration of the C_5 conformer and to include other regions of the RR spectrum, in particular the 500–700-cm^{-1} range, in the correlation between conformers and spectral signatures.

To date, approximately 10 *N*-acylglycine dithioester derivatives have been crystallized as single crystals. All but one of these give a Raman spectral signature, in the 1050–1200-cm^{-1} region, characteristic of a B-type conformer. The exception is *N*-(*p*-nitrobenzoyl)glycine ethyl dithioester which crystallized in the A form (Huber et al., 1982). The Raman spectra of *N*-benzoylglycine and *N*-(*p*-nitrobenzoyl)glycine ethyl dithioesters, seen for the single crystals in Figures 3 and 4, therefore allow us to discuss the characteristic signatures of conformers B and A, respectively. Usually, the corresponding peaks can be identified in the Raman and RR spectra; thus, unless specifically stated, information can be transferred from the Raman to the RR case and vice versa. For the unlabeled, ^{13}C and ^{15}N derivatives of compound **2** (Figure 3) band II appears at 1120, 1103, and 1119 cm^{-1}, respectively. In the Raman spectrum of the unlabeled compound there is a composite peak near 1160 cm^{-1} and a weak band at 1192 cm^{-1}. RR spectra of the solid compounds (not shown) were obtained by grinding up crystals and pressing them into a KBr matrix in a rotatable Raman cell designed for absorbing materials (Kiefer & Bernstein, 1971). Due to luminescence the data are of poorer quality than those seen for the Raman spectra of the single crystals in Figure 3. However, in the RR spectra there is no discernible band near

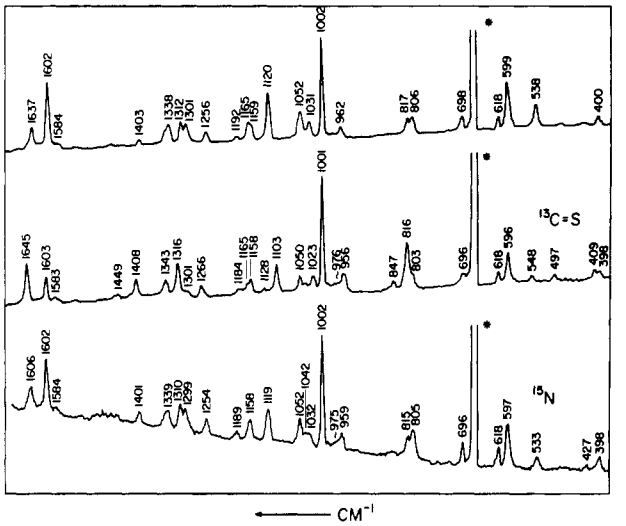

FIGURE 3: Raman spectra of single crystals of *N*-benzoylglycine ethyl dithioester: unlabeled (top), $PhC(=O)NHCH_2{}^{13}C(=S)SC_2H_5$ (middle), $PhC(=O)^{15}NHCH_2C(=S)SC_2H_5$ (bottom). 647.1-nm excitation, 80 mW, 5-cm^{-1} spectral slit. Asterisk denotes laser plasma line.

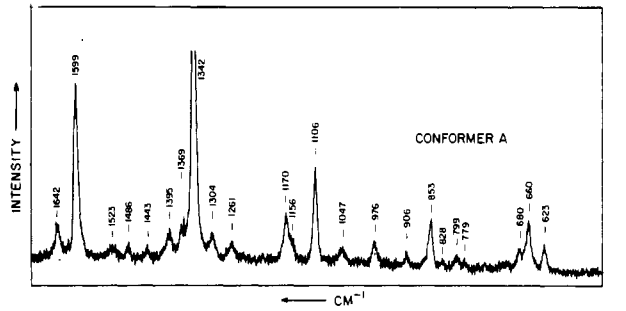

FIGURE 4: Raman spectrum of a single crystal of *N*-(*p*-nitrobenzoyl)glycine ethyl dithioester. 514.5-nm excitation, 10 mW, 8-cm^{-1} spectral slit.

1190 cm^{-1}, but a weak feature is observed near 1165 cm^{-1}. From this it is apparent that conformer B can give rise to a weak RR peak near 1165 cm^{-1}, and this should not be confused with band I which is an intense conformer A mode occurring near 1170 cm^{-1}. The weak conformer B feature near 1165 cm^{-1} probably consists of more than one band and shows complex behavior upon ^{15}N and ^{13}C substitution (in Figure 3 the situation is complicated further by the presence of an additional feature at 1158 cm^{-1}; however, the 1165-cm^{-1} feature appears to decrease in intensity upon ^{13}C substitution and to shift under the 1159-cm^{-1} peak upon ^{15}N substitution). It may, in part, be assigned to a mode analogous to a coupled $N–C^{\alpha}$ and $C–C^{\alpha}$ stretch reported in this region for polyglycine by Painter et al. (1982). It is not a group frequency and is probably vibrationally delocalized throughout the glycine $NH–CH_2–C(=S)$ moiety. This feature assumes importance in the RR studies of dithioacylpapains because the above analysis shows that a weak peak seen in the enzyme spectra near 1170 cm^{-1} does not have to be assigned to a minor conformer A population. Indeed, the resemblance in the spectral pattern of the ^{13}C derivative (Figure 3) and the analogous ^{13}C-labeled dithioacylpapain (Storer et al., 1983) strongly suggests that the 1170-cm^{-1} peak observed in the latter is a conformer B mode. In the 500–700-cm^{-1} region of Figure 3 three features at 698, 599, and 538 cm^{-1} are associated with the dithioester group. The peak at 618 cm^{-1} is assigned to the phenyl ring mode designated 6b by Varsanyi (1969). The corresponding peaks for the single crystals of (*p*-methoxy-

Table I: Characteristic Raman Signatures, Associated with the -C(=S)S- Group, from Single Crystals in B or A Type Conformations

N-acylglycine ethyl dithioester, acyl =	conformer	band I (cm^{-1})	band II (cm^{-1})	peaks (cm^{-1}) in 700–500-cm^{-1} region			
benzoyl	B[a]		1120	698		599	538[b]
p-methoxybenzoyl	B[a]		1115	680		584	546
p-methylbenzoyl	B[a]		1114	678		583	538
p-chlorobenzoyl	B[a]		1122	680		584	546
acetyl	B		1141	698		584	502
N-(β-phenylpropionyl)	B[a]		1132	692		612, 570	532
p-nitrobenzoyl	A	1170		680	660		

[a] These crystals give rise to a weak composite feature near 1170 cm^{-1} in both Raman and RR, which is probably unrelated to band I and is of complex origins (see text). [b] Values are in cm^{-1}.

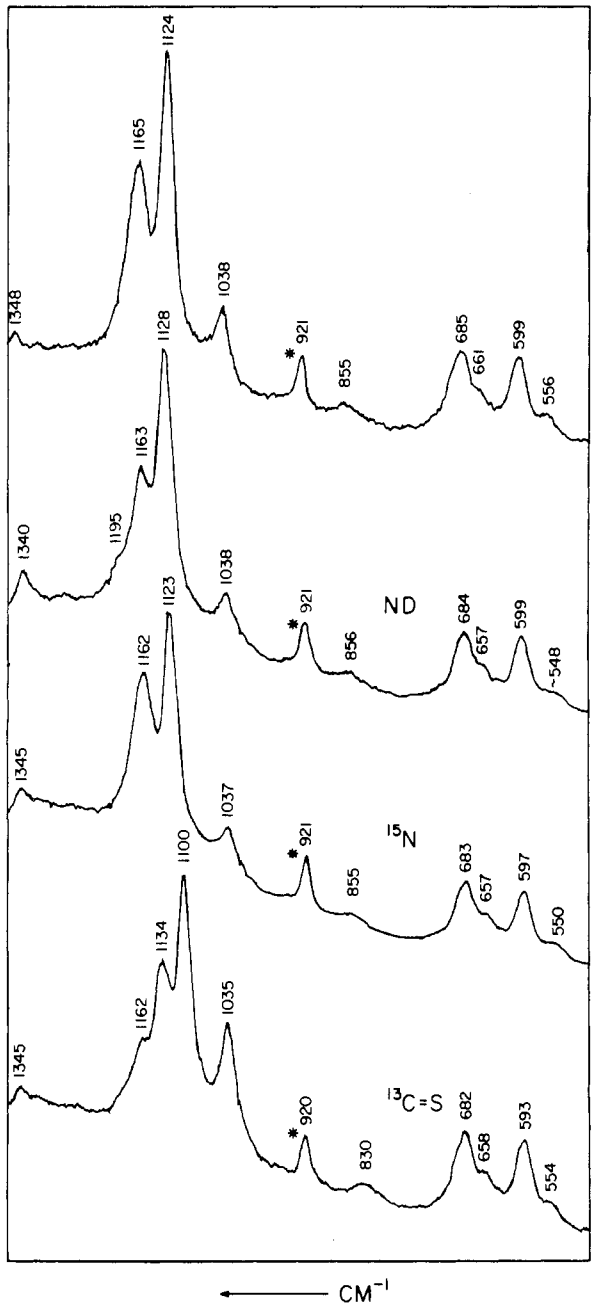

FIGURE 5: Multiplex resonance Raman spectra of unsubstituted (top) and isotopically substituted (in the positions denoted) *N*-benzoylglycine ethyl dithioester, 3×10^{-3} M in CH_3CN/H_2O (30:70 v/v). Solvent peaks are marked with an asterisk. Excitation 324 nm, ~50 mW; 12-cm^{-1} spectral slit, 1-min total acquisition (3 s × 20).

benzoyl)-, (*p*-methylbenzoyl)-, and (*p*-chlorobenzoyl)glycine dithioesters are listed in Table I. They agree closely with the values for the unsubstituted benzoyl compound.

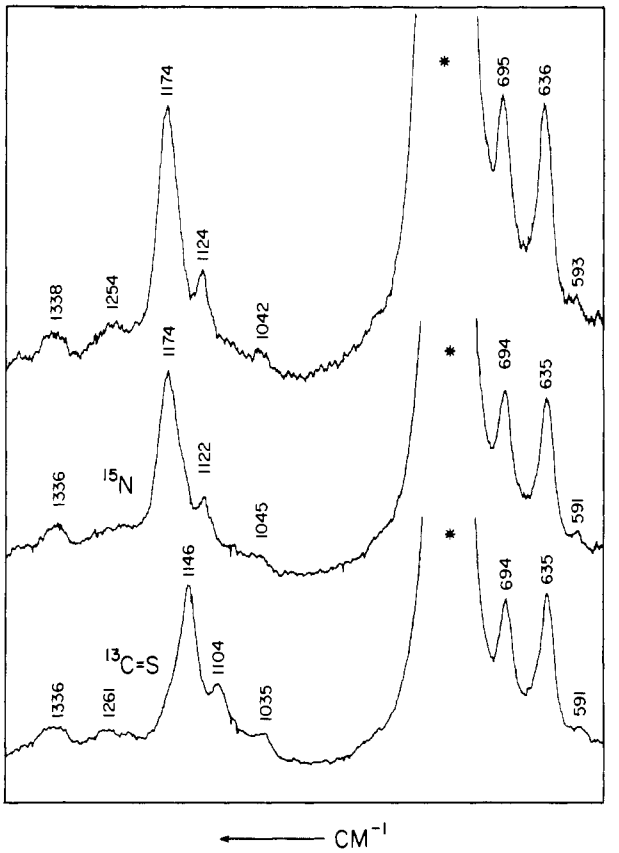

FIGURE 6: Multiplex resonance Raman spectra of unsubstituted (top) and isotopically substituted (in the positions denoted) *N*-benzoylglycine ethyl dithioester, ~10^{-3} M in CCl_4. Solvent peak is marked with an asterisk. Excitation 324 nm, ~50 mW, 12-cm^{-1} spectral slit, 1-min acquisition (3 × 20).

In the spectrum of conformer A seen in Figure 4, band I appears at 1170 cm^{-1} [the 1156-cm^{-1} shoulder and the 1106 cm^{-1} features in Figure 4 are not seen in the 324-nm excited RR spectrum of the crystalline *p*-nitrobenzoyl derivative (Huber et al., 1982)]. In the 500–700-cm^{-1} range, peaks associated with the dithioester appear at 680 and 660 cm^{-1} with the 623-cm^{-1} band again being associated with the benzene 6b mode. These observations, taken with earlier data, assign the spectral signatures in the solid phase and can now serve as a basis point for understanding the RR spectra–conformer correlations in solution.

The RR spectra of **2** and **1**, seen in the top traces in Figures 5–8, show that the relative intensity of the band near 1130 cm^{-1} increases upon going from CCl_4, to CH_3CN solutions. In keeping with the above data on the crystalline compounds and our earlier analysis (Storer et al., 1982; Huber et al., 1982), this change is ascribed to an increase in population of conformer B in CH_3CN solution. Although, heretofore, we assigned the feature near 1175 cm^{-1}, in CH_3CN and CCl_4

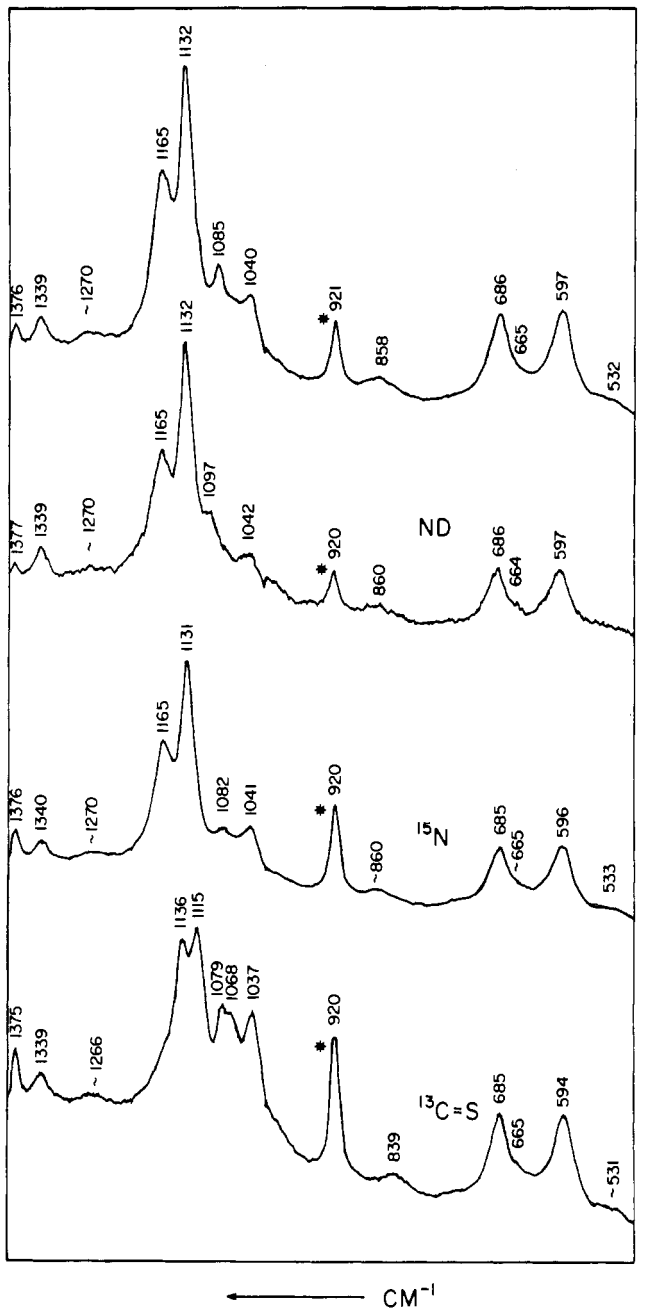

FIGURE 7: Multiplex resonance Raman spectra of unsubstituted (top) and isotopically substituted (in the positions indicated) *N*-(β-phenylpropionyl)glycine ethyl dithioester, 3×10^{-3} M in CH_3CN/H_2O (30:70 v/v). Solvent peaks are marked with an asterisk. Excitation 324 nm, ~50 mW, 12-cm^{-1} spectral slit, 1-min acquisition (3 s × 20).

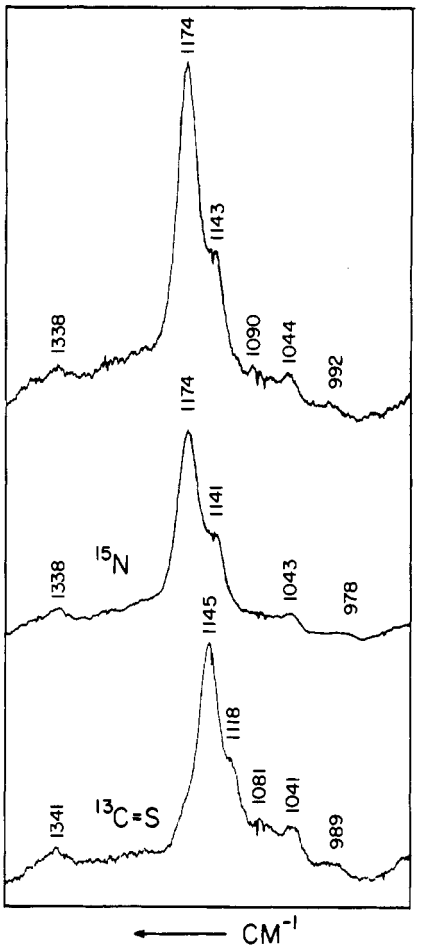

FIGURE 8: Multiplex resonance Raman spectra of unsubstituted (top) and isotopically substituted (in the positions indicated) *N*-(β-phenylpropionyl)glycine ethyl dithioester, 10^{-3} M in CCl_4. Excitation 324 nm, 50 mW, 12-cm^{-1} spectral slit, 1-min acquisition time (3 s × 20). At this concentration the 600-cm^{-1} region of the solute's RR spectrum is obscured by CCl_4 bands; in other experiments solute bands detected at 689, 631, and 590 (weak) cm^{-1} (unlabeled), 686 and 632 cm^{-1} (^{13}C), and 632 cm^{-1} (^{15}N).

solutions, to an A conformer, we must now take into account the FTIR evidence that a substantial population of a C_5 conformer occurs in CCl_4 (and other non-H-bonding solvents). This in no way alters the earlier conclusions concerning enzyme-bound substrates (Ozaki et al., 1982a; Huber et al., 1982) since the spectral signatures of the A and C_5 conformers are very similar in the 1050–1200-cm^{-1} region. The 1174-cm^{-1} features seen in the CCl_4 solutions of **1** and **2** (Figures 8 and 6) are due to predominately C_5 conformers while the corresponding peaks in CH_3CN solution at 1165 cm^{-1} are due to predominately A type conformers. The same trend to slightly higher cm^{-1} for band I in C_5 conformers is seen also in the FTIR spectra (Figures 9 and 10). In the FTIR spectra the shoulders near 1185 and 1160 cm^{-1} may be related to the features at 1192 and 1159 cm^{-1} in Figure 3 and discussed above. In the unlabeled compounds the feature seen near 1174 cm^{-1} in the FTIR spectrum corresponds to band I while the feature near 1124 cm^{-1} corresponds to band II in the RR spectra. Bands I and II show similar solvent dependencies in both the RR and FTIR spectra. For the non-B conformers there is a switch from a predominance of conformer A in CH_3CN to a predominance of conformer C_5 in CCl_4, but we cannot rule out the possibility of a minor population of conformer A existing in CCl_4, or a minor population of C_5 existing in CH_3CN solutions.

As has been pointed out in earlier studies, some *N*-acylglycine dithioesters give rise to a characteristic B conformer mode near 1090 cm^{-1}, designated band III. Compound **2** does not give rise to a band III, whereas compound **1** does; it is observed in CH_3CN solution (Figure 7) but is weak or absent in CCl_4 solution (Figure 8) wherein the B population has diminished. Band III shows a very high degree of RR intensity enhancement: it is barely perceptible in the normal Raman spectra of the crystalline conformer B (data not shown) but is of medium intensity in the RR spectra in solution (Figure 7) or the solid (not shown). An additional assignment, which has not been discussed before, concerns the RR feature observed near 1040 cm^{-1} in the solution spectra of all the *N*-acylglycine derivatives (including the *p*-nitrobenzoyl compound). This band increases in intensity with increasing B population. It is present in the RR spectra of the solid forms

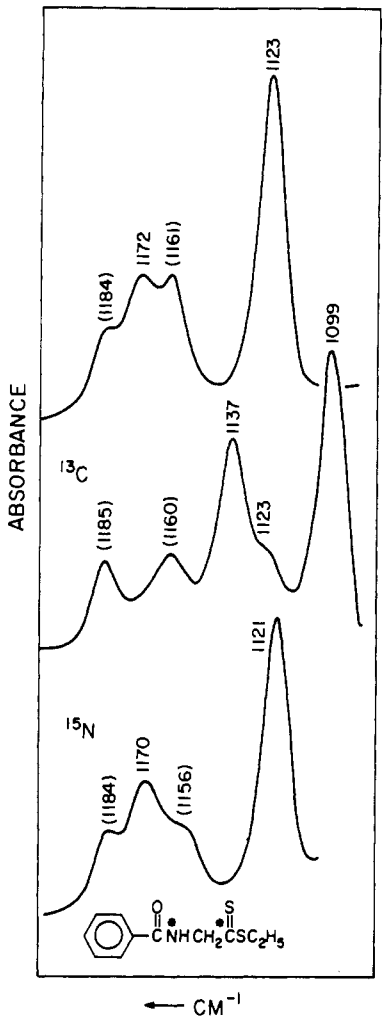

FIGURE 9: FTIR spectra, in the 1100–1200-cm^{-1} region, of unsubstituted (top) and isotopically labeled (in the positions indicated) *N*-benzoylglycine ethyl dithioester, 2×10^{-3} M in CH_3CN.

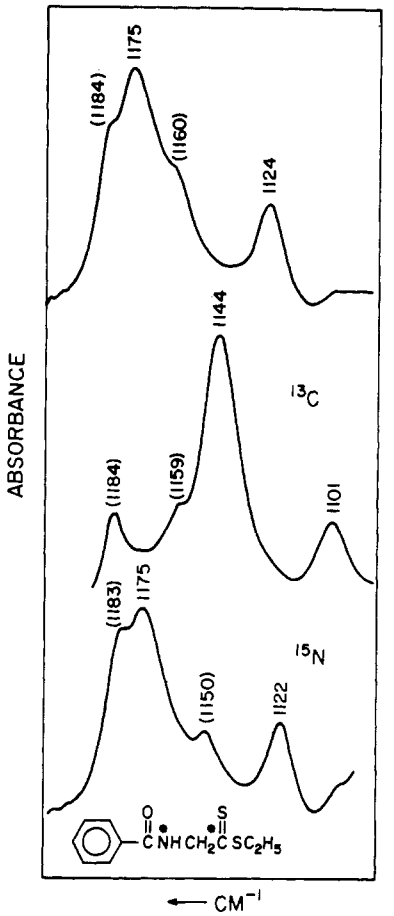

FIGURE 10: FTIR spectra, in the 1100–1200-cm^{-1} region, of unsubstituted (top) and isotopically labeled (in the positions indicated) *N*-benzoylglycine ethyl dithioester, 10^{-3} M in CCl_4.

of **1** and **2** (unpublished work, this laboratory) but absent in the RR spectrum of the crystalline *p*-nitrobenzoyl derivative (Huber et al., 1982). This strongly suggests that the 1040-cm^{-1} feature is associated with a B type conformer. The normal mode origin of this band is not certain; it shows, at most, small shifts upon ^{13}C or ^{15}N substitution (below). However, the fact that the 1040-cm^{-1} peak is absent in the RR spectrum of *N*-benzoylglycine *methyl* ester in solution (Figure 11) and that for $CH_3C(=S)SC_2H_5$ a weak feature of 1050 cm^{-1} was assigned to the C–C stretch from the ethyl group (Teixeira-Dias et al., 1982) suggests that the 1040-cm^{-1} band in *N*-acylglycine ethyl dithioesters is associated with the ethyl moiety. It may be tentatively assigned to the C–C stretch although it is probably coupled, to some extent, to motions of the C–S atoms in the C–S–C–C skeleton. RR enhancement of the 1040-cm^{-1} mode appears to require a B type conformation in the glycinic NH–C–C bonds. Although we do not known if there is a conformational requirement in the C(=S)–S–C–C fragment, we note that the three conformer B *N*-acylglycine ethyl dithioesters we have presently examined as single crystals (Huber et al., 1982; unpublished work, this laboratory) all have a moderately intense RR band near 1040 cm^{-1} and all have a planar zig-zag C–S–C–C skeleton. Interestingly, the 1040-cm^{-1} band appears to show the greatest degree of intensity enhancement in the ^{13}C-substituted analogues (Figures 5–8), and this is of possible relevance to the enzyme work (Storer et al., 1983).

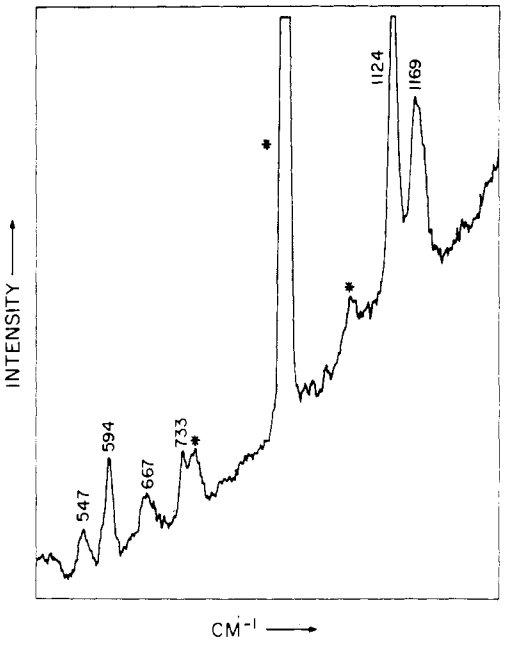

FIGURE 11: RR spectrum of *N*-benzoylglycine methyl dithioester (6×10^{-3} M) in CH_3CN solution. Spectral conditions: flow cell 40 mW, 337.5 nm, 10-cm^{-1} spectral slit. Asterisks denote solvent features.

Turning to the 500–700-cm^{-1} range it is apparent that the B conformer gives rise to bands near 595 and 690 cm^{-1} and, in some cases, a band near 540 cm^{-1}. The 595-cm^{-1} feature is particularly characteristic and, as can be seen in Figure 6, diminishes in relative intensity with the diminution of con-

Table II: Correlation between RR Spectra and Conformational State of *N*-Acylglycine Dithioesters

conformer B	545–564[a,b] (w)	585–606 (m)	680–705 (m)	1038–1049 (m)	1089–1100[b]	1115–1150 (s)
conformer A		660–667 (m)	~680 (m)			1167–1181 (s)
conformer C_5		631–650 (m)	683–703 (m)			1170–1181 (s)

[a] Peak position in cm^{-1}, s = strong, m = medium, and w = weak; data from this work and Storer et al. (1982). [b] Sometimes absent.

Table III: Resonance Raman and FTIR Data for ^{13}C-, ^{15}N-, and ND-Substituted *N*-Benzoylglycine Ethyl Dithioester in Solution[a,b]

in CH_3CN					in CCl_4			
unlabeled	^{13}C	^{15}N	ND	conf	unlabeled	^{13}C	^{15}N	conf
1165	1134	1162	1163	A[c]	1174	1146	1174	C_5[c]
(1172)	(1137)	(1170)			(1175)	(1144)	(1175)	
1124	1100	1123	1128	B[d]	1124	1104[e]	1122	B[d]
(1123)	(1099)	(1121)			(1124)	(1101)	(1122)	
1038	1035	1037	1038	B	1042	1035	1045	B
685	682	683	684	B, A	695	694	694	C_5, B
661	658	657	657	A				
599	593	597	599	B	636	635	635	C_5
~556	554[e]	550[e]	~548[e]	B				

[a] FTIR values appear in parentheses. [b] Values are in cm^{-1}. [c] Band I. [d] Band II. [e] Broad.

Table IV: Resonance Raman Data for ^{13}C-, ^{15}N-, and ND-Substituted *N*-(β-Phenylpropionyl)glycine Ethyl Dithioester in Solution[a]

in CH_3CN					in CCl_4			
unlabeled	^{13}C	^{15}N	ND	conf	unlabeled	^{13}C	^{15}N	conf
1165	1136	1165	1165	A[b]	1174	1145	1174[a]	C_5[b]
1132	1115, 1079	1131	1132	B[c]	1143	1118, 1081	1141	B[c]
1085	1068	1082	1097	B[d]	1090			B[d]
1040	1037	1041	1042	B	1044	1041	1043	B
686	685	685	686	B, A	689	686		C_5, B
665	665	665	664	A	631	632	632	C_5
597	594	596	597	B	590			B

[a] Values are in cm^{-1}. [b] Band I. [c] Band II; alternatively the 1080-cm^{-1} peak may be associated with band III; see text. [d] Band III.

former B population in CCl_4 solutions. For the C_5 conformer the band corresponding to the B mode near 595 cm^{-1} appears near 635 cm^{-1}. As in the case of conformer B, C_5 gives rise to a peak near 695 cm^{-1}. In the analysis below a case will be made for the assignment of the conformationally insensitive feature near 695 cm^{-1} to a vibration involving the S–C bond in the C–C(=S)–S–C– group. We know from the single crystal data for *N*-(*p*-nitrobenzoyl)glycine ethyl dithioester that conformer A gives rise to dithioester peaks near 660 and 680 cm^{-1}. In the spectra of compounds **1** and **2** in CH_3CN (Figures 7 and 5), the 660-cm^{-1} peak is seen as a shoulder on the S–C peak near 685 cm^{-1}. However, as shown in our earlier studies concerning ethyl and methyl dithioacetate, the S–C peak can be shifted to 730 cm^{-1} by replacing $-SC_2H_3$ with $-SCH_3$ (Teixeira-Dias et al., 1982). The RR spectrum of the $-SCH_3$ analogue of compound **2** (Figure 11) clearly shows the conformer A peak at 667 cm^{-1}. In summary, we have precise data on the structure of conformers A, B, and C_5 from X-ray crystallographic and theoretical analyses, and by combining these with spectroscopic studies on the crystalline materials and the molecules in solution, we are able to define the conformational states of the *N*-acylglycine derivatives in solution. Moreover, we are able to assign a characteristic RR signature for each of the conformers in solution, and these relationships are given in Table II.

RR Spectra of the ^{13}C- and ^{15}N-Substituted Dithioesters in Solution. The RR spectra of $PhC(=O)NHCH_2{}^{13}C(=S)SC_2H_5$ and $PhC(=O)^{15}NHCH_2C(=S)SC_2H_5$ are compared with the spectrum of the unlabeled compound in the crystalline phase (Figure 3) in CH_3CN solution (Figure 5) and in CCl_4 solution (Figure 6). The analogous solution spectra for compound **1** appear in Figures 7 and 8. Data for the ND derivatives in CH_3CN are also shown in Figures 5 and 7. The results are summarized in Tables III and IV, which also include the FTIR data from Figures 9 and 10. In addition, Tables III and IV assign each band to conformer A, or B, or C_5.

As might be expected the RR spectra are more sensitive to ^{13}C than to ^{15}N or ND substitution. However, the effects of substituting any isotope are seen only in the 1040–1180-cm^{-1} region. Although the 538-cm^{-1} peak of solid **2** is sensitive to $^{13}C{=}S$ substitution (Figure 3), the solution RR spectra between 500 and 700 cm^{-1} are virtually unchanged by either ^{15}N, ^{13}C, or ND replacement. The insensitivity of peaks in the 500–700-cm^{-1} range to ^{13}C substitution resembles the behavior of methyl and ethyl dithioacetate upon placing ^{13}C in the C=S group (Teixeira-Dias et al., 1982). Additionally, in this range there is a similarity in the shift of a band near 680–730 cm^{-1} upon going from the ethyl to methyl esters in both classes of compound. By analogy with ethyl and methyl dithioacetate, the RR feature occuring near 690 cm^{-1} in conformers A, B, and C_5 is assigned to a mode possessing a degree of S–C [in –C(=S)S–C–] stretching character. The band between 590 and 660 cm^{-1}, which is more sensitive to conformational changes, is attributed to a highly coupled mode which may involve C–C, C–S, and C=S stretches coupled to skeletal deformations. The origin of the conformer B feature occuring for some *N*-acylglycine dithioesters near 540 cm^{-1} is presently unknown.

For compound **2** in CH_3CN, band I shifts from 1165 to 1134 cm^{-1} upon ^{13}C substitution (Figure 5). Band II shows a smaller shift of 24 cm^{-1} upon ^{13}C substitution. The same situation is found in CCl_4 (Figure 6) and for the FTIR data (Figures 9 and 10). The normal mode analyses of ethyl and

methyl dithioacetate show that the intense RR feature for these molecules near 1190 cm^{-1} has major contributions from $\nu_{C=S}$ and ν_{C-C} [from C–C(=S)S], and the present results are consistent with bands I and II having a similar origin. However, it is likely that the differences between conformers A and B, which are mainly due to a rotation of ~150° about the C–C bond, modulate the vibrational coupling in and around the –C–C(=S)S– skeleton. This would change the quantitative normal mode description of bands I and II and account for their differential sensitivity to ^{13}C substitution. The shoulder at 1162 cm^{-1} seen in Figure 5 for the ^{13}C derivative in CH_3CN may contain some contribution from residual ^{12}C compound and probably has a contribution from the complex conformer B peak seen in this region in Figure 3. For compound **1** band I shows a shift of 29 cm^{-1} in both CH_3CN and CCl_4 (Figures 7 and 8). This is quite similar to the shift seen for band I in compound **2** but the response of band II to ^{13}C substitution is quite different in the two molecules. Band II shows a simple shift in **2**, but in the RR spectrum of the ^{13}C derivative of **1** (Figure 7, bottom) it is necessary to account for an additional feature. In the ^{13}C spectrum, band I is at 1136 cm^{-1}, band II has apparently moved to 1115 cm^{-1} and diminished in intensity relative to band I, and finally there are two features at 1068 and 1079 cm^{-1} (the 1037-cm^{-1} peak apparently shifting very slightly to lower wavenumbers in the ^{13}C derivative). Remarkably similar behavior is observed in the corresponding dithioacylpapain (Storer et al., 1983), although no evidence is found for band I from a conformer A population in the enzyme spectra. For the present ethyl esters, the *sum* of the integrated intensities of the 1079- and 1115-cm^{-1} features relative to band I is the same in the ^{12}C and ^{13}C compounds. Hence, one explanation is that for compound **1** band II shifts to the 1100-cm^{-1} region in the ^{13}C analogue and is then split into the 1079- and 1115-cm^{-1} components by an interaction within the B type molecule. The origin of the splitting is presently unknown, but one candidate would be a Fermi type interaction with the overtone of a fundamental occurring near 550 cm^{-1}. An alternative explanation is that, in the ^{13}C analogue, band II shifts to 1115 cm^{-1} and that the 1068- and 1079-cm^{-1} peaks originate from band III. Band III in the unlabeled compound may contain two unresolved features which shift differentially upon ^{13}C substitution, or it may contain a single feature which is split by a vibrational interaction in the ^{13}C compound. It is difficult to favor strongly one explanation, but the important point is that the B form spectrum for the ^{13}C substituted molecule is highly characteristic and may be used as a benchmark for the dithioacyl enzyme work.

The reason for synthesizing the ^{15}N and ND derivatives was to gauge the extent to which motions in the amide portion of the *N*-acylglycine molecules must be taken into account in the normal mode description of the RR features. Attention is focused on the amide by the finding that the position of band III is sensitive to –NH being replaced by –ND and by the fact that the nitrogen atom plays an important stereochemical role in conformer B. In the latter conformer the nitrogen atom approaches the thiol sulfur at less than van der Waals contact, and this changes the electron distribution in the dithioester moiety compared to that of conformer A. The data (Figures 5–8) show that the RR spectra are only slightly perturbed by ^{15}N substitution. In repeated experiments band III of compound **1** showed a reproducible shift of 3–4 cm^{-1} to lower frequency in the ^{15}N derivative. In crystal spectra of compound **2** (Figure 3) there is an apparent increase in the relative intensity of the 1158-cm^{-1} peak in the ^{15}N analogue, probably due to a decrease in frequency of the 1164-cm^{-1} component seen in the unlabeled compound. A similar situation is encountered in the corresponding dithioacyl enzymes (Storer et al., 1983). Band III in the RR spectra (Figure 7) is also sensitive to ND substitution, moving ~10 cm^{-1} to higher frequency and decreasing in relative intensity in this compound. The sensitivity of band III to $^{13}C(=S)$ substitution, discussed above, indicates that the normal mode giving rise to this feature involves the motion of the thiocarbonyl carbon, but at the same time, the ND and ^{15}N results suggest that there must be fairly extensive vibrational coupling into the NH–C–C(=S) portion.

In summary, in the 1000–1200-cm^{-1} range it appears that conformer A gives rise to one RR feature near 1170 cm^{-1} that is sensitive only to $^{13}C{=}S$ substitution. In contrast, conformer B has a suite of bands in this region; band II, the 1045-cm^{-1} feature, and sometimes band III. Bands II and III, in particular, show complex behavior on ^{15}N, ND, and $^{13}C{=}S$ substitution, suggesting that the modes giving rise to these bands are vibrationally delocalized. While this prohibits simple explanations based on a group frequency approach, the peculiar sensitivity to isotopic replacements provides a unique set of standards by which to compare the behavior of the corresponding dithioacylpapains in the following paper (Storer et al., 1983).

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Registry No. $PhC(=O)NHCH_2{}^{13}C(=S)SC_2H_5$, 86584-14-7; $PhC(=O)^{15}NHCH_2C(=S)SC_2H_5$, 86584-15-8; $PhCH_2CH_2C(=O)NHCH_2{}^{13}C(=S)SC_2H_5$, 86584-16-9; $PhCH_2CH_2C(=O)^{15}NHCH_2C(=S)SC_2H_5$, 86584-17-0; $PhC(=O)NHCH_2C(=S)SC_2H_5$, 24748-71-8; $PhCH_2CH_2C(=O)NHCH_2C(=S)SC_2H_5$, 81309-43-5; $CH_3OC_6H_4$-*p*-$CONHCH_2C(=S)SC_2H_5$, 86584-18-1; $CH_3C_6H_4$-*p*-$CONHCH_2C(=S)SC_2H_5$, 86584-19-2; ClC_6H_4-*p*-$CONHCH_2C(=S)SC_2H_5$, 86584-20-5; $AcNHCH_2C(=S)SC_2H_5$, 77055-32-4; $O_2NC_6H_4$-*p*-$CONHCH_2C(=S)SC_2H_5$, 81811-79-2.

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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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