Precise Structural Information for Transient Enzyme–Substrate Complexes by a Combined X-ray Crystallographic–Resonance Raman Spectroscopic Approach†

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ABSTRACT: A combined resonance Raman (RR) spectroscopic and X-ray crystallographic analysis of single crystals of dithioester model compounds is used to set up precise structure–spectra correlations. In turn, these correlations allow us to obtain detailed information on the acyl group structure in some acylpapain intermediates. According to spectral criteria, *N*-acetylglycine ethyl dithioester crystallizes in a form closely resembling the conformation (conformer B) taken up by the majority of acyl groups in an acylpapain. Similarly, *N*-(*p*-nitrobenzoyl)glycine ethyl dithioester crystallizes in a form (conformer A) which has some RR spectral properties resembling those of the acyl groups in the minor population of acylpapain. However, there is insufficient RR spectral data for the minor population to identify the conformation of its acyl group with certainty. X-ray crystallographic analysis shows that the major conformational difference between the *N*-acetyl and *p*-nitrobenzoyl derivatives is a rotation (~150°) about the [$NHCH_2$–C(=S)S] C–C bond in the glycine moiety. In conformer B (as exemplified by the *N*-acetyl derivative), the amide nitrogen atom comes into close contact with the thiol sulfur atom, an interaction which can account for the unusual spectral properties of conformer B and the fact that the C(=S)–S single bond is appreciably (0.027 Å) shorter in conformer B than in conformer A. In the case of conformer B the RR data allow us to extrapolate the crystallographic results for the model compound to the structure of the enzyme-bound acyl group. This process demonstrates that for the majority of the acyl-enzyme molecules (those assuming conformation B) the C–S bond undergoing cleavage is unexpectedly shortened and is on the order of 0.03 Å shorter than the same bond in the minor conformational population. The possible causes and consequences of this *strengthening* of the bond undergoing cleavage are discussed.

An understanding of the molecular nature of enzymic catalysis remains a major goal of biochemical and biophysical investigations. An important step toward this goal is a detailed structural and dynamical description of the intermediates formed during catalytic turnover. For certain intermediates we have shown recently that it is possible, by using the resonance Raman (RR) technique, to monitor the vibrational spectrum of the scissile bonds in the enzyme's active site (Storer et al., 1979; Ozaki et al., 1982). This observation assumes importance due to the fact that while the vibrations of a molecule are by nature a dynamic property, the vibrational spectrum can, in a number of ways, provide detailed information on molecular structure.

The RR technique provides the means by which we can obtain the vibrational spectrum associated with the bonds undergoing transformation in the active site. RR spectra were obtained for a series of specific acylpapains in the preceding paper (Ozaki et al., 1982) by utilizing the chromophoric properties of dithioesters. The cysteine protease catalyzed hydrolysis of a thionoester proceeds via the formation of a chromophoric dithioacyl-enzyme intermediate (Lowe & Williams, 1965) in which the –C(=S)–S– group linking the acyl and enzyme moieties has an intense electronic absorption band near 315 nm. When this band is excited with laser radiation of 324.0 or 337.5 nm in wavelength, a RR spectrum of the labile –C(=S)–S– group is obtained. Consequently, a vibrational description of those bonds undergoing transformation is accessible. Qualitative interpretation of the RR spectra of six dithioacyl-enzymes in the preceding paper (Ozaki et al., 1982) allowed us to identify the presence of two acyl group conformations in the active site. In any acyl-enzyme population the majority of acyl groups assume the conformation designated conformer B, by analogy to the spectral properties of related model compounds, in which there is a strong intramolecular interaction in the acyl group between the amide and the dithioester moieties. The acyl conformation of the minor population of acyl-enzymes is not known due to the lack of RR spectral data on this population. The minor population, however, does lack the intramolecular interaction characterizing conformer B and does have certain RR spectral properties analogous to those of model compounds in a conformation we have designated conformer A (Ozaki et al., 1982; Storer et al., 1982). Conformers A and B, in either model compounds or acyl-enzymes, thus far have only been partially characterized by spectroscopic means, and the purpose of the present paper is to precisely define the nature of the two conformers by a joint X-ray crystallographic and Raman spectroscopic study on single crystals of suitable model compounds.

The results allow us to precisely define conformers A and B for two dithioester model compounds and to identify the origin of the important intramolecular interaction of conformer B that perturbs the properties of the dithioester group. The model compound *N*-acetylglycine ethyl dithioester mimics closely the RR properties of the major population of the transient acyl-enzyme, and consequently the RR spectra can be used as a vector to transfer the precise structural information obtained by X-ray crystallographic techniques on the "small" molecule to the biological site that is less readily accessible to such detailed scrutiny. In this manner we have been able to detect changes in lengths of the scissile bond in the active site of the acylpapains which may have important consequences for the intermediates' kinetic properties.

Experimental Procedures

Both compounds crystallized as clear, yellow prisms.

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Crystals of the *N*-acetyl and *p*-nitrobenzoyl compounds were formed by diffusing hexane vapor into solutions of the compounds in ether or CH_3CN (5%)/ether (95%), respectively. Preliminary precession photographs gave approximate cell dimensions and showed that in both cases the symmetry was monoclinic and the space group was $P2_1/c$. Accurate cell dimension and relative intensity measurements were made on an Enraf-Nonius CAD-4 diffractometer. The $\omega/2\theta$ scan method was used for measuring intensities.

N-Acetylglycine ethyl dithioester ($C_6H_{11}NOS_2$, M_r 177.29) had the following parameters: a = 17.865(4), b = 4.829(19), and c = 15.423(3) Å, β = 135.46(2)°, V = 933.24 Å^3, Z = 4, d_x = 1.26 Mg m^{-3}, specimen dimensions 0.2 × 0.3 × 0.2 mm, Ni-filtered Cu Kα radiation [λ(Cu Kα_1) = 1.54056 Å, μ(Cu Kα) = 4.64 mm^{-1}]. Two check reflections were monitored after every 4000 s of X-ray exposure, and their intensities, which decreased about 4% during the data collection, were used to scale the data. Of the 1902 independent reflections (*hkl* and *hkl̄*) in the range $2\theta \leq 150°$, 1185 had $I \geq 1.43\sigma(I)$. The remainder were classified as unobserved.

N-(*p*-Nitrobenzoyl)glycine ethyl dithioester ($C_{11}H_{12}N_2O_3S_2$, M_r 284.36) had the following parameters: a = 14.767(3), b = 9.202(3), and c = 10.199(2) Å, β = 106.93(1)°, V = 1326.0 Å^3, Z = 4, d_x = 1.424 Mg m^{-3}, specimen dimensions 0.5 × 0.3 × 0.5 mm, graphite-monochromatized Mo Kα radiation [λ(Mo Kα_1) = 0.70930 Å, μ(Mo Kα) = 0.39 mm^{-1}].

Two check reflections, monitored after every 4000 s of X-ray exposure, showed only random fluctuations (±2.2%) in intensity. Out of 2330 measured independent reflections (*hkl* and $\bar{h}kl$) in the range $2\theta \leq 50°$, 1587 had intensities $\geq 2\sigma(I)$.

Both structures were solved routinely with MULTAN78 (Main et al., 1978). Refinement was by block-diagonal least squares, minimizing $\Sigma w(\Delta F)^2$, and using isotropic temperature factors initially and anisotropic parameters subsequently for the non-hydrogen atoms. The hydrogen atoms were located on difference maps and were refined isotropically. Scattering factor values for S, O, N, and C and a correction for the real part of anomalous scattering for the S curve were taken from *International Tables for X-ray Crystallography* (1974). Scattering factor values for H were taken from Stewart et al. (1965). At convergence $R(F) = 0.035_2$ and $R_w(F^2) = 0.0423$ for the *p*-nitrobenzoyl compound and $R(F) = 0.062_8$ and $R_w(F^2) = 0.075$ for the *N*-acetyl compound, in both cases for all observed reflections. Difference maps were calculated for both compounds from the final structure factors [tables of observed and calculated structure factors and of anisotropic thermal parameters for both compounds are available (see paragraph at end of paper regarding supplementary material)], and no residual density was observed above 0.18 (for the *p*-nitrobenzoyl compound) and 0.40 e/Å^3 (for the *N*-acetyl compound). All calculations, unless otherwise mentioned, were done with the NRC set of crystallographic programs (Ahmed et al., 1973).

The equipment used to record the Raman and RR spectra has been described elsewhere (Ozaki et al., 1982; Storer et al., 1982). The RR spectrum of crystalline *N*-(*p*-nitrobenzoyl)-glycine ethyl dithioester was obtained by crushing single crystals of the compound and pressing the resultant powder into a KBr matrix within a rotatable Raman cell designed for solid samples.

Results

The *N*-acetylglycine and *N*-(*p*-nitrobenzoyl)glycine ethyl dithioesters were selected on the basis that single crystals of these compounds gave rise to Raman spectroscopic signatures of the B conformer and the A conformer, respectively. We have surveyed the spectral properties of single crystals of several *N*-acylglycine dithioesters (unpublished work), and thus far only the *N*-(*p*-nitrobenzoyl) compound has crystallized in the "A" form, an observation that is consonant with the greater thermodynamic stability of the B conformer (Storer et al., 1982). For either conformer A or conformer B the Raman and RR spectra tend to be similar in appearance (although the absolute intensities of the RR spectra are much higher), and thus conclusions reached for the Raman spectra can be transferred to the RR case and vice versa. The Raman spectroscopic signatures of conformers A and B have been dealt with in the preceding paper (Ozaki et al., 1982), and the description here will rely on a knowledge of the preceding paper. In that paper and in an earlier publication dealing with model compounds (Storer et al., 1982) the discussion focused on the so-called C=S stretching region between 1050 and 1200 cm^{-1}, and it was shown that the A conformer gives rise to an intense Raman or RR band between 1160 and 1180 cm^{-1}. In the B conformer this band is replaced by a feature between 1120 and 1155 cm^{-1} and, sometimes, a second feature between 1080 and 1100 cm^{-1}.

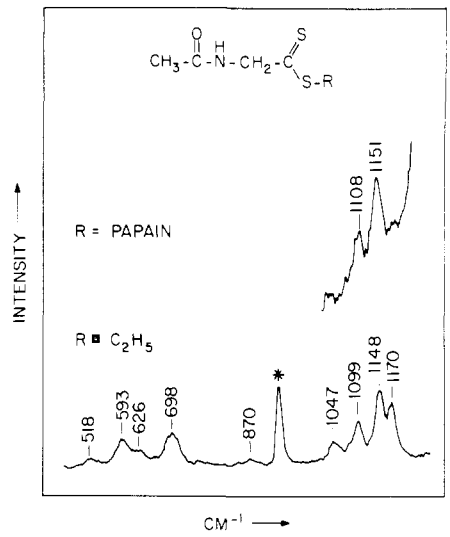


FIGURE 1: RR spectra of $CH_3C(=O)NHCH_2C(=S)S$-papain (top) and 4 × 10^{-4} M $CH_3C(=O)NHCH_2C(=S)SC_2H_5$ in CH_3CN/H_2O (1:4 v/v) (bottom). The conditions used for obtaining the acyl-enzyme RR spectrum are given in Ozaki et al. (1982). Spectral conditions for both spectra: ~20 mW, 324-nm excitation, spectral slit ~10 cm^{-1}. Asterisk denotes feature due to CH_3CN.

The RR spectrum of *N*-acetylglycine ethyl dithioester in aqueous acetonitrile contains the characteristic spectral signatures of the two conformers A and B (Figure 1). In the "C=S stretching region" between 1050 and 1200 cm^{-1}, band I at 1170 cm^{-1} is assigned to conformer A while band II at 1148 cm^{-1} and band III at 1099 cm^{-1} are signature frequencies for conformer B. Between 500 and 700 cm^{-1} there are two main features at 593 and 698 cm^{-1}. For the corresponding dithioacylpapain, a partial RR spectrum scanning only the C=S stretching region contains the RR signature bands of conformer B, namely, bands II and III at 1151 and 1108 cm^{-1}, respectively (Figure 1). A band near 1170 cm^{-1} cannot be reliably detected above the spectral background. The strong preference for conformer B in the native dithioacylpapain has been established for this and other dithioacylpapain intermediates (Ozaki et al., 1982).

Crystallization of *N*-acetylglycine ethyl dithioester from ether/hexane mixtures afforded crystals suitable for both Raman and X-ray crystallographic investigations. With 514.5-nm laser excitation, the Raman spectrum of a single

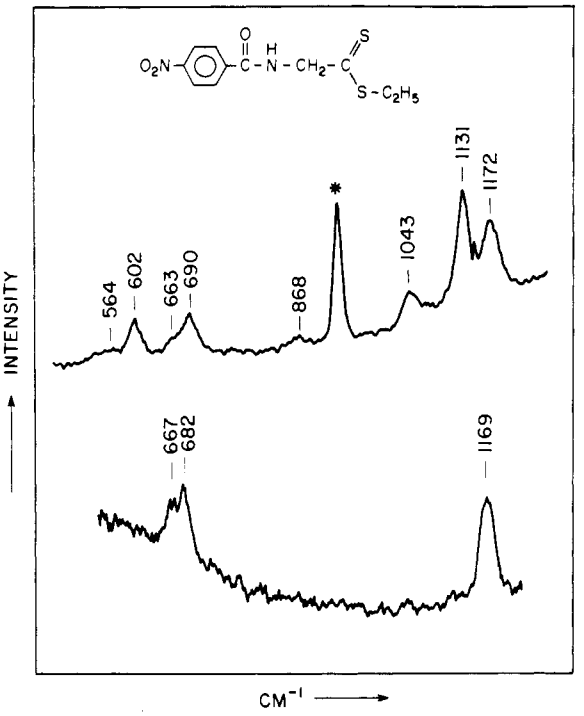


FIGURE 2: RR spectra of ≈4 × 10^{-4} M $pNO_2C_6H_4C(=O)NHCH_2C(=S)SC_2H_5$ in CH_3CN/H_2O (1:4 v/v) (top) and in a crystalline form (bottom). For the dithioester moiety, the RR spectrum closely resembles the Raman spectrum of the single crystal used for X-ray examination. Asterisk denotes feature due to CH_3CN.

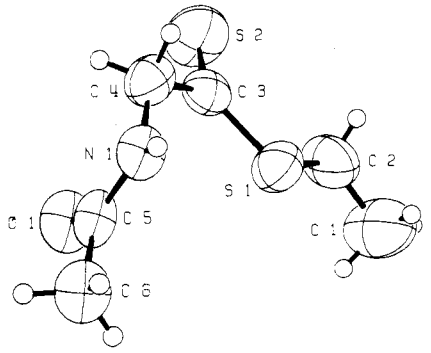


FIGURE 3: Conformer B. The structure of crystalline $CH_3C(=O)NHCH_2C(=S)SC_2H_5$.

crystal of the *N*-acetyl derivative contains the vibrational features characteristic of conformer B, namely, band II at 1141 cm^{-1} and band III at 1088 cm^{-1} (Storer et al., 1982). There is no evidence for band I in the spectrum of the crystals, and similarly in the 500–700-cm^{-1} range there is a major feature at 584 cm^{-1} but no major band near 695 cm^{-1}. A Raman spectrum of the model compound after dissolution of the crystals in aqueous acetonitrile shows significant populations of both conformers A and B. In particular, band I at 1170 cm^{-1} appears after dissolution of the crystals, as does an intense feature at 692 cm^{-1} (Storer et al., 1982).

The RR spectrum of crystalline *N*-(*p*-nitrobenzoyl)glycine ethyl dithioester indicates that this molecule has crystallized as the A conformer. The RR spectrum (Figure 2) contains band I at 1169 cm^{-1}, and band II at 1131 cm^{-1} is present only upon dissolution in aqueous acetonitrile (Figure 2, top spectrum). Furthermore, a band appears in the solution spectrum at 602 cm^{-1} which is absent in the RR spectrum of the crystalline form.

The so-called C–S stretching region, between 500 and 700 cm^{-1}, has been little discussed hitherto, partly due to our lack of confidence in transferring normal mode data concerning this region from simple dialkyl dithioesters (Teixeira-Dias et al., 1982) to *N*-acylglycine derivatives. However, a tentative correlation is beginning to emerge in that B conformers seem to be characterized by an intense feature near 595 cm^{-1} and no bands near 680 cm^{-1}, while A conformers have two intense bands near 660 and 680 cm^{-1} but lack a feature near 595 cm^{-1}. This correlation is borne out by the results for the two compounds discussed here and has been observed also for several other *N*-acylglycine ethyl dithioesters (unpublished work).

Detailed molecular structures of the *N*-acetylglycine ethyl dithioester (conformer B) (Figure 3) and of the *N*-(*p*-nitrobenzoyl)glycine ethyl dithioester (conformer A) (Figure 4)

Table I: Fractional Coordinates (×10^4, except ×10^3 for Hydrogen) for *N*-Acetylglycine Ethyl Dithioester

	x	*y*	*z*
S(1)	2697 (1)	7937 (3)	301 (1)
S(2)	3535 (1)	10418 (4)	2621 (1)
O(1)	320 (3)	10863 (5)	−768 (3)
N(1)	850 (3)	6479 (6)	−197 (3)
C(1)	3907 (5)	9914 (22)	6 (6)
C(2)	3872 (4)	9760 (16)	904 (5)
C(3)	2671 (3)	8567 (9)	1364 (4)
C(4)	1760 (4)	7202 (10)	1083 (4)
C(5)	163 (3)	8382 (8)	−1027 (4)
C(6)	−798 (4)	7360 (11)	−2300 (5)
H(1)	66 (3)	464 (10)	−39 (4)
H(11)	330 (5)	1095 (17)	−61 (6)
H(12)	453 (5)	1049 (15)	27 (6)
H(13)	390 (7)	778 (21)	−3 (8)
H(21)	385 (6)	1191 (18)	105 (7)
H(22)	437 (4)	868 (12)	152 (5)
H(41)	156 (3)	814 (10)	141 (4)
H(42)	204 (4)	558 (11)	157 (4)
H(61)	−138 (4)	774 (12)	−244 (4)
H(62)	−95 (4)	539 (13)	−243 (5)
H(63)	−75 (5)	783 (14)	−280 (5)

Table II: Fractional Coordinates (×10^4, except ×10^3 for Hydrogen) for *N*-(*p*-Nitrobenzoyl)glycine Ethyl Dithioester

	x	*y*	*z*
S(1)	7653 (0)	1493 (1)	10357 (1)
S(2)	6722 (1)	636 (1)	7450 (1)
O(1)	4530 (1)	−171 (2)	8142 (2)
O(2)	498 (2)	1036 (3)	2890 (3)
O(3)	1099 (2)	2938 (4)	2301 (3)
N(1)	5024 (1)	2112 (2)	7996 (2)
N(2)	1137 (2)	1904 (3)	3039 (3)
C(1)	9110 (2)	1785 (5)	9192 (5)
C(2)	8633 (2)	731 (4)	9892 (4)
C(3)	6710 (2)	1354 (3)	8889 (2)
C(4)	5836 (2)	1969 (3)	9180 (3)
C(5)	4427 (2)	1011 (3)	7554 (2)
C(6)	3590 (2)	1280 (3)	6341 (2)
C(7)	3547 (2)	2406 (3)	5413 (3)
C(8)	2743 (2)	2595 (3)	4335 (3)
C(9)	1996 (2)	1686 (3)	4196 (3)
C(10)	2017 (2)	562 (3)	5077 (3)
C(11)	2825 (2)	367 (3)	6154 (3)
H(1)	499 (2)	288 (3)	757 (3)
H(11)	866 (2)	219 (4)	841 (3)
H(12)	956 (2)	128 (4)	879 (4)
H(13)	938 (2)	245 (4)	982 (4)
H(21)	899 (2)	39 (3)	1073 (3)
H(22)	841 (2)	−10 (4)	929 (3)
H(41)	596 (2)	286 (3)	960 (2)
H(42)	568 (2)	130 (3)	983 (3)
H(71)	404 (2)	300 (3)	549 (2)
H(81)	269 (2)	327 (3)	382 (3)
H(101)	150 (2)	0 (3)	498 (3)
H(111)	285 (2)	−34 (3)	675 (3)

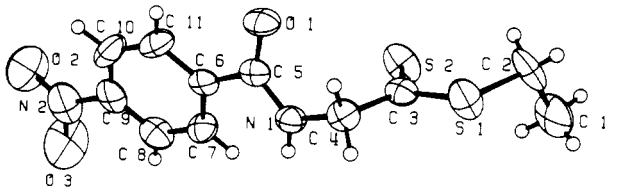


FIGURE 4: Conformer A. The structure of crystalline $pNO_2C_6H_4C(=O)NHCH_2C(=S)SC_2H_5$.

Table III: Molecular Dimensions for *N*-Acetylglycine Ethyl Dithioester (Conformer B)[a]

(a) Bond Distances (Å)

S(1)-C(2)	1.807 (9)	N(1)-C(5)	1.323 (6)
S(1)-C(3)	1.700 (6)	C(1)-C(2)	1.433 (13)
S(2)-C(3)	1.635 (5)	C(3)-C(4)	1.504 (11)
O(1)-C(5)	1.231 (5)	C(5)-C(6)	1.497 (7)
N(1)-C(4)	1.444 (6)		

(b) Bond Angles (Deg)

C(2)-S(1)-C(3)	103.7 (3)	S(2)-C(3)-C(4)	119.7 (4)
C(4)-N(1)-C(5)	121.2 (5)	N(1)-C(4)-C(3)	115.7 (5)
S(1)-C(2)-C(1)	111.3 (6)	O(1)-C(5)-N(1)	121.5 (5)
S(1)-C(3)-S(2)	126.1 (4)	O(1)-C(5)-C(6)	122.0 (5)
S(1)-C(3)-C(4)	114.2 (4)	N(1)-C(5)-C(6)	116.5 (5)

(c) Selected Torsional Angles (Deg)

S(1)-C(3)-C(4)-N(1)	−22.2
S(2)-C(3)-C(4)-N(1)	158.7
C(1)-C(2)-S(1)-C(3)	−168.5
C(3)-C(4)-N(1)-C(5)	−76.3
C(4)-N(1)-C(5)-O(1)	6.4

(d) Selected Mean Plane Calculations: Deviations (Å) of Atoms from Least-Squares Planes

S(1),[b] 0.000 (2); S(2),[b] 0.000 (2); C(2), −0.020 (7); C(3),[b] 0.006 (5); C(4),[b] −0.003 (5)
O(1),[b] 0.008 (6); N(1),[b] 0.018 (6); C(4),[b] −0.017 (9); C(5),[b] −0.030 (8); C(6), −0.105 (9); H(1), −0.14 (7)

(e) Hydrogen Bonding Geometry

Distances (Å)

N(1)-H(1)	0.92 (5)	O(1)···H(1)[c]	1.88 (5)
O(1)···N(1)[c]	2.799 (4)		

Angles (Deg)

C(4)-N(1)-H(1)	118 (3)	N(1)-H(1)···O(1)[c]	178 (5)
C(5)-N(1)-H(1)	119 (3)	C(5)-O(1)···H(1)[c]	174 (2)

[a] The standard deviations (in parentheses) refer (throughout) to the least significant digit. [b] Atoms used to define the plane. [c] Atom belonging to an adjacent molecule.

Table IV: Molecular Dimensions for *N*-(*p*-Nitrobenzoyl)glycine Ethyl Dithioester (Conformer A)[a]

(a) Bond Distances (Å)

S(1)-C(2)	1.791 (4)	C(1)-C(2)	1.497 (6)
S(1)-C(3)	1.727 (3)	C(3)-C(4)	1.516 (4)
S(2)-C(3)	1.615 (3)	C(5)-C(6)	1.493 (4)
O(1)-C(5)	1.230 (3)	C(6)-C(7)	1.393 (4)
O(2)-N(2)	1.212 (4)	C(6)-C(11)	1.377 (4)
O(3)-N(2)	1.205 (5)	C(7)-C(8)	1.374 (4)
N(1)-C(4)	1.439 (3)	C(8)-C(9)	1.359 (5)
N(1)-C(5)	1.331 (3)	C(9)-C(10)	1.364 (4)
N(2)-C(9)	1.472 (4)	C(10)-C(11)	1.378 (4)

(b) Bond Angles (Deg)

C(2)-S(1)-C(3)	104.5 (2)	O(1)-C(5)-C(6)	120.4 (2)
C(4)-N(1)-C(5)	121.5 (2)	N(1)-C(5)-C(6)	117.4 (2)
O(2)-N(2)-O(3)	123.3 (3)	C(5)-C(6)-C(7)	123.4 (2)
O(2)-N(2)-C(9)	117.9 (3)	C(6)-C(7)-C(8)	119.7 (3)
O(3)-N(2)-C(9)	118.7 (3)	C(7)-C(8)-C(9)	119.5 (3)
S(1)-C(2)-C(1)	113.7 (3)	N(2)-C(9)-C(8)	118.9 (3)
S(1)-C(3)-S(2)	126.8 (2)	N(2)-C(9)-C(10)	118.7 (3)
S(1)-C(3)-C(4)	108.7 (2)	C(8)-C(9)-C(10)	122.4 (3)
S(2)-C(3)-C(4)	124.5 (2)	C(9)-C(10)-C(11)	118.2 (3)
N(1)-C(4)-C(3)	114.8 (2)	C(6)-C(11)-C(10)	121.1 (3)
O(1)-C(5)-N(1)	122.2 (2)		

(c) Selected Torsional Angles (Deg)

S(1)-C(3)-C(4)-N(1)	−171.7
S(2)-C(3)-C(4)-N(1)	10.6
O(1)-C(5)-C(6)-C(11)	−18.5
C(1)-C(2)-S(1)-C(3)	−83.9
C(3)-C(4)-N(1)-C(5)	−86.2
C(4)-N(1)-C(5)-O(1)	−1.1

(d) Selected Mean Plane Calculations: Deviations (Å) of Atoms from Least-Squares Planes

S(1),[b] 0.000 (1); S(2),[b] 0.000 (1); C(2), 0.020 (4); C(3),[b] 0.017 (3); C(4),[b] −0.006 (3)
O(1),[b] −0.001 (2); N(1),[b] −0.003 (2); C(4),[b] 0.002 (3); C(5),[b] 0.005 (3); C(6), −0.041 (3); H(1), 0.07 (3)

(e) Hydrogen Bonding Geometry

Distances (Å)

N(1)-H(1)	0.83 (3)	O(1)···H(1)[c]	2.13 (3)
O(1)···N(1)[c]	2.912 (3)		

Angles (Deg)

C(4)-N(1)-H(1)	115 (2)	N(1)-H(1)···O(1)[c]	158 (2)
C(5)-N(1)-H(1)	123 (2)	C(5)-O(1)···H(1)[c]	125.7 (7)

[a] The standard deviations (in paretheses) refer (throughout) to the least significant digit. [b] Atoms used to define the plane. [c] Atom belonging to an adjacent molecule.

were determined by X-ray crystallographic studies. Final positional parameters are listed in Tables I and II. Tables III and IV each give bond distances, bond angles, selected torsional angles, mean plane calculations, and the hydrogen bonding geometry for the *N*-acetyl and *p*-nitrobenzoyl derivatives, respectively. The C–H distances (not listed) vary from 0.80 to 1.07 Å with a mean of 0.93 Å.

In the model compounds the torsional angles N(1)–C(4)–C(3)–S(1) and C(5)–N(1)–C(4)–C(3) are important since it is here that the major differences between conformers A and B are found. These angles are somewhat analogous to the main chain dihedral angles ψ and ϕ, respectively, defined for polypeptide chains, and we shall designate the corresponding angles in the present compounds ψ' and ϕ'. By use of the corresponding nomenclature recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (1970) for polypeptides, the torsional angle N(1)–C(4)–C(3)–S(1) of the *N*-(*p*-nitrobenzoyl) derivative (ψ') = −171.7° while C(5)–N(1)–C(4)–C(3) (ϕ') = −86.2°. However, for the *N*-acetylglycine ethyl dithioester model compound, the corresponding ψ' and ϕ' angles are −22.2° and −76.3°, respectively.

For both model compounds, the dithioester groups defined by C(2), S(1), C(3), and S(2) are planar to within 0.02 Å and adopt the S-cis conformation typical of esters (Jones & Owen, 1973). In particular, there is no evidence for pyramidalization of the C(3) carbon in the *N*-acetyl molecule. The amide groups in both compounds are slightly nonplanar; in the *N*-acetyl compound there is evidence (Table III) for slight pyramidalization at the amide nitrogen while for the *p*-nitrobenzoyl derivative (Table IV) there appears to be a slight pyramidalization about the C=O carbon atom. For the *N*-(*p*-nitrobenzyl) derivative the aromatic ring is twisted out of the plane of the amide by −18.5° [torsional angle O(1)–C(5)–C(6)–C(11)]. The bond lengths and angles within the amide moieties of both compounds are markedly similar and agree satisfactorily with those of the usual trans peptide group (Sutton, 1965).

There is no evidence for intramolecular or intermolecular hydrogen bonding between the hydrogen of the amide and either of the sulfur atoms of the dithioester moiety. Instead, in the crystal structure, the hydrogen bonding is to the carbonyl of an adjacent molecule. The hydrogen bonding distances between the donor and acceptor atom are typical for amide–

carbonyl hydrogen bonds (2.9 ± 0.1 Å).

For the *N*-acetyl derivative the nonbonded contact distance between N(1) and the sulfur of the ester link S(1) is unusually short. This N(1)···S(1) nonbonded distance of 2.891 Å is approximately 0.45 Å shorter than the sum of the van der Waals radii (3.35 Å). Rotation about the C(3)–C(4) bond by 148.7° converts conformer B to a conformation that closely resembles conformer A and in which N(1) is in close contact with the other sulfur atom; in the *p*-nitrobenzoyl structure the N(1)···S(2) nonbonded distance is 3.043 Å.

In order to estimate the degree of π bonding between divalent sulfur and an sp^2 carbon, Hordvick (1970) proposed a bond length/π-bond order relationship using C–S single bond and double bond values of ($C_{(sp^2)}$–S) = 1.82 Å and ($C_{(sp^2)}$=S) = 1.61 Å for π-bond orders of 0 and 1, respectively. From our crystallographic data on the *p*-nitrobenzoyl derivative, the C(3)–S(2) bond length of 1.615 Å indicates a π-bond order of 1 for C=S, whereas the slightly longer C(3)–S(2) bond of 1.635 Å for the *N*-acetyl derivative suggests a marginally diminished π-bond order of approximately 0.9.

For the *N*-acetyl derivative, the C(3)–S(1) bond length of 1.700 Å is considerably shorter than the S(1)–C(2) single bond length of 1.807 Å. This difference of 0.107 Å is presumably due to electron delocalization between the thiocarbonyl and the ester sulfur atom. Consequently, the C(3)–S(1) bond possesses some double bond character, and from the bond length/π-bond order relationship proposed by Hordvick, this C–S single bond has a π-bond order of 0.6–0.65. In comparison, the C(3)–S(1) bond in the *N*-(*p*-nitrobenzoyl) derivative is 1.727 Å, which suggests a reduced π-bond order of 0.5. These crystallographic findings are fully consistent with the RR results which show that the C–S linkage must be considered as part of the chromophore in dithioesters giving rise to the 305-nm absorbance band (Teixeira-Dias et al., 1982).

Discussion

A combined Raman and X-ray crystallographic investigation has allowed us to identify the molecular structures of conformers A and B for two *N*-acylglycine ethyl dithioester derivatives. The major conformational difference arises from rotation by ~150° about the C(3)–C(4) bond together with a small rotation of ~10° about the C(4)–N(1) bond. In both molecules there are unusually short contacts between the amide nitrogen atom and a dithioester sulfur atom, the thiol sulfur in the case of the *N*-acetyl derivative and the thiono sulfur in the case of the *N*-(*p*-nitrobenzoyl) derivative. The N···S interaction in conformer B is particularly interesting because this is the predominant conformer found in the native active site and, in addition, the interaction can be analyzed according to the precepts set out by Rosenfield et al. (1977). These authors have considered the directional preferences of nonbonded atomic contacts between a heteroatom X and the divalent sulfur atom in the Y–S–Z grouping, and they proposed two classifications defined by the directional preference of the X···S nonbonded contact. In general, electrophilic heteroatoms (type I) approach S roughly 20° from the perpendicular to the plane through the atoms Y–S–Z whereas nucleophilic heteroatoms (type II) tend to approach along the extension of one of the covalent bonds to S and from a direction 30° above the plane. In the *N*-acetylglycine ethyl dithioester (Figure 3), the N(1)···S(1) contact approach is only 22° above the plane defining C(2)–S(1)–C(3) and the [C(2)-S(1)···N-(1)] contact angle is 161°. Accordingly, the attractive nonbonded interaction that gives rise to the unusually short N···S contact possesses nucleophilic (type II) character. The preferred thermodynamic stability of conformer B over conformer A (Storer et al., 1982) is probably in part due to this favorable nonbonded interaction. It is also likely that the atypical RR spectroscopic properties of conformer B (Storer et al., 1982) result from the N(1)···S(1) contact. A complete vibrational analysis of a conformer B fragment is required to provide a quantitative demonstration, but clearly the changes in bond lengths and the need to introduce the N···S interaction into the force field calculations will perturb the vibrational frequencies from the "normal" values found for the A conformer.

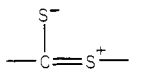
Table V: Comparison of C-S and C=S Bond Lengths from the Literature

fragment	C-S and S-C bond lengths in C(=X)-S-C structures: C-S	S-C
$CC(=O)SC^a$	1.780	1.814
$C_1S_1C_2(=S)C_3(=O)S_2C_4{}^b$	1.719 (C_2-S_1), 1.767 (C_3-S_2)	1.801 (S_1-C_1), 1.809 (S_2-C_4)
$C_6H_5C(=S)SC^c$	1.724	1.788
$C_6H_5SC(=O)C(=O)SC_6H_5{}^d$	1.753	
$OC(=S)SSC(=S)O^e$	1.729	
$CH_3C(=O)NHCH_2C(=S)SC_2H_5{}^g$	1.700	1.807
$pNO_2C_6H_4C(=O)NHCH_2C(=S)SC_2H_5{}^g$	1.727	1.791

fragment	C=S bond lengths
$CSC(=S)C(=O)SC^b$	1.631
$C_6H_5C(=S)SC^c$	1.630
$OC(=S)SSC(=S)O^e$	1.620
$C_6H_5C(=S)OC^f$	1.631
-C(=S)N-	1.67-1.69
$H(CH_3)C=S^h$	1.610
$RRC=S^i$	1.608
$HC(=S)OCH_3{}^j$	1.612
$CH_3C(=O)NHCH_2C(=S)SC_2H_5{}^g$	1.635
$pNO_2C_6H_4C(=O)NHCH_2C(=S)SC_2H_5{}^g$	1.615

[a] Guy & Hamor (1974). [b] Niemer et al. (1978). [c] Adiwidjaja & Voss (1977a). [d] Pellinghelli et al. (1974). [e] Watanabe (1971). [f] Adiwidjaja & Voss (1977b). [g] This work. [h] Kroto & Landsberg (1976). [i] Shirrell & Williams (1973). [j] Johnson et al. (1971).

A detailed discussion of bond lengths within the dithioester group of the two compounds studied here is hampered by a lack of suitable reference compounds. Listed in Table V are a selection of C–S and C=S bond lengths found in the literature, and as is evident from the table, there are no previously analyzed structures consisting of simple dialkyl dithioesters. For this reason we do not know what constitutes normal C–S and C=S bond lengths in dialkyl dithioesters, and the discussion will therefore focus on the differences between the structures determined in the present work. A direct comparison of the bond lengths and angles in the *N*-acetyl derivative (conformer B) with those in the *N*-(*p*-nitrobenzoyl) derivative (conformer A) suggests a polarization of electron density toward the sulfur of the thiocarbonyl in conformer B. In valence bond terms, conformer B contains a greater contribution of the canonical form



Accordingly, in comparison with the corresponding bonds in conformer A, the C(3)–S(1) single bond (1.700 Å) in conformer B is shorter by 0.027 Å (4σ) while the C(3)–S(2) double bond (1.635 Å) is longer by 0.020 Å (>3σ). The C(3)–S(1) single bond in conformer B possesses a high degree of π-bond order (0.6–0.65) and is shorter than any of the

corresponding C–S bonds in Table V. The C(3)=S(2) double bond in conformer B is comparable in length to those C=S bonds listed in Table V which are part of a conjugated π-electron system (footnotes *b*, *c*, and *f* of Table V) and which, therefore, would be expected to have increased single bond character compared to an unconjugated C=S moiety. It is interesting to note that for the *N*-(*p*-nitrobenzoyl) derivative, the short C=S bond length of 1.615 Å corresponds to a π-bond order of 1, typical for simple thioaldehydes, thioketones, and some thionoesters (Table V). Thus the C=S bond length for conformer A suggests very little polarization of the thiocarbonyl.

In conformer B, despite the close contact distance N(1)···S(1), the bond angles about the thiocarbonyl group indicate no significant deformations due to steric repulsion between these atoms. Indeed, the value for the S(1)–C(3)–C(4) bond angle of 114.2(4)° is typical for corresponding bond angles in unhindered dithioesters [112.5° (Adiwidjaja & Voss, 1977a)] and thiol esters [113.0° (Niemer & Mattes, 1978); 115° (Mattes et al., 1977); 112.1° (Guy & Hamor, 1974)].

Significance for the Structures of the Acylpapains. As described above and in detail elsewhere (Storer et al., 1982; Ozaki et al., 1982), in solution *N*-acylglycine ethyl dithioesters exist in two major conformations, A and B, whereas in the corresponding dithioacylpapains between pH 4 and 9 the *N*-acylglycine dithioester is predominantly in the B form. The crystallization of the two compounds described herein, possessing spectral signatures characteristic of the A and B conformers, provided the opportunity to define the conformers precisely by X-ray crystallographic methods. The detailed crystallographic results on the "small" model compounds can now be "carried into" the active site of the transient acylpapains to allow detailed conformational conclusions on the major population in which the acyl group assumes a B-type conformation. Hence, the detailed molecular structure for conformer B of the model compound (Figure 3, Table III) also provides a description of the structure of the predominant dithioacylpapain intermediate. Indeed, the structure in Figure 3 bears a remarkable resemblance to the conformation of an acylpapain intermediate previously proposed by Drenth et al. (1976) on the basis of extrapolation from the X-ray results on a papain–inhibitor complex. The three-dimensional structure of papain has been defined by X-ray diffraction (Drenth et al., 1968), and by using a difference–Fourier technique, Drenth and his co-workers (1976) determined the binding mode for the benzyloxycarbonyl-L-phenylalanyl-L-alanine chloromethyl ketone inhibitor within the active site. Using an electron-density difference map, they obtained a detailed picture of the binding of the R–C(=O)NHCH$_2$–C(=O)–CH$_2$– moiety to the active site. Then, by hypothetically removing the extra methylene group between the carbonyl and the S$_\gamma$ of Cys-25, they constructed a structural model for the acyl moiety (Drenth et al., 1976). The agreement between the results of Drenth and his co-workers, based on an inactive complex, and our results, based on the spectroscopic properties of an active complex, is gratifying. However, as discussed below, the present work goes further in detecting a mixture of conformations in the active acyl-enzyme population and in delineating small but kinetically significant changes in the length of the bond undergoing cleavage.

The presence, in the dithioacylpapain RR spectra, of a weak peak near 1175 cm^{-1} was taken as evidence for a small percentage of the acyl groups existing in a second conformation (Ozaki et al., 1982). The peak appears in the region regarded as normal for $\nu_{C=S}$ in an unperturbed *N*-acylglycine dithioester (Storer et al., 1982). Conformer A gives rise to band I in this same region, but conformer A is but one of many possible unperturbed conformers possessing different ψ' and ϕ' angles. We expect each of these conformers to have a signature in the 1165–1180-cm^{-1} range. Hence, we can only designate the minor conformer as being in a conformation which does not possess a strong intramolecular interaction. A peak due to the minor population in the C–S region is expected to provide further information on the conformation, but this peak is too weak to be detected with our existing equipment.

To form an initial estimate of the difference between a normal and perturbed electron distribution in a dithioester group, we take the crystallographic results for the *p*-nitrobenzoyl compound as representing a normal, unperturbed dithioester group and the *N*-acetyl compound as representing a perturbed dithioester moiety. These results can then be used to estimate the differences between the perturbed (conformer B type) and unperturbed dithioester linkages in the active site. Surprisingly this analysis leads to the conclusion that the scissile C–S bond in the predominant conformer B population is shorter and thus stronger than the same bond in the acyl enzyme in the minor conformer possessing the unperturbed C(=S)S group. Thus the favored acyl-enzyme population possesses a conformation which appears to strengthen the bond undergoing hydrolytic cleavage. The C(3)–S(1) linkage in conformer B represents a very short C–S single bond length (Table V) and appears to be shortened as a result of the N(1)···S(1) intramolecular interaction. The lone pair of electrons in the p orbital of N(1) are directed toward S(1), and this increases the contribution of the canonical form –C(–S$^-$)=S$^+$– with a corresponding increase in double-bond character of the C–S single bond. In molecular orbital terms, the nitrogen pπ electrons, in conformer B, cannot be interacting with the σ^* [C(3)–S(1)] orbital since this would weaken, and hence lengthen, the linkage. The difference in the C(3)–S(1) bond lengths in conformers A and B may also reflect the N(1)···S(2) interaction in conformer A suppressing canonical forms of the type –C(–S$^-$)=S$^+$–, but the very short length of C(3)–S(1) in conformer B (Table III) suggests that the N(1)···S(1) intramolecular interaction in B is the major factor responsible for shortening the C–S bond.

The kinetic consequences of two acyl group structures possessing significantly different bond lengths at the point of cleavage are, of course, of considerable interest. The difference in C(3)–S(1) lengths, corresponding to the acyl group-to-enzyme linkage, is ≈0.03 Å, and while we cannot, at present, quantitatively relate this difference to kinetic parameters for dithioesters, it is possible to argue, by analogy with other systems, that the difference in bond length must give rise to very significant changes in kinetic properties. Jones & Kirby (1979) have recently demonstrated that a linear relationship exists between bond length and reactivity for the hydrolysis of a series of compounds derived from 2-phenoxytetrahydropyran. The length of the C–O bond undergoing cleavage was found to be linearly related to the free energy of activation of hydrolysis. The rates of hydrolysis (log k_{hyd}) could be related to the C–O bond length by using the finding that both quantities are linearly dependent on the pK_a of the leaving group. A change of 0.03 Å in bond length of the labile C–O bond (Jones & Kirby, 1979) corresponds to a change of about 6 in log k_{hyd} (Craze & Kirby, 1978). These conclusions cannot be carried over to enable us to quantitatively express the kinetic effect of the 0.03-Å difference in C(3)–S(1) bond length between the major and minor populations in dithioacylpapain.

However, it does seem reasonable to state, on this basis, that the shorter C(3)–S(1) is intrinsically less susceptible to cleavage.

In the present work, combined X-ray and RR studies have enabled us to follow changes in the lengths of the scissile linkage in transient acyl-enzyme complexes to the accuracy of a few hundredths of an angstrom. Surprisingly it appears that the bond undergoing scission is shortened, and thus strengthened, in the major acyl-enzyme population.

Supplementary Material Available

Thermal vibration parameters for *N*-(*p*-nitrobenzoyl)glycine ethyl dithioester and for *N*-acetylglycine ethyl dithioester in the form of U_{ij} values for non-hydrogen atoms and *B* values for hydrogen atoms as well as tables of observed and calculated structure factors for both crystal structures (22 pages). Ordering information is given on any current masthead page.

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