Vibrational Spectra of Scissile Bonds in Enzyme Active Sites: A Resonance Raman Study of Dithioacylpapains†

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ABSTRACT: The resonance Raman (RR) spectra of six transient dithioacylpapains have been obtained. For five of these intermediates the dithioacyl group comprises an *N*-acylglycine dithioester, $RC(=O)NHCH_2C(=S)S-$, while the sixth is a benzoyloxyacetic acid dithioester, $C_6H_5C(=O)OCH_2C(=S)S-$. The RR spectra indicate that, for the great majority of molecules in each dithioacylpapain population, the vibrational properties of the dithioester center undergoing catalytic attack are markedly perturbed. By analogy with the RR spectra of models (the corresponding dithioacyl ethyl esters) and by noting the effects of amide hydrogen–deuterium exchange, the source of the perturbation is identified as an intramolecular interaction between the acyl's amide (for the five *N*-acylglycine dithioester groups) or ester (for the benzoyloxyacetic acid dithioester group) moiety and the dithioester that forms the dithioacylpapain linkage. The RR and absorption spectra of the dithioacyl-enzymes are unchanged between pH 4 and 9, but below pH 4 there are marked spectral alterations which occur with a pK_a of 3.2. Below pH ≈3.2 the RR spectra of the dithioacylpapains closely resemble the spectra of their corresponding model compounds. In aqueous acetonitrile solution these models exist in almost equal proportions of two conformers, one of which possesses an intramolecular interaction that perturbs the vibrational properties of the dithioester group, while in the other conformer this interaction is absent. For the dithioacylpapains between pH 4 and 9, there is RR evidence that only a very small population of the bound dithioacyl groups is in the nonintramolecularly interacting conformation. Hence, in this pH range the effect of the active site is to change the population distribution in favor of the conformer in which the intramolecular interaction is occurring.

It was demonstrated recently (Storer et al., 1979) that the resonance Raman (RR) spectrum of a dithioacylpapain enables us to monitor the vibrational spectrum of the bonds undergoing catalytic transformation in the enzyme's active site. During catalysis, a transient $-C-C(=S)S-$ linkage is formed in which the substrate and the enzyme contribute the $-C-C(=S)-$ and $-S-$ moieties, respectively (Lowe & Williams, 1965). The dithioester group has a λ_{max} near 315 nm, and by use of laser excitation in the 330-nm region, RR bands can be detected that contain contributions from the stretching motions of the C=S bond, which is undergoing nucleophilic attack in the enzyme's active site, and the C–S bond, which is being cleaved during enzymolysis. The RR spectrum of a dithioacyl-enzyme can be used to form a detailed understanding of the structure of the labile group in the active site. However, spectral interpretation was hampered initially by the lack of background spectroscopic information concerning dithioesters. Thus, we instigated a series of infrared, Raman, and RR spectroscopic studies aimed at providing the material required to interpret the RR spectra of dithioacyl-enzymes. The present paper can only be understood by reference to the spectroscopic work and we summarize the important conclusions from these studies as follows:

(1) Simple dithioesters of the type $CH_3C(=S)SCH_3$ have an intense $\pi \rightarrow \pi^*$ electronic transition near 307 nm, and laser excitation within this transition gives rise to intense RR features near 590 and 1195 cm^{-1}. By means of extensive isotopic substitution and a normal coordinate treatment (Teixeira-Dias et al., 1982), these bands are assigned to modes which, for the 590-cm^{-1} feature, have high contributions from stretching motions of the C–C, C=S, and C–S bonds about the $C-C(=S)-S$ group and, for the 1195-cm^{-1} feature, have a major contribution from C=S stretching coupled to a smaller contribution from C–C stretching. The vibrational spectral regions from 550 to 700 cm^{-1} and from 1050 to 1200 cm^{-1} are sometimes, rather loosely, referred to as the C–S and C=S stretching regions, respectively.

(2) The effect of rotational isomerism on the RR spectra of dithioesters was investigated by considering compounds of the type

(Verma et al., 1981; Ozaki et al., 1982). The key conclusion was that isomers which differ by rotations about the bonds indicated have different spectral signatures in the C–S stretching region but identical spectral signatures in the C=S stretching region.

(3) Since the dithioacyl portions of the dithioacylpapains are usually derivatives of *N*-acylglycine, a series of *N*-acylglycine ethyl dithioesters, $RC(=O)NHCH_2C(=S)SC_2H_5$, were synthesized and studied as models for the dithioacyl-enzymes (Storer et al., 1982). The relevant properties of these compounds can be explained by reference to Figure 1. In solution, the *N*-acylglycine ethyl dithioesters exists in two major conformational states, designated conformers A and B. In the C=S stretching region conformer A gives rise to a spectral signature designated band I between 1160 and 1185 cm^{-1}. Conformer B, however, has a different signature and gives rise to band II between 1115 and 1155 cm^{-1} and, in some compounds, band III between 1080 and 1100 cm^{-1} (Figure 1). Additionally, conformer B has a peak in the C–S stretching region near 590 cm^{-1}. Band I occurs at the predicted frequency of the "C=S stretching" mode, taking into account through bond inductive effects. However, the frequency of band II is highly perturbed, and this, taken with other evidence, such as the dependence of band III on NH to ND exchange, led to the conclusion that, in conformer B, a marked intramolecular interaction is occurring between the amide and dithioester groups (Storer et al., 1982). An additional important finding was that a similar interaction also exists in a model compound in which the amide group has been replaced by an ester group.

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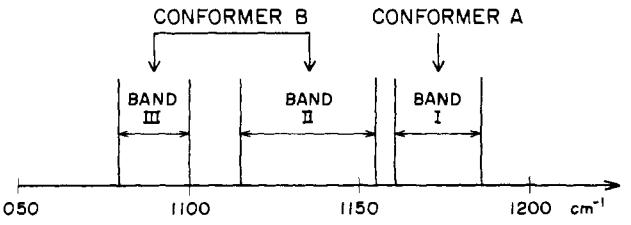

FIGURE 1: Relationship between the RR "signatures" and conformers in solution of *N*-acylglycine ethyl dithioesters.

Table I: Thionoester Substrates

no.	compound	formula
1	methyl thionohippurate	$C_6H_5CONHCH_2CSOCH_3$
2	benzoyloxyacetic acid methyl thionoester	$C_6H_5COOCH_2CSOCH_3$
3	*N*-acetylglycine cyclohexylmethyl thionoester	$CH_3CONHCH_2CSOCH_2C_6H_{11}$
4	*N*-phenylacetylglycine methyl thionoester	$C_6H_5CH_2CONHCH_2CSOCH_3$
5	*N*-(β-phenylpropionyl)glycine methyl thionoester	$C_6H_5CH_2CH_2CONHCH_2CSOCH_3$
6	*N*-carbobenzoxyglycine methyl thionoester	$C_6H_5CH_2OCONHCH_2CSOCH_3$

Although this observation proved to be useful in the discussion of the nature of the intramolecular interaction, the interaction is as yet incompletely understood. It is known that it is not due to simple hydrogen bonding or enethiol tautomerism, but factors such as through space dipole–dipole and sulfur dπ–amide electron interactions are thought to be important.

In this paper we have synthesized the substrates shown in Table I and characterized the RR spectra of the six corresponding dithioacyl-enzymes. The results show that the replacement of $CH_2C(=O)OCH_3$ in the specific substrates with $CH_2C(=S)OCH_3$, i.e., using a single atom substitution, provides a general means by which the vibrational spectra of the scissile bonds in acylpapains may be obtained. The RR spectra, by reference to the spectra of model compounds, allow us to monitor the conformation of the enzyme-bound acyl group in short-lived complexes. The following paper (Huber et al., 1982) deals with joint X-ray and Raman spectroscopic studies on single crystals of model compounds and sets up detailed structure–spectra correlations. These are used with the dithioacyl-enzyme data to provide precise structural information on the acyl-enzyme during catalytic turnover.

Experimental Procedures

Materials. Papain was purchased from the Sigma Chemical Co. as a suspension in sodium acetate. The papain was further purified by affinity chromatography (Blumberg et al., 1970). After elution from the column, the enzyme was converted to inactive mercuripapain by the addition of 1 equiv of mercuric chloride. The inactive enzyme was then concentrated to approximately 10 mg/mL by the partial dehydration of dialysis bags containing the enzyme, using poly(ethylene glycol). The concentrated papain was reactivated as required by the method of Soejima & Shimura (1961). The thiol content of the enzyme was determined by using 5,5′-dithiobis(2-nitrobenzoic acid) as described by Ellman (1959). The enzyme prepared in this way was found to contain 0.97 ± 0.02 mol of active cysteine per mol of protein.

The thionoester substrates (given in Table I) were synthesized by a modification of the procedure outlined for the corresponding dithioesters (Storer et al., 1982). The synthetic route used in this study was essentially the same; however, methanol or cyclohexylmethanol was used in place of the ethanethiol. The action of dry HCl gas on mixed solutions of the corresponding nitriles (Storer et al., 1982) and alcohols produced imido ester hydrochlorides which were then converted to the thionoesters by the action of H_2S in pyridine. The substrates were purified by crystallization and column chromatography on silica gel with acetonitrile–ether mixtures (1:9) as eluant. The results of elemental analysis of all the compounds synthesized agreed within acceptable limits (±0.02 × calculated percentage) with the theoretical values, and the purity of the samples was checked by NMR.

Methods. UV absorption spectra were obtained by using Cary 118 or 219 spectrophotometers. Raman measurements were made by using a Spex 0.5-m double spectrometer with direct current detection. The excitation source was a Coherent Radiation 3000K or 2000K Kr^+ laser, and near-UV laser lines were separated by using a Pellin-Broca prism. Peak frequencies were calibrated by using emission lines from a Ne lamp and are believed to be accurate to ±2 cm^{-1} for well-resolved bands. A rotating cell assembly was employed to prevent photodegradation of the sample in the laser beam. All samples were prepared at room temperature and contained typically 150–175 μM enzyme and 5.5–10 mM substrate (depending on its solubility) in a buffer solution containing 20% acetonitrile, 5 mM EDTA, and 50 mM sodium phosphate, pH 6.8. Under these conditions a steady-state level of dithioacyl-enzyme was produced for several minutes with an optical density at 315 nm of 0.4–1.5. RR spectra of the intermediate were obtained by using 25–40 mW of 324-nm laser light. Each partial spectrum shown in Figure 2 was recorded by using a fresh reaction mixture. Each section took approximately 5 min to scan.

For the variable pH study the dithioacylpapain was produced at pH 6.8 and then titrated down to the required pH by the addition of the necessary amount of 0.1 N HCl or 1 N HCl.

Results

Resonance Raman spectra (Figure 2) were obtained for the dithioacyl-enzyme intermediates produced during the papain-catalyzed hydrolysis of the six thionoester substrates shown in Table I. Spectra were obtained in both H_2O and D_2O solutions containing 20% (v/v) acetonitrile. The organic solvent was included to enhance the solubility of the substrates in predominantly aqueous solutions. RR spectra were obtained for dithioacylpapain (**1**) in 5% and 30% acetonitrile, and they were the same as that observed in 20% acetonitrile. The λ_{max}'s of the near-ultraviolet absorption peaks of the dithioacylpapain intermediates are given in Table II. These differ from the λ_{max}'s for the corresponding *N*-acylglycine ethyl dithioester models by as much as +6 nm for **1** to −2 nm for **3** (Table II).

In order to obtain the RR spectra, we used a large excess of substrate over enzyme such that a "steady-state" concentration of the intermediate was produced for the 4–5 min required to record the sections of spectra shown in Figure 2. Only those portions of the spectra that were found to contain features due to the intermediates are shown. For any one substrate the intensities of the features in the RR spectra and of the absorption peak in the electronic spectra show the same time dependency. This correlation, taken with the fact that the spectral features in Figure 2 are absent from spectra obtained for mixtures in which either substrate or enzyme has been omitted (data not shown), confirms the assignments of the RR peaks to the dithioacylpapain intermediates. Although

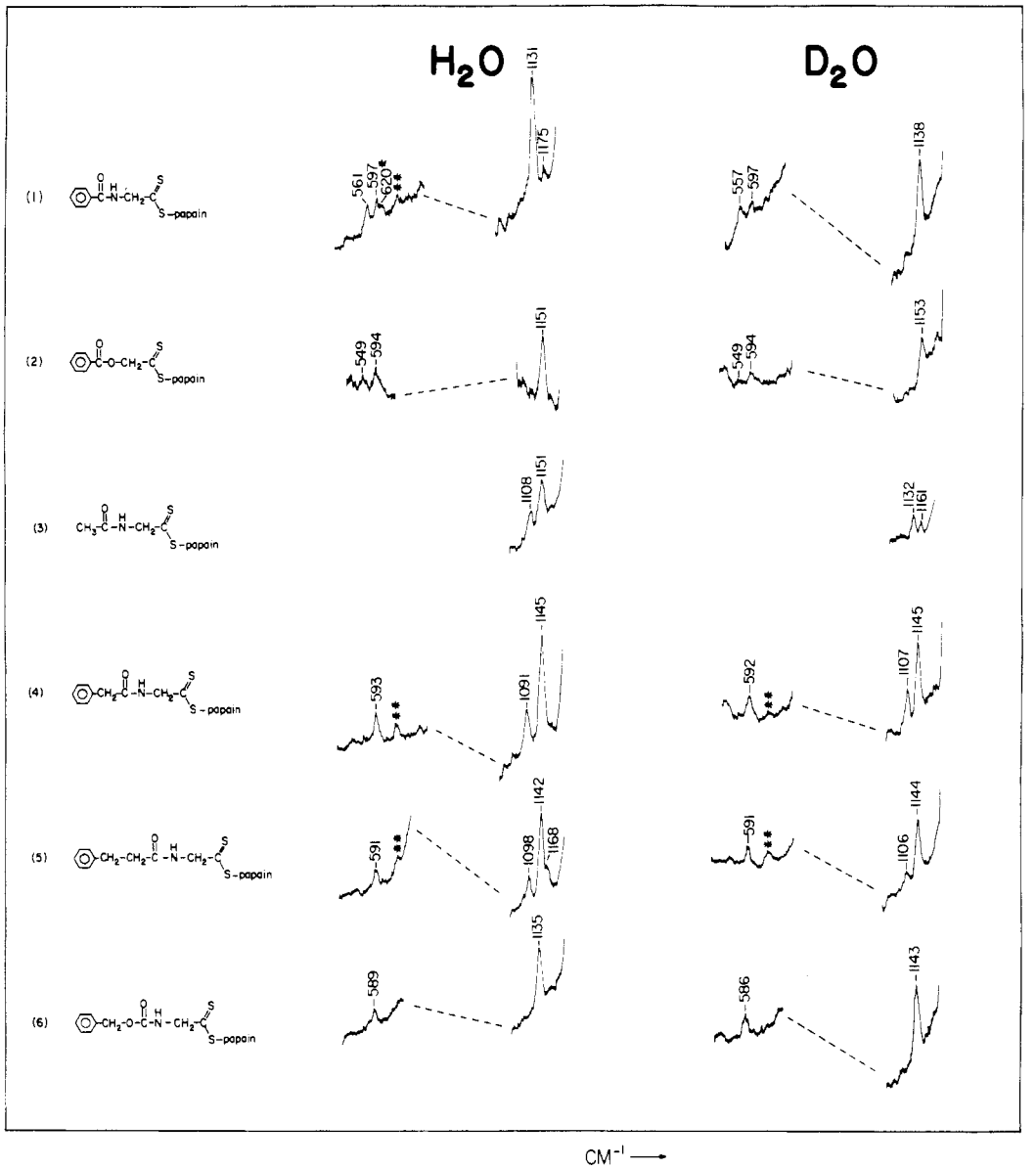

FIGURE 2: Resonance Raman spectra of dithioacylpapains **1–6** obtained at pH 6.8 in 20% (v/v) CH_3CN and either 80% H_2O or 80% D_2O. The concentration of papain was approximately 150 μM and the concentrations of the thionoester substrates were 10 (**1**, **4**, and **5**), 7.5 (**3** and **6**), and 5.5 mM (**2**). 40 mW of 324-nm laser excitation was used with a scan speed of 0.75 cm^{-1} s^{-1} and a spectral slit width of 10 cm^{-1}. One asterisk denotes a peak due to thionoester substrate **1**, and two asterisks denote a possible laser plasma line.

no Raman peaks are observed that are assignable to papain, the peak at 620 cm^{-1} in the spectrum of the dithioacyl-enzyme (**1**) (Figure 2) can be assigned to substrate **1** (Storer et al., 1979). Other minor Raman features due to the buffer or solvent are observed in some spectra.

As in the case of dialkyl dithioesters (Teixeira-Dias et al., 1982) and *N*-acylglycine ethyl dithioesters (Storer et al., 1982), the RR bands of the dithioacylpapains appear in the regions 500–700 and 1050–1200 cm^{-1}. Moreover, in the discussion below we will make a strong case for assigning the dithioacyl-enzyme bands in the 1050–1150-cm^{-1} range to the bands designated II and III in Figure 1 for the corresponding dithioester models. Thus, the most intense feature in each spectrum of the dithioacylpapains (Figures 2 and 3) corresponds to band II of the *N*-acylglycine ethyl dithioester models (Figure 1). Band III of the models is also present for dithioacyl-enzymes **3**, **4**, and **5**; however, for **4** and **5** it is more intense, relative to band II, than it was in the model spectra. In the spectra of dithioacylpapains **1** and **5**, a weak feature is also present near 1170 cm^{-1}. For all the different dithioacyl groups studied, small differences are seen in the positions of bands II and III when the dithioacyl-enzyme spectra are compared to their corresponding model spectra. In addition, the spectra of intermediates **1** and **2** possess a feature near 550 cm^{-1} which is more intense relative to the 590-cm^{-1} peak than in the spectra of the model compounds.

When the RR peak positions of the dithioacylpapains in H_2O and D_2O solutions are compared, changes are observed that parallel the solvent isotope effects reported for the *N*-acylglycine ethyl dithioester model compounds (Table II). For dithioacylpapains **1** and **6** band II shifts to higher wavenumbers by 7–8 cm^{-1} in D_2O, for **4** and **5** band III shifts by +16 and +8 cm^{-1}, respectively, and for intermediate **3** both bands II and III shift (by +24 and +10 cm^{-1}, respectively) and there is a reversal in the relative intensities of these bands. Importantly, there is no change, within the experimental error of ± 2 cm^{-1}, for band II of dithioacylpapain **2**.

Three of the dithioacylpapain intermediates (**1**, **4**, and **6**) were chosen for a study of the effect of pH on the RR spectrum. Lowe & Williams (1965) have already reported a

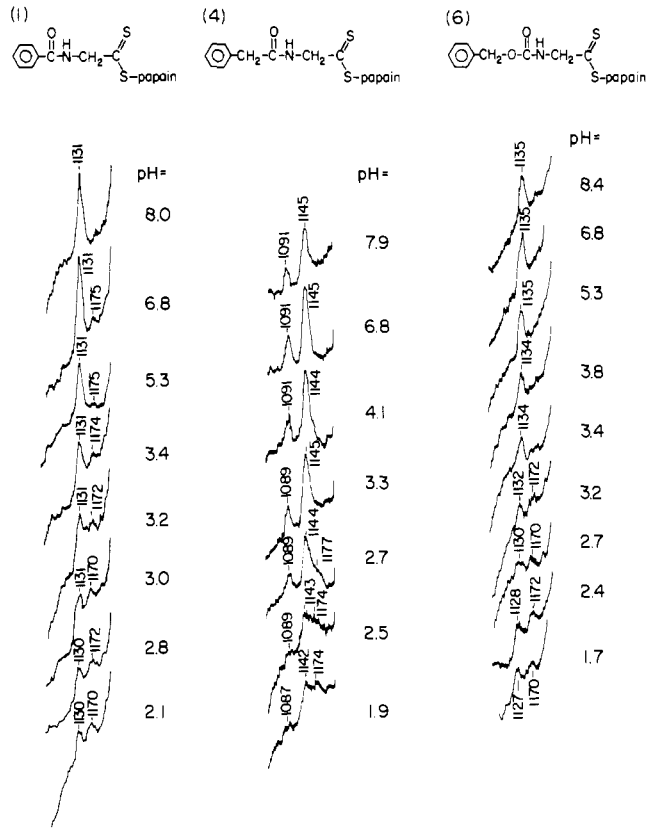

FIGURE 3: pH dependencies of the 1050–1200-cm^{-1} regions of the resonance Raman spectra of dithioacylpapains **1**, **4**, and **6**. The numbers at the side of the spectra refer to pH. The instrument conditions were as for Figure 2, and the experimental conditions are described in the text.

change in the λ_{max} of dithiohippurylpapain (**1**), from 313 to 309 nm, upon acidification of a solution of substrate **1** and papain. They attributed this shift to a denaturation of the acylated enzyme. In the present study, acidification of dithioacylpapain solutions shortly after the substrate was mixed with enzyme at pH 6.8 produced marked changes in the RR spectra (Figure 3). At a final pH of 3.4 or lower, a band appeared in the 1170–1180-cm^{-1} region, resembling band I of the models, and became more intense as the pH of the solution was lowered further (Figure 3). In addition, for intermediate **4**, the intensity of the 1091-cm^{-1} feature decreased relative to that of the 1145-cm^{-1} band. For dithioacylpapain **6**, concomitant with the increase in intensity of band I, band II shifted from 1135 to 1127 cm^{-1} as the pH was lowered from 6.8 to 1.7 and consequently moved closer to the position of band II at 1121 cm^{-1} observed for model **6** (Table II). Band II for intermediates **1** and **4** is apparently less sensitive to pH, although this probably reflects the fact that the differences between the band positions for these intermediates and their models are smaller (Table II). Although not shown in Figure 3, the band at 561 cm^{-1} for intermediate **1** decreases in intensity with pH, such that at lower pHs this band is much less intense than the 590-cm^{-1} feature, as is the case for the ethyl dithiohippurate model (**1**) (Storer et al., 1982). The overall effect of low pH on the RR spectra of the dithioacylpapains is to make them resemble the spectra of the corresponding model dithioesters.

Concomitant with the changes in the RR spectra, acidification of dithioacylpapain solutions modifies the adsorption spectra. The λ_{max}'s of the *N*-acylglycine ethyl dithioester models are independent of pH over the measured range pH 2–7. However, the λ_{max}'s of the dithioacylpapain intermediates

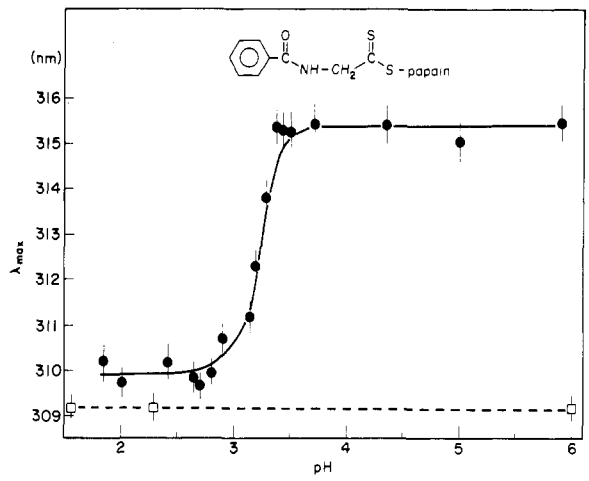

FIGURE 4: λ_{max} as a function of pH for the dithioacylpapain **1**: (●) after titrating with dilute aqueous HCl, dithioacylpapain solutions produced at pH 6. Also shown (□) is the lack of effect of pH on the λ_{max} of model **1**.

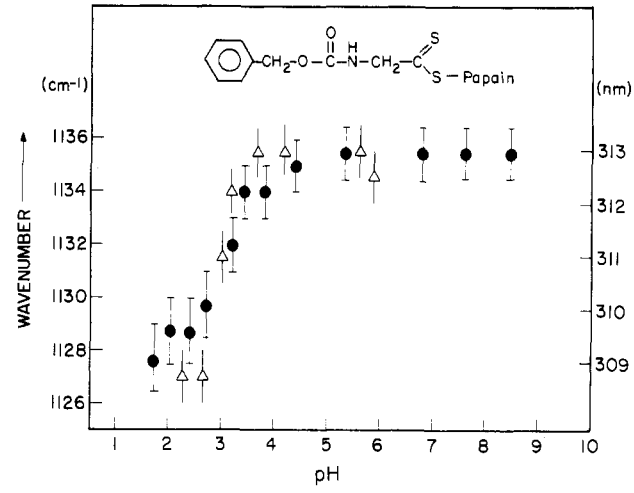

FIGURE 5: pH dependencies of the frequency of band II (●) and the λ_{max} (△) of dithioacylpapain **6**.

vary considerably with pH (e.g., Figure 4). At low pH (2.8) the absorption maximum for intermediate **1** is almost identical with that of the dithioester model **1** (Figure 4). The correspondence between the effects of acidification on the RR and absorption spectra is demonstrated by Figure 5 in which, for dithioacylpapain **6**, the blue shift in λ_{max}, accompanying a reduction in pH, is closely paralleled by the shift in frequency of band II in the RR spectrum.

Discussion

Between pH 4 and 9 the RR spectra of the dithioacylpapains closely resemble the RR spectra of conformer B (Figure 1 and introduction) of the corresponding model *N*-acylglycine ethyl dithioesters (Table II). The main features in the RR spectra of the dithioacylpapains are band II, band III when present, and a peak between 590 and 600 cm^{-1}, which are characteristic properties of the B conformer of the models. The B conformer is the most stable form of the *N*-acylglycine models in aqueous acetonitrile solution (Storer et al., 1982), and in keeping with this preferred stability, model **3** crystallizes out entirely in the B state. There is close similarity between the Raman spectrum of the crystalline form of **3** (Storer et al., 1982) and the RR spectrum of the dithioacylpapain of **3**. These facts strongly suggest that the dithioacyl group in native dithioacylpapains

Table II

RC(=S)S-, R =	species	solvent[a]	λmax (nm)	conformer A, band I (cm^{-1})	conformer B (cm^{-1}) band II	band III
$C_6H_5CONHCH_2$- (1)	model[b]	CCl_4	309	1177	1130	
		H_2O/CH_3CN	309.5	1169	1128	
		D_2O/CH_3CN		1169	1133	
	dithioacylpapain	H_2O/CH_3CN, pH 6.8	315	1175[c]	1131	
		H_2O/CH_3CN, pH 2.1	309.5	1170[c]	1130	
		D_2O/CH_3CN, pD 6.8			1138	
$C_6H_5COOCH_2$- (2)	model[b]	CCl_4		1184	1153	
		H_2O/CH_3CN	310.5	1181	1148	
		D_2O/CH_3CN		1183	1148	
	dithioacylpapain	H_2O/CH_3CN, pH 6.8	310		1151	
		D_2O/CH_3CN, pD 6.8			1153	
$CH_3CONHCH_2$- (3)	model[b]	CCl_4		1173	1151	1100
		H_2O/CH_3CN	309	1170	1148	1098
		D_2O/CH_3CN		1177	1157	1128
	dithioacylpapain	H_2O/CH_3CN, pH 6.8	307		1151	1108
		D_2O/CH_3CN, pD 6.8			1161	1132
$C_6H_5CH_2CONHCH_2$- (4)	model[b]	CCl_4	309.5	1181	1151 (sh)	1089
		H_2O/CH_3CN	310	1173	1138	1087
		D_2O/CH_3CN		1174[c]	1139	1101
	dithioacylpapain	H_2O/CH_3CN, pH 6.8	312		1145	1091
		H_2O/CH_3CN, pH 1.9		1174	1142	1087
		D_2O/CH_3CN, pD 6.8			1145	1107
$C_6H_5CH_2CH_2CONHCH_2$- (5)	model[b]	CCl_4	309.5	1178	1142	1090
		H_2O/CH_3CN	310	1172	1139	1093
		D_2O/CH_3CN		1175	1139	1106
	dithioacylpapain	H_2O/CH_3CN, pH 6.8	312	1168[c] (sh)	1142	1098
		D_2O/CH_3CN, pD 6.8			1144	1106
$C_6H_5CH_2OCONHCH_2$- (6)	model[b]	CCl_4	309	1176	1125	
		H_2O/CH_3CN	309.5	1167	1121	
		D_2O/CH_3CN		1169	1128	
	dithioacylpapain	H_2O/CH_3CN, pH 6.8	313		1135	
		H_2O/CH_3CN, pH 1.7	309	1170[c]	1127	
		D_2O/CH_3CN, pD 6.8			1143	

[a] All H_2O/CH_3CN and D_2O/CH_3CN mixtures contain 20% (v/v) CH_3CN. [b] The data for the model compounds were taken from Storer et al. (1982). [c] This dithioacylpapain peak occurs in the range for band I. However, we do not know the conformation of the enzyme-bound acyl group giving rise to the weak feature near 1170 cm^{-1}. See the text.

assumes a B-type conformation which, as in the case of the model compounds, is characterized by an intramolecular interaction between the acyl's amide function and the dithioester moiety. Furthermore, the spectral analogies between the model and dithioacyl-enzyme forms of **2**, in which the amide is replaced by an ester group, also suggest the presence of a B-type conformer. Thus, in the enzyme's active site the great majority of the dithioacyl groups are in a conformation that is very similar to conformer B found in the model compounds. Nevertheless, there is a small population of enzyme-bound dithioacyl groups in a second conformation as witnessed by the weak spectral features near 1170 cm^{-1} in the RR spectra of dithioacyl-enzymes **1** and **5** (Figure 2). The presence of these weak features is discussed further below.

The differences in peak positions reported in Table II between the dithioacyl-enzymes and the B conformers of the models in H_2O/CH_3CN become smaller when the models are dissolved in CCl_4. This suggests that the active site about the dithioester bonds is of low dielectric constant, and the very close similarity in peak positions strongly indicates that the conformation of the enzyme-bound dithioacyl group is little perturbed from that of the B conformer of the models. Thus, there is no evidence for additional perturbations due to a proximal charge or the α-helix dipole (Hol et al., 1978) in the active site. In addition, we find no evidence for the dithioester bonds being markedly distorted from the geometry of conformer B in solution. Hydrogen bonding of the protein to the dithioester group cannot be discounted, however, since the position of bands containing a substantial proportion of $\nu_{C=S}$ seems to be fairly insensitive to changes in hydrogen bonding (Teixeira-Dias et al., 1982; Storer et al., 1982). It must be emphasized that the RR spectrum of conformer B is markedly different from that predicted for an *N*-acylglycine dithioester (Storer et al., 1982), indicating that the intramolecular interaction in B appreciably changes the properties of the dithioester moiety. By this token, the distribution of electrons in the scissile bonds is quite different from that in a form in which an intramolecular B-type interaction is absent. However, we have no evidence for a major perturbation of the dithioester group by the enzyme, over and above that found in the B conformer in solution.

The close similarity between dithioacyl-enzyme and corresponding model RR spectra is in marked contrast to earlier results from this laboratory concerning acylpapains in which the acyl group is a substituted cinnamoyl moiety (Carey et al., 1976, 1978). The spectral properties of [4-(dimethylamino)-3-nitrocinnamoyl]papain, for example, were greatly perturbed compared to any model compounds based on the 4-(dimethylamino)-3-nitrocinnamoyl group, and the perturbation was ascribed to strong charge polarization within the π electrons of the cinnamoyl system (Carey et al., 1978). The question then arises as to why the active-site forces which substantially perturb the properties of substituted cinnamoylpapains appear to be inoperative in the dithioacyl-enzymes. The answer probably lies in the nature of the acyl groups and their orientations in the active site. Compared to

the dithioacyl chromophore, groups based on cinnamoyl are large, less specific, and possess easily polarizable π-electron chains. Any or all of these effects can render the cinnamoyl chromophore more susceptible to active-site forces such as the juxtaposition of a charge, or a dipole, or to the hydrogen-bonding properties of neighboring side chains. The results of the cinnamoylpapains clearly show that the active site has the potential for strongly perturbing the bound substrate. An intriguing question remains as to whether or how this same potential is realized for specific acyl-enzymes.

Since conformer B in the dithioester models and presumably in the dithioacylpapains usually involves an intramolecular interaction between an amide group and a dithioester group, it is necessary to discuss the possibility that in the dithioacylpapains the interaction may be between a protein amide group and the dithioester group. Two pieces of evidence point to the fact that the peaks we have labeled bands II and III arise from an interaction within the dithioacyl group very similar to that found in conformer B of the models and are not from an interaction between the dithioester and protein amide groups. Importantly, the lack of any deuteration effect on the RR spectrum of dithioacylpapain **2** [which contains the $C(=O)OCH_2CS_2$ group and not the $C(=O)NHCH_2CS_2$ group] is strong evidence against a protein–amide and dithioester interaction since this, as in the case of **1** and **3–6**, would then be sensitive to deuteration. Second, there is marked parallelism between the RR spectra in H_2O and D_2O of the six dithioacyl-enzymes and the RR spectra of conformer B of the corresponding dithioester models. It seems improbable that such close similarities would be observed if the RR bands for the dithioacyl-enzymes and for the model esters derived from different origins.

Each dithioacylpapain population appears to contain a small percentage of molecules in which the acyl group is not in a B-type conformation. Although the band near 1170 cm^{-1} is a small feature in the RR spectra of the intermediates, it is not an artifact, and its presence was shown to be reproducible. This feature has the highest relative intensity in the RR spectra of dithioacyl-enzymes **1** and **5** but was also detected in the best quality spectra of the other dithioacylpapains. The relative intensity of the band near 1170 cm^{-1} in the RR spectra of the other dithioacylpapains is so low that the peak is frequently "lost" in the noisy background of the spectra. It is not visible in the spectra of some of the dithioacyl-enzymes shown in Figure 2 since this figure illustrates spectra run on the same day, and consequently individual spectra do not necessarily have the best signal-to-noise ratio achieved. The weak band near 1170 cm^{-1} is assigned to the presence of a small percentage of the acyl groups *not* in a B-type conformation. The band appears in the range regarded as normal for $\nu_{C=S}$ of a *N*-acylglycine dithioester and may be the signature of any of a number of possible conformers (of which conformer A is but one) possessing a relatively unperturbed dithioester group. Significant rotation about the NHC–C(=S) single bond of conformer B (Huber et al., 1982) will likely attenuate the intramolecular interaction which gives rise to conformer B's atypical RR properties. Thus, with the present data, we can only say that the minor population differs from the major population by a significant (probably >30°) rotation about the NHC–C(=S), and/or possibly the NH–CC(=S), single bond. The occurrence of this second low population conformer in the enzyme active site could result from two effects: the first is a partial denaturation of the dithioacylpapain during the course of the experiment, while the second is due to a certain amount of conformational flexibility in the enzyme's active site. Since dithioacyl-enzymes **1** and **5** invariably showed the highest relative intensity of the 1170-cm^{-1} feature in their RR spectra, it seems reasonable to assume that these intermediates contain a higher population of the minor conformer compared to the other dithioacyl-enzymes. However, if partial denaturation is the cause of the weak 1170-cm^{-1} band, it is difficult to understand why there are differing degrees of denaturation depending upon the nature of the dithioacyl group involved. Moreover, in the approximately 200 active dithioacyl-enzyme preparations we characterized by RR spectroscopy, we did not observe a measurable change in the ratio of the intensities of bands between 1050 and 1200 cm^{-1} for a given dithioacyl-enzyme. For these reasons we prefer to ascribe the presence of the 1170-cm^{-1} band to a dithioacyl-enzyme that is in a second conformation but in which the enzyme is not denatured. Conformational flexibility, allowing a mixed population of conformers, is a known property of enzymes (Lakowicz & Weber, 1973; Caughey et al., 1981), and this property has often been ascribed to papain with its bilobar structure (Lowe, 1976). In the present case the enzyme apparently changes the equilibrium between the thermodynamically accessible states of the dithioacyl group in favor of conformer B, which for the dithioester ester models is the most stable form (Storer et al., 1982).

Below pH 3.4 the population of conformers reverts to that of the corresponding model dithioesters. The pK_a ($\simeq$3.2) of this effect (Figures 4 and 5) is close to that proposed by Johnson et al. (1981) on the basis of NMR studies for histidine-159 in the *S*-methylthio derivative of papain at low ionic strength. However, below pH 3.5 papain becomes irreversibly denatured with time, and further work is required to establish if the spectral effects seen in Figures 3–5 are completely reversible in a short time period (and are thus ascribable simply to the protonation of histidine-159) or if the spectral changes reflect, in part, an irreversible denaturation.

On the basis of our work on model dithioesters (Teixeira-Dias et al., 1982; Ozaki et al., 1982; Storer et al., 1982) we can tentatively describe the normal mode origins of the dithioacyl-enzyme bands. The description is only tentative because while we have arrived at a satisfactory normal mode analysis of molecules of the type $CH_3C(=S)SC_2H_5$, analysis of the fragment $-C(=O)NCH_2C(=S)SC_2H_5$ is still under way. With this rider and considering the pH range from 4 to 9, we assign the weak band near 1170 cm^{-1} (from the minor dithioacyl-enzyme population in this pH range) to a mode which has a high degree of C=S stretching character and a substantial contribution from C–C stretching of the $-NHCH_2-C(=S)$ moiety. Band II is the corresponding normal mode due to the major population. Additionally, in some molecules band II may have a contribution from a motion of the NH hydrogen atom. Although band III is clearly associated with the major population, its normal-mode origin is unclear, but on the basis of the sensitivity to NH to ND exchange, it does contain a contribution from a motion of the NH hydrogen or the ND deuteron. The sensitivity of bands II and III to change in solvent from H_2O to D_2O represents changing participation of the compounds' NH (ND) group in the modes and does not reflect changes in inter- or intramolecular hydrogen bonding (Storer et al., 1982). The appearance of modes in the RR spectra of the *N*-acylglycine ethyl dithioester models and the dithioacyl-enzymes which are sensitive to NH to ND exchange was surprising, since only modes localized around the dithioester chromophore were expected. The atypical position of band II and the appearance of modes containing some amide character are ascribed to an

intramolecular interaction between the dithioester and amide (or, in the case of **2**, ester) which is described in the following paper (Huber et al., 1982). The dithioacyl-enzyme band near 595 cm^{-1} (Figure 2) may correspond to a peak found in this region for dialkyl dithioesters (Teixeira-Dias et al., 1982) which has its origin in a blend of C–C, C–S, and C=S stretching motions of the C–C(=S)–S group.

Although we have emphasized the similarity between the dithioacyl-enzyme RR spectra and those of conformer B in the corresponding models, it is important to point out that certain spectral differences do exist and that these may provide additional information on the nature of the binding site and the interactions between substrate and protein. There are three main differences between the spectra of the dithioacyl-enzymes and their corresponding models. First, dithioacylpapain and model **6** show a substantial difference of 16 cm^{-1} in the position of band II; second, for dithioacyl-enzymes **4** and **5** band III has a higher relative intensity than in the spectra of the corresponding models; third, in the RR spectra of dithioacyl-enzyme **1**, and to a lesser extent **2**, there is a marked intensity increase of a band near 550 cm^{-1} over that found in the models.

The atypical behavior of band II for compound **6** may reflect the fact that the intramolecular interaction in this case is between –OC(=O)NH– (carbamate) and dithioester chromophores rather than an amide and dithioester interaction. For dithioacyl-enzyme **6**, interactions between the dithioacyl group and the protein may substantially perturb the geometry of the atypical conformer B from that found for its model in solution and give rise to the large observed difference in the position of band II.

Band III probably contains a contribution from a motion of the NH proton, and this, taken with the observation of high intensity enhancement of band III in the RR spectrum, led us to propose (Storer et al., 1982) that band III results from a strong intramolecular interaction between the amide and dithioester chromophores. For this reason the observed intensity change of band III in the dithioacyl-enzyme spectra is a good candidate for monitoring the effects of enzyme–dithioacyl group interactions on the conformer of the bound dithioacyl group.

The origin of the band near 550 cm^{-1} in the spectra of the dithioacylpapains and models **1** and **2** is at present uncertain. No features are seen at this frequency in the Raman spectra of *N*-methylbenzamide (0.25 M in CH_3CN), ethyl hippurate (0.25 M in CH_3CN) or methyl thionohippurate (0.03 M in CH_3CN) in solution (data not shown). The only structural feature that dithioacylpapains **1** and **2** and models **1** and **2** have in common and which is absent from the other models is the close proximity of the benzene ring to the dithioester group. Thus, a possible source of the 550-cm^{-1} feature is a benzene ring and sulfur interaction which gives rise to an intensity-enhanced phenyl ring mode in the RR spectrum. There has been considerable recent interest in the biochemistry of such interactions in proteins (Bodner et al., 1980; Némethy & Scheraga, 1981). In any event, an assignment and understanding of the 550-cm^{-1} band will also add to our knowledge of interactions in the dithioacyl-enzyme.

The intramolecular interaction of conformer B in the model compounds does not involve simple hydrogen bonding or enethiol tautomerism (Storer et al., 1982). Instead, probable interactions within conformer B are dipole–dipole forces or amide π electrons interacting through space with sulfur $d\pi$ orbitals. Since both these effects have been shown to operate in thioesters (Nyquist & Potts, 1959; Storer et al., 1981), we believe it is important to look for intramolecular interactions, akin to those of the B conformer, in natural thioacyl-enzymes.

In this study, the resonance Raman spectra of dithioacylpapain intermediates formed during catalytic turnover have allowed us to identify important intramolecular interactions within the acyl moiety that significantly perturb the chemical properties of the dithioester linkage. To precisely define the nature of these intramolecular interactions in conformer B and to answer the questions raised by the spectral differences discussed above, we have instigated a series of studies by using Raman and X-ray crystallographic techniques on single crystals of the *N*-acylglycine ethyl dithioesters. The initial findings of these studies are reported in the following paper (Huber et al., 1982) which deals with *N*-acetylglycine and *N*-(*p*-nitrobenzoyl)glycine ethyl dithioesters. The *N*-acetyl derivative formed crystals which exhibited spectral properties of the B conformer and hence allowed us to precisely characterize this form while it was possible to crystallize the *N*-(*p*-nitrobenzoyl) derivative as an A conformer. By use of these "standards" the conformation of the major population of the papain-bound acyl groups may be derived and the effect of this conformation on the bond undergoing scission characterized.

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