Resonance Raman spectroscopic and kinetic consequences of a nitrogen · · · sulphur enzyme-substrate contact in a series of dithioacylpapains

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ABSTRACT The resonance Raman (RR) spectroscopic, conformational, and kinetic properties of six dithioacylpapain intermediates have been examined. Five of the intermediates are of the form *N*-(methyloxycarbonyl)—X—glycine—C(=S)S-papain, where X is L-phenylalanine, D-phenylalanine, glycine, L-phenylglycine, or D-phenylglycine. The sixth intermediate is *N*-phenylacetyl-glycine—C(=S)S-papain. Throughout the series there is an ~50-fold variation in k_{cat}, the rate constant for deacylation, and a 1750-fold variation in k_{cat}/K_M. Existing RR spectra structure correlations allow us to define the torsional angles in the NH—CH_2—C(=S)—S—CH_2—CH fragment of the functioning intermediates. The values of these angles for each bound substrate appear to be very similar, with the substrates assuming a B-type conformer such that the nitrogen atom of the P_1 glycine residue is *cis* to the thiol sulphur atom of cysteine-25. For each intermediate, the C(=S)S—CH_2CH torsional angle is approximately −90°, whereas for the SCH$_2$—CH torsional angle the cysteine-25 thiol sulphur (S) and cysteine-25 C_α hydrogen (H) atoms are approximately *trans*. The three acyl-enzymes with the lowest catalytic rate constants, viz. *N*-(methyloxycarbonyl)-glycine-glycine-, *N*-(methyloxycarbonyl)-L-phenylglycine-glycine-, or *N*-(phenylacetyl)-glycine-dithioacylpapains, have atypical RR spectra in that they show a feature of medium intensity in the 1,085-cm^{-1} region. This band is sensitive to NH to ND exchange of the P_1 glycine residues' (-NH-) function and, thus, the corresponding mode involves an excursion of the NH hydrogen. It is hypothesized that the high intensity is due to a particularly strong interaction between the P_1 glycine nitrogen atom and the thiol sulphur of cysteine-25, which also has the effect of retarding deacylation, because the nitrogen · · · sulphur contact has to be broken in the rate-determining step.

INTRODUCTION

Relating structure to reactivity is a major challenge for contemporary protein biochemistry. Attempts at redesigning enzymes and at creating new catalysts by protein engineering all require an improvement in our definition of the structural and dynamical determinants of protein reactivity. An approach instigated in our laboratory has involved the use of chromophoric enzyme-substrate complexes to generate the resonance Raman (RR) spectra of functioning, catalytically active intermediates (Carey and Tonge, 1990). Interpretation of these spectra, often by recourse to a library of structure-spectra correlations built up for 'small' model compounds, provides detailed structural information on the complex which is then related to kinetic parameters.

The cysteine protease papain catalyzes the hydrolysis of ester or peptide linkages by a three-step mechanism: e.g.,

$$R_1C(=O)-OR_2 + \text{HS-papain} \rightarrow R_1C(=O)-\text{S-papain} \xrightarrow{H_2O} R_1C(=O)-OH + \text{HS papain.}$$

The use of thionoesters, $R_1C(=S)OR_2$, enables us to form chromophoric (λ_{max} 315 nm) dithioester enzyme-substrate complexes, $R_1C(=S)$S-papain. These may be monitored by absorption spectroscopy or can provide resonance Raman data associated with the dithioester moiety. The latter are a source of detailed conformational information on the dithioester and neighboring bonds. With the proviso that the C=S has replaced C=O, intermediates of the kind $R_3C(=O)NHCHR_4C(=S)$S-papain are very close to those formed by the enzyme's physiological substrates. Detailed analyses have been undertaken on dithioacyl intermediates of the form $R_3C(=O)NHCHR_4C(=S)$S-papain. For *N*-acylglycine dithioacylpapain complexes, where R_4=H, ~12 substrates have been examined, and the acyl group from each binds in the active site as a so-called B conformer in which glycine's nitrogen atom and the cysteine sulphur atom are *cis* (Carey et al., 1984b; Storer et al., 1983; Varughese et al., 1984). Analysis also reveals the torsional angles in cysteine-25's S—CH_2—CH linkages (Kim and Carey, 1991), and this conformation is depicted in Fig. 1.

This work focusses on the effect of changing R_3 in structures of the form $R_3C(=O)NHCH_2C(=S)$S-papain. R_3 binds in the so-called S_2 site[1] in papains extended active site (Berger and Schecter, 1970). It is known that although variations in R_3 alter enzyme-substrate contacts distant from the point of catalytic attack there is a marked effect on the rate of catalysis. It is thus of considerable interest to explore the chemical rationale

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[1] Nomenclature of the subsites (S) in the active site of papain and of the respective positions of the amino acids in the substrate (P) is according to Berger and Schecter (1970). P_1 is the substrate's amino acid covalently attached to the sulphur of the active site's cysteine-25; S_2 binds the subsequent amino acid on the acyl side.

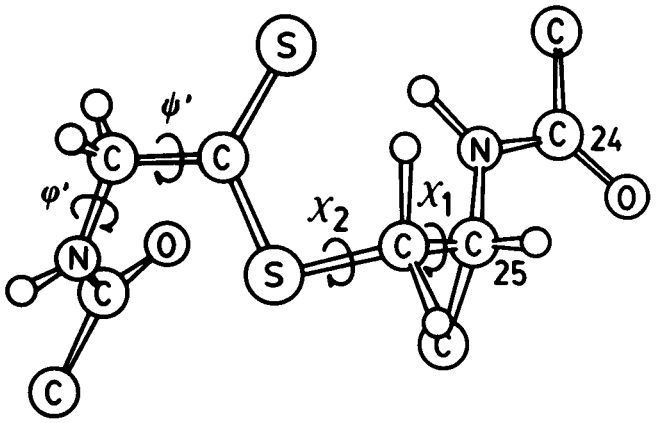

FIGURE 1 Conformation B—G⁻—P_H for an *N*-acylglycine dithioacylpapain. The C_α main chain carbon atoms of papain residues Cys-25 and Ser-24 are labeled 25 and 24, respectively. This conformation is defined by the angles ϕ', ψ', χ_1, and χ_2 shown in the figure.

for this (Angus et al., 1986) and to see if, for example, a change in distant enzyme-substrate contacts changes the conformation about bonds in the immediate region of catalytic transformation. In spite of the differing enzyme-substrate contacts consequent upon changing the R_3 residue, each dithioacyl group appears to take up a B-type conformer with, in addition, similar torsional angles in the cysteine bonds. However, some of the observed variation of up to 50-fold in k_{cat}, the rate of hydrolysis of the intermediate, may be accounted for by changes in the strength of the nitrogen (P_1 glycine) · · · sulphur (cysteine-25) contact seen within the conformer B framework.

EXPERIMENTAL PROCEDURES

Materials

D_2O (99.8% D) was from MSD Isotopes (Merck Frosst Canada Inc., Pointe Claire, Dorval, Québec, Canada H9R 4P7). Papain 2× crystallized suspension in 0.05 M sodium acetate, pH 4.5, was from Sigma Chemical Co. (St. Louis, MO). The enzyme was prepared, activated, and assayed as described previously (Carey et al., 1984a). Titration with 5,5'-dithiobis(2-nitrobenzoic acid) gave 0.95 active thiol groups per mole protein.

Synthesis

The *N*-(methyloxycarbonyl)- derivatives of L or D phenylalanine, L or D phenylglycine, and glycine were prepared by treatment with methylchloroformate and the derivatized amino acids were coupled to aminoacetonitrile hydrochloride using isobutylchloroformate and *N*-methylmorpholine (Carey et al., 1984a). *N*-phenylacetyl-aminoacetonitrile was prepared from the acid chloride of phenylacetic acid (Storer et al., 1982). The nitriles were converted into the corresponding methyl thiono esters as described previously (Carey et al., 1984a).

All compounds were checked for purity either by HPLC or TLC and then subjected to either high resolution Exact Mass analysis or elemental analysis to confirm their identities.

Resonance Raman (RR) instrumentation

RR spectra were obtained at room temperature with 324-nm Kr^+ excitation (Coherent Radiation 3,000 K; Palo Alto, CA). Scattered light was collected from the sample in 180° back scattering geometry using a Cassegrain type collection optic (Anagrain; Anaspec Research Laboratories Ltd., Berkshire, UK) and focused onto the entrance slit of a Spex 1877 Triplemate (Edison, NJ). Raman spectra were detected and processed using an intensified diode array (PAR 1421B-1024-HQ) and an OMA III data collection system (PAR 1460; Princeton Applied Research, Princeton, NJ). The spectral resolution was 7 cm^{-1}. The sample, 0.5 ml volume, was contained in a 1 × 0.5-cm quartz cuvette and agitated using a small magnetic stirring bar.

Kinetics

Steady-state kinetic parameters (k_{cat} and k_{cat}/K_M) for *N*-(methyloxycarbonyl)-glycine-glycine methyl thiono ester were obtained with the use of a Radiometer RTS 822 pH-stat (Westlake, OH) as described in Tonge et al. (1991). The reaction mixture contained 0.3 M NaCl, 1.0 mM EDTA, and 20% acetonitrile. The papain concentration was 1 μM and the substrate concentration was varied from 0–10 mM. Initial rates (v) were obtained directly from the recorder trace and kinetic parameters were calculated by linear regression of plots [S]/v against [S]. All the other kinetic data presented herein are available from previous studies in this laboratory.

RESULTS AND DISCUSSION

Conformation in the active-site

The RR spectra of the six dithioacylpapains in H_2O and D_2O are compared in Fig. 2. In general, they are similar to the RR spectra of other *N*-acylglycine dithioacylpapains discussed in detail before (Angus et al., 1986; Storer et al., 1983). They all have an intense band near 1,140 cm^{-1} and features near 590 and 660 cm^{-1}. Detailed analysis of model compounds, based on a library of RR spectra-x-ray structure correlations (Varughese et al., 1984; Huber et al., 1982) supported by isotopic substitutions (Storer et al., 1983), has shown that spectra of this type are due to the acyl group assuming the so-called B conformation shown in Fig. 1. This is characterised by a small, $\approx \pm 20°$, $NHCH_2—CS_{(thiol)}$ torsional angle (ψ'), such that the glycine nitrogen atom is *cis* to the thiol sulphur atom. Thus, we conclude that each acyl group of the six substrates giving rise to the data in Fig. 2 binds in the active-site as a B conformer. Moreover, for the spectra taken in H_2O, there is no evidence for features in the 1,150–1,200 cm^{-1} region which emanate from A-like (characterized by glycine nitrogen *trans* to the thiol sulphur) or other non-B conformers (Lee et al., 1983). Thus, each acyl group is binding in a single B-like population. These findings are the same as those reached for other *N*-acylglycine dithioacylpapains (Angus et al., 1986; Storer et al., 1983).

Recently, spectra-structure correlations have been developed for the cysteine-25 torsional angles χ_1 and χ_2 shown in Fig. 1 (Kim and Carey, 1991). This analysis indicates that the band seen in each spectrum in Fig. 2 near 665 cm^{-1}, which has a substantial component from

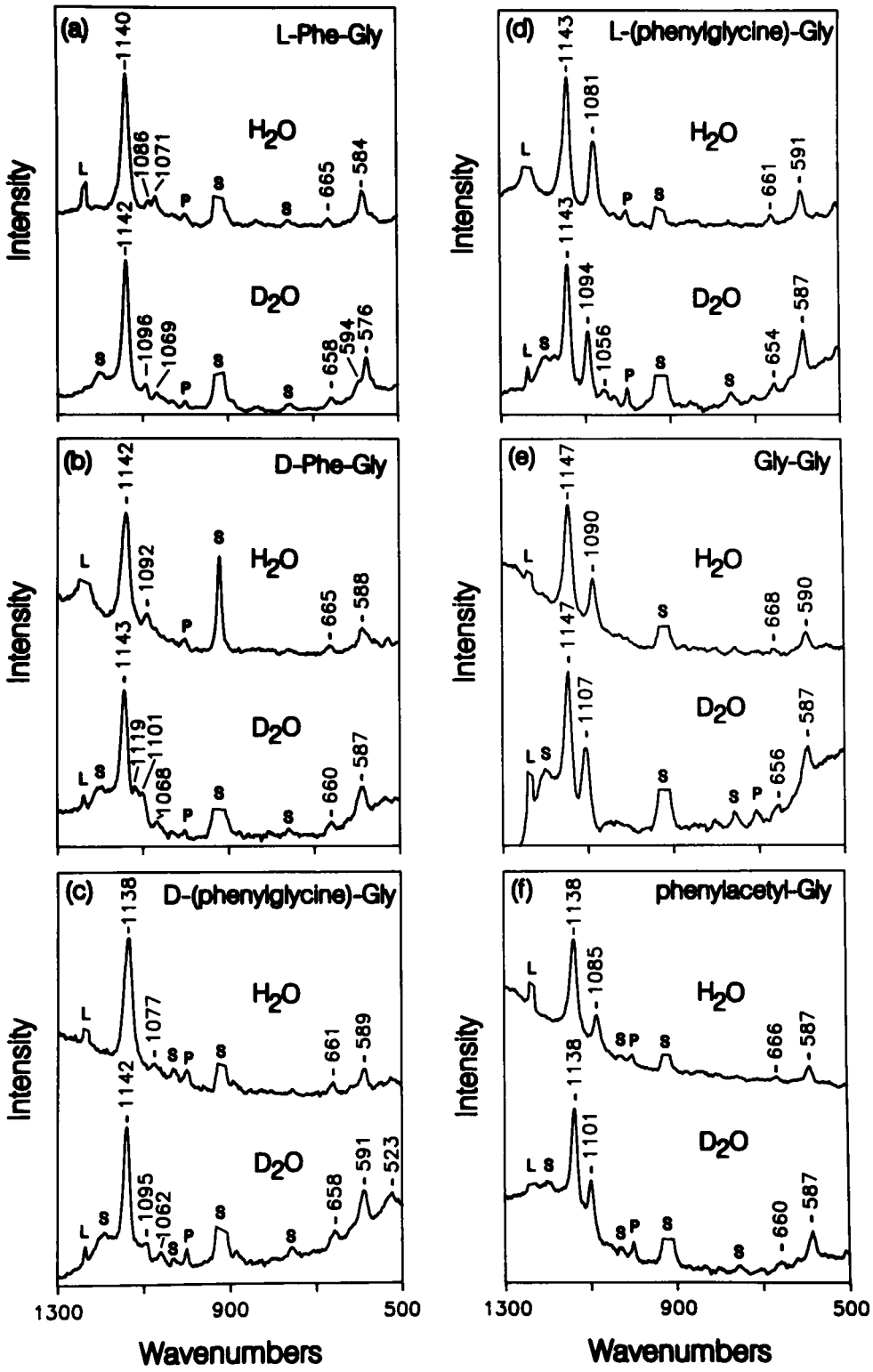

FIGURE 2 Resonance Raman spectra of dithioacylpapains. In each case the reaction mixture contained 20% acetonitrile, 30 mM sodium acetate buffer (H_2O or D_2O), 0.11 mM papain (H_2O or D_2O), and 5 or 10 mM methyl thiono ester substrate (see below). The final pH or pD was 6.0. Spectra were obtained using 100 mW laser radiation and data were acquired over 200 s in 40 s blocks. Identical memory blocks obtained before substrate exhaustion were coadded to give total acquisition times of 120 or 200 s (see below). No further processing of the data was performed. S, solvent: acetonitrile 1,030, 922, 750 cm^{-1}, D_2O 1,200 cm^{-1}. P, substrate 1,000, 708 cm^{-1}. L, laser line, 1,239 cm^{-1}. (*a*) L-Phe-Gly *N*-(Methyloxycarbonyl)-L-phenylalanyl-glycine dithioacylpapain in H_2O and D_2O. 5 mM substrate, 120 s acquisition time. (*b*) D-Phe-Gly *N*-(Methyloxycarbonyl)-D-phenylalanyl-glycine dithioacylpapain in H_2O and D_2O. 5 mM substrate, 120 s acquisition time. (*c*) L-(phenylglycine)-Gly *N*-(Methyloxycarbonyl)-L-phenylglycinyl-glycine dithioacylpapain in H_2O and D_2O. 10 mM substrate, 200 s acquisition time. (*d*) D-(phenylglycine)-Gly *N*-(Methyloxycarbonyl)-D-phenylglycinyl-glycine dithioacylpapain in H_2O and D_2O. 10 mM substrate, 200 s acquisition time. (*e*) Gly-Gly *N*-(Methyloxycarbonyl)-glycinyl-glycine dithioacylpapain in H_2O and D_2O. 10 mM substrate, 200 s acquisition time. (*f*) phenylacetyl-Gly *N*-(Phenylacetyl)-glycine dithioacylpapain in H_2O and D_2O. 10 mM substrate, 200 s acquisition time.

TABLE 1 **Kinetic data for the methyl ester (C=O) and methyl thiono ester (C=S) substrates**

Substrate	k_{cat} s^{-1} (C=S)	k_{cat}/K_M $M^{-1}s^{-1}$ (C=S)	k_{cat} s^{-1} (C=O)	k_{cat}/K_M $M^{-1}s^{-1}$ (C=O)
L-Phe-Gly	0.61 ± 0.02	4.41 (±0.98) × 10^4	6.2 ± 0.7	3.58 (±0.35) × 10^4
D-Phe-Gly	0.39 ± 0.01	2.92 (±0.35) × 10^3	6.7 ± 0.3	1.89 (±0.10) × 10^3
D-(phenylglycine)-Gly	0.27 ± 0.01	1.03 (±0.11) × 10^3	10.2 ± 0.5	1.80 (±0.18) × 10^3
L-(phenylglycine)-Gly	0.075 ± 0.001	3.18 (±0.32) × 10^2	0.95 ± 0.03	1.29 (±0.06) × 10^2
Gly-Gly	0.11 ± 0.01	50.0 ± 6.0	2.0 ± 0.2	35.0 ± 1.4
phenylacetyl-Gly	0.013 ± 0.001	25.2 ± 2.7	0.59 ± 0.05	22.7 ± 2.9

Abbreviations for the substrates used are as in the legend to Fig. 2. Values of k_{cat} and k_{cat}/K_M for *N*-(methyloxycarbonyl)-glycinyl-glycine methyl thiono ester were obtained in this study and represent the average of three separate experiments. The other kinetic parameters were available from previous studies in this laboratory, wherein C=S refers to methyl thiono ester substrates and C=O refers to oxygen methyl ester substrates.

the stretching motion of the cysteine S—C bond ν_{S-C}, is a sensitive monitor of conformation about χ_1 and χ_2. In this instance there are two pertinent conclusions. Firstly, the conformation about χ_1 and χ_2 giving rise to the 665 cm^{-1} feature is $G^- — P_H$. That is, χ_2 is $\approx -90°$ and in the cysteine fragment $\underline{\underline{S}}CH_2 — C\underline{\underline{H}}$, χ_1 is such that the $\underline{\underline{S}}$ and $\underline{\underline{H}}$ atoms are approximately *trans*. This configuration is shown in Fig. 1. The second conclusion stems from the fact that the ν_{S-C}s seen in Fig. 2 only span the range 665 ± 4 cm^{-1}. Given the sensitivity of ν_{S-C} to changes in χ_1 and χ_2 (Kim and Carey, 1991), this means that the χ_1 and χ_2 values are very similar for the six acyl-enzymes seen in Fig. 1.

Discussion of the feature seen in the 1,080–1,100 cm^{-1} range in the spectra in Fig. 2, the so-called Band III (Ozaki et al., 1982), is deferred until the final section of Results and Discussion.

Kinetic parameters

Values of the kinetic parameters, k_{cat} and k_{cat}/K_M, are given in Table 1 for the ester (C=O) and thiono ester (C=S) derivatives of each of the six substrates reacting with papain. For reactions of this kind there is evidence that deacylation is rate limiting such that $k_{cat} = k_3$, the rate constant for deacylation (Storer et al., 1988).

The kinetic data in Table 1 indicate that the substrates fall into two classes, 'good' and 'poor'. Thus, for ester (C=O) hydrolysis, *N*-(methyloxycarbonyl)-L-phenylalanine-glycine-, *N*-(methyloxycarbonyl)-D-phenylalanine-glycine-, and *N*-(methyloxycarbonyl)-D-(phenylglycine)-glycine- methyl ester are 'good' substrates with k_{cat}/K_Ms in the range 1,800 to 36,000 M^{-1} s^{-1} and k_{cat} from 6.2 to 10.2 s^{-1}, while *N*-(methyloxycarbonyl)-L-(phenylglycine)-glycine-, *N*-(methyloxycarbonyl)-glycine-glycine-, and *N*-(phenylacetyl)-glycine methyl ester are 'poor' substrates with k_{cat}/K_M s in the range 23 to 130 M^{-1} s^{-1} and k_{cat} from 0.6 to 2.0 s^{-1}.

A further interesting observation is that k_{cat} (C=O)/k_{cat}(C=S) varies over a fairly narrow range, viz. 10 to 46. Thus, the delineation into 'good' and 'poor' substrates also holds for the methyl thiono ester substrates; for *N*-(methyloxycarbonyl)-L-phenylalanine-glycine-, *N*-(methyloxycarbonyl)-D-phenylalanine-glycine-, and *N*-(methyloxycarbonyl)-D-(phenylglycine)-glycine-methyl thiono ester k_{cat} = 0.61, 0.39, and 0.27 s^{-1}, respectively, whereas for *N*-(methyloxycarbonyl)-L-(phenylglycine)-glycine-, *N*-(methyloxycarbonyl)-glycine-glycine-, and *N*-(phenylacetyl)-glycine methyl thiono ester k_{cat} = 0.075, 0.11, and 0.013 s^{-1}, respectively.

Appearance of an intense Band III correlates with low k_{cat}

An intriguing observation stemming from the kinetic classification of 'good' and 'poor' substrates is that the 'good' substrates have weak features in the RR spectra at ~1,080–1,100 cm^{-1}, whereas 'poor' substrates have a feature of medium to high intensity in that region (Table 2). This is apparent in Fig. 2, where the spectra in *a*, *b*, and *c* are due to good substrates, whereas those in *d*, *e*, and *f* are from poor substrates.

The feature occurring between 1,080 and 1,100 cm^{-1} has previously been designated as Band III (Ozaki et al., 1982). In dithioacyl-enzymes and model compounds, the position of Band III changes upon the P_1 glycine-NH being exchanged to -ND (Ozaki et al., 1982; Storer et al., 1983; Lee et al., 1983). Thus, the mode giving rise to Band III involves displacement of the -NH hydrogen atom. Additionally, Band III only occurs for B conformers of *N*-acylglycine ethyl dithio esters (model compounds or dithioacyl-enzymes) and is usually of weak to medium intensity (Angus et al., 1986; Carey and Storer, 1983). It does not occur for non-B conformers such as conformers A or C_5 (where the glycinic nitrogen and cysteine thiol sulphur atoms are essentially *trans*). For the dithioacylpapains characterized in this study, a band found in the RR spectra ~1,080–1,090 cm^{-1} in H_2O is observed to shift up in frequency by 10–18 cm^{-1} upon transfer of the dithioacylpapain to D_2O (Fig. 2, Table 2). This band is assigned to Band III. A semi-quantitative estimation of the intensity of Band III is given in Table 2 relative to the corresponding intensity of the band observed ~1,140–1,150 cm^{-1} in each spectrum and previously designated as Band II (Ozaki et al., 1982). Thus, the 'good' substrates give a Band II/Band III intensity ratio of ~7–11, whereas the 'poor' substrates give a ratio of ~2–3.

Early analysis of the forces in play within a B-type conformer suggested that, because the P_1 glycine nitro-

TABLE 2 **Resonance Raman band positions and intensities for the dithioacylpapains**

Substrate	H_2O			D_2O		
	Band II	Band III	II/III‡	Band II	Band III	II/III‡
L-Phe-Gly	1140	1086	11.5	1142	1096	11
D-Phe-Gly	1142	1092	7.5	1143	1119/1101	ND*
D-(phenylglycine)-Gly	1138	1077	13	1142	1095	10
L-(phenylglycine)-Gly	1143	1081	1.8	1143	1094	1.7
Gly-Gly	1147	1090	2.3	1147	1107	1.9
Phenylacetyl-Gly	1138	1085	3.0	1138	1101	2.0

Abbreviations for the substrates used are as in the legend to Fig. 2. *ND, not determined. ‡Intensity ratio Band II/Band III.

gen and cysteine-25 sulphur atoms were in less than van der Waals contact in structures of *N*-acylglycine ethyl dithio esters derived by x-ray crystallography (Varughese et al., 1984), a weak but favorable N···S contact existed. Moreover, a preferred line of approach of N to S in the N···S nonbonded contact was identified (Rosenfield et al., 1977), wherein maximal nitrogen to sulphur orbital interactions occurred. It was proposed that this consisted of a HOMO-LUMO type interaction between the nitrogen's lone pair electrons and low lying empty orbitals associated with the S—C linkage. Further insight into this interaction has been obtained from ab initio SCF-MO calculations on *N*-formylglycine dithio acid (Fausto et al., 1991). For the B-form of this acid, Fausto et al. (1991) found a N···$S_{(thiol)}$ distance of 289.8 pm, in close agreement with values derived by x-ray analysis on similar dithio esters (Varughese et al., 1984; Huber et al., 1982). The ab initio results indicated that the N···$S_{(thiol)}$ interaction involves predominantly the nitrogen 2p orbital (containing the lone pair) perpendicular to the amide linkage and the sulphur 3p orbital nearly parallel to the nitrogen 2p orbital. Importantly, the theoretical results confirmed that one consequence of the interaction is a shortening of the C(=S)—S single bond as a result of increased electron delocalisation in the B-form dithio ester group.

That the N···$S_{(thiol)}$ contact might modulate kinetic parameters has been discussed by Carey et al. (1984b). For a series of *para*-substituted *N*-benzoylglycine dithioacylpapains, Y—C_6H_5C(=O)NHCH_2C(=S)S-papain, the rate constant for deacylation k_3 was found to correlate with the strength of the N···S contact in the B conformation. The deacylation rate varied sixfold for substituents Y = H, OCH_3, CH_3, Cl, and NO_2. When Y was strongly electron withdrawing, e.g., NO_2, the strength of the N···S interaction in a model compound decreased while the rate of deacylation of the corresponding *N*-benzoylglycine dithioacylpapain increased. This gave rise to the notion that increasing the strength of the N···S interaction leads to a small but significant decrease in deacylation because the N···S contact has to be broken in the rate determining step (upon converting the acyl enzyme to the transition-state for deacylation [Storer and Carey, 1985]). The idea received further support in the work of Lee et al. (1989), where the use of the strongly electron withdrawing pentafluorophenyl ring in *N*-(pentafluorobenzoyl)glycine dithioacylpapain extended the range in k_3 from 0.03 (*p*-methylbenzoyl) to 0.3 s^{-1} (pentafluorobenzoyl).

The above indicates that one of the factors accounting for the low deacylation rates of the 'poor' thiono ester substrates, *N*-(methyloxycarbonyl)-L-(phenylglycine)-glycine-, *N*-(methyloxycarbonyl)-glycine-glycine-, and *N*-(phenylacetyl)-glycine methyl thiono ester, may be a strong N···S interaction, because this interaction has to be broken in the rate-determining step for deacylation. Moreover, a strong N···S interaction may lead to strengthening of the C—S bond which is being broken in the deacylation step (Fausto et al., 1991). A further consideration is that close N···S contact will bring about greater interaction of the amide and dithio ester π-electron systems. In particular, a strong N···S interaction optimizing the overlap between amide and dithio ester orbitals could provide a mechanism for an increase in RR intensity of the amide modes. This would be especially important for the NH group lying close to the cysteine sulphur and could provide a means for intensity enhancement for modes involving the NH group. In other words, the slow deacylation rate for the poor substrates and the appearance of an intense Band III (Tables 1 and 2) may both follow from the same cause, viz., a strong interaction between the amide and dithio ester chromophores via effective N···S contact.

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