intensity, spectra are taken with a pulse interval of 4.0 s, which is longer than 5 times the longest T_1 of the H1 protons. For calculation of recoveries, reaction mixtures at $t = 0$ and at equilibrium are measured also with a pulse interval of 36.0 s, which is longer than the T_1's of the methylene protons of diglyme. One hundred scans serve this purpose, the total area of the two methylene signals being used as standard. Ratios of *α-anti*-7 to *α-syn*-7 and *β-anti*-7 to *β-syn*-7 are obtained as follows: two areas, A_{anti} (6.35 and 6.38 ppm) and A_{syn} (6.57 and 6.59 ppm), are measured by computer integration of the spectra. Because the α- and β-components in each of A_{anti} and A_{syn} overlap slightly (overlapping area ca. 0.9 and 2.0% of A_{anti} and A_{syn}, respectively), they are resolved by the cut-and-weigh method (generally, two cuts are taken, standard deviations being less than 0.7% of the total area). The resulting data, from which the kinetic parameters of Table V are derived, are available in Table IX as supplementary materials. Rate and equilibrium constants are optimized simultaneously by the nonlinear least-squares method to fit the reversible first-order kinetic equation to the observed data.

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Supplementary Material Available: Unprocessed kinetic data from the thermal rearrangements of **3**, **5**, and **7** (Tables VII-IX, respectively) (4 pages). Ordering information is given on any current masthead page.

Markedly Different Acyl Papain Structures Deacylate at Similar Rates: Resonance Raman Spectroscopic and Kinetic Evidence†

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Abstract: Resonance Raman (RR) spectroscopy has been used to determine the structure of the acyl group in a series of dithioacyl papains in which the side chain of the substrates P_1 amino acid residue has been extended from 2 (CH_3CH_2–) to 3 ($CH_3CH_2CH_2$–), and to 4 ($CH_3CH_2CH_2CH_2$–) carbon atoms in a linear chain. A conformational analysis was carried out on the corresponding ethyl ester model compounds, *N*-(methyloxycarbonyl)-L-phenylalanyl-L-ethylglycine, *N*-(methyloxycarbonyl)-L-phenylalanyl-L-norvaline, and *N*-(methyloxycarbonyl)-L-phenylalanyl-L-norleucine ethyl dithio esters, based on the RR spectra and known conformational states of glycine and alanine-based dithio esters. Comparison of the RR spectra of the model compounds with those of the corresponding *N*-(methyloxycarbonyl)-L-phenylalanyl-L-ethylglycine, -L-norvaline, and -L-norleucine dithioacyl papains shows that the acyl fragments adopt an A-like structure in the active site. An A-like structure is characterized by a large (near ±160°) nitrogen to thiol sulfur torsional angle about the NHCHR′–CS single bond. This conformation is in marked contrast to that found for *N*-acylglycine dithioacyl papains which have a small (near ±15°) $NHCH_2$–CS(thiol) torsional angle in the P_1 residue giving rise to the so-called B conformer. Thus we have evidence that the two classes of substrate give rise to two substantially different acyl group structures in the active site. However, for the ethylglycine, norvaline, and norleucine dithioacyl papains the deacylation rate constants (k_{cat}'s) are only ca. 3 times greater than k_{cat} for the most reactive *N*-acylglycine substrate. Thus deacylation can occur from both A- and B-type dithioacyl papains with only a small kinetic penalty for the latter. The existence of an A-type conformer in the active site and the need to maintain binding in the oxyanion hole raise the possibility that the acyl group is binding backwards, i.e. in the S_1' and S_2' binding sites.

Introduction

Relating enzyme and enzyme–substrate structure to catalytic reactivity remains a central challenge in biochemistry. Among the plethora of techniques brought to bear on this problem, resonance Raman (RR) spectroscopy offers the advantage of providing detailed molecular information on functioning enzyme–substrate complexes. The RR spectrum of the chromophoric center, e.g. the dithio ester in RC(=O)NHCHR′C(=S)S-papain, enables us to monitor the vibrational spectrum of the bonds undergoing catalytic transformation. Structural detail is accessed by interpreting the spectra usually by reference to vibrational and vibrational-crystallographic analyses of model compounds, e.g. RC(=O)NHCHR′C(=S)SC_2H_5.

Detailed RR studies, combined with X-ray crystallographic analysis, of *N*-acylglycine[1,2] and *N*-acylalanine[3-5] ethyl dithio esters have shown that these compounds are present in aqueous solution as an equilibrium mixture of two conformational populations which have been termed the A and B conformers (Figure 1). In the A conformation the nitrogen atom of the first dithio ester amino acid residue eclipses the thiono sulfur atom (C=S) of the dithio ester moiety. This structure is characterized by an angle of ca. 160° about the $NHCH_2$–CS bond (ψ'). In contrast the B con-

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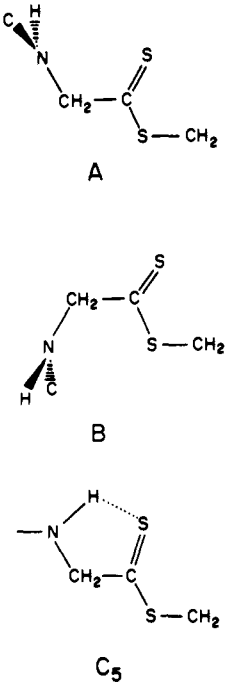

Figure 1. A, B, and C_5 structures for a glycine-based dithio ester.

former is characterized by a small ψ angle (near ±15°) such that the nitrogen atom is close to the thiol sulfur atom, with the distance between the atoms being less than the sum of their van der Waals radii (Figure 1). This interaction between the nitrogen and sulfur atoms involves a HOMO-to-LUMO donation of electrons from the nitrogen atom to the sulfur atom.[6] Subsequently, RR spectroscopy of a series of *N*-acylglycine dithioacyl papains has revealed that in each case the acyl moiety is bound in the active site in a B-type conformation.[7,8] In contrast the acyl moiety in *N*-acylalanine dithioacyl papains is bound in the active site in a non-B conformation.[9]

The work presented in this paper represents an extension of the studies on dithio esters to include dithioacyl papains generated from a series of *N*-(methyloxycarbonyl)-L-phenylalanyl-L-amino acid thiono methyl esters in which the side chain of the substrates P_1 amino acid residue has been linearly extended from two (ethylglycine) to three (norvaline) and to four (norleucine) carbon atoms. In contrast to glycine and alanine-based dithio esters the RR spectroscopic studies reveal that in aqueous solution the ethylglycine, norvaline, and norleucine dithio esters are present in only a single conformational population. Reference to the spectra–structure correlations established for the alanine and glycine dithio esters, in particular for *N*-(methyloxycarbonyl)-L-phenylalanyl-L-alanine ethyl dithio ester, strongly suggests that the single conformation observed for the ethylglycine, norvaline, and norleucine dithio esters is A-like. RR spectroscopy of the corresponding dithioacyl papains shows that the acyl groups in these complexes adopt the same conformation in the active site as that observed for the dithio esters in solution, viz. conformer A. Furthermore the deacylation rate constants for the A-type conformer acyl–enzyme complexes are only ca. 3 times greater than the corresponding limiting rate constant for the B conformer glycine-based dithioacyl papains. We thus have evidence for similar rates of deacylation from two substantially different acyl–enzyme structures. The apparent lack of a structure–rate correlation for acyl–papains is discussed with reference to previously published X-ray crystallographic structures of papain–inhibitor complexes.

Experimental Procedures

Materials. Sodium [^{13}C]cyanide (99% ^{13}C) was from MSD Isotopes (Merck Frosst Canada Inc., Montreal, Canada). Papain 2× crystallized suspension in 0.05 M sodium acetate, pH 4.5, was from Sigma Chemical Co. The enzyme was prepared, activated, and assayed as described previously.[10] Commonly titration with 5,5′-dithiobis(2-nitrobenzoic acid) gave 0.95–1.0 active thiol groups per mole of protein.

Synthesis. *N*-(Methyloxycarbonyl)-L-phenylalanine was synthesized as described previously.[10] L-Ethylglycine (L-2-aminobutyric acid), L-norvaline, and L-norleucine were converted into their respective methyl esters by treatment with thionyl chloride in methanol.

L-Ethylglycine Methyl Ester. L-Ethylglycine (5.15 g, 0.05 mol) was added to 30 mL of absolute methanol in a 250-mL round-bottomed flask and cooled to −10 °C on an ice–salt–methanol bath. Thionyl chloride (3.65 mL, 5.95 g, 0.05 mol) was then added dropwise with stirring while the temperature was maintained at −5 °C. After an additional 10 min at −10 °C the reaction mixture was allowed to come to room temperature and then heated to 40 °C on a water bath for 1 h. The solvent was removed by rotary evaporation, and the resulting solid was redissolved in 20 mL of absolute methanol. Addition of ether resulted in the formation of a white precipitate which was collected by filtration, washed with ether, and dried in vacuo. This gave 5.32 g (0.045 mol, 90%) of L-ethylglycine methyl ester.

The methyl esters were coupled to *N*-(methyloxycarbonyl)-L-phenylalanine with use of isobutylchloroformate and *N*-methylmorpholine.[10] In order to synthesize the methyl thiono ester substrates and dithio ester model compounds, the *N*-(methyloxycarbonyl)-L-phenylalanyl-L-amino acid methyl esters were first converted into the corresponding nitrile derivatives. Conversion of the nitriles into methyl thiono esters and ethyl dithio esters was as described previously.[10,11] *N*-(Methyloxycarbonyl)-L-phenylalanylglycine methyl thiono ester was synthesized as described previously.[10] All analyses agreed with calculated values within ±0.4%.

***N*-(Methyloxycarbonyl)-L-phenylalanyl-DL-norleucine ^{13}C=S Methyl Thiono Ester and Ethyl Dithio Ester.** In order to synthesize the title compounds [1-^{13}C]-DL-norleucine nitrile was first synthesized from valeraldehyde and sodium [^{13}C]cyanide (Na[^{13}C]N).[12]

Valeraldehyde (8.6 g, 10.6 ml, 0.1 mol) was dissolved in 30 mL of acetonitrile. This solution was then added dropwise to a suspension of 10.4 g (0.1 mol) of $NaHSO_3$ in 12 mL of H_2O and the mixture was stirred overnight at room temperature. The white solid that formed was obtained by filtration, washed with 100 mL of acetonitrile, and dried in vacuo to give 16.91 g (0.089 mol, 89%) of the bisulfite addition product [CH_3-$(CH_2)_3CH(OH)$-SO_3Na] which was used without further purification for the next step.

The bisulfite addition product (3.8 g, 0.02 mol) was dissolved in 8 mL of H_2O and placed in a 100-mL 3-necked flask fitted with a dropping funnel and reflux condenser. The flask was cooled on an ice–water bath and a solution of 2.0 g (0.04 mol) of Na[^{13}C]N in 6 mL of H_2O was then added dropwise with stirring over 30 min. During the course of the addition refluxing was observed. After standing for a further 1 h on the ice–water bath followed by 2 h at room temperature the reaction mixture was extracted with 5 × 10 mL of CH_2Cl_2. The CH_2Cl_2 extracts were combined, dried over anhydrous Na_2SO_4, and filtered. Evaporation of the solvent gave the cyanohydrin product as a yellow oil (2 g, 0.018 mol, 90%) which was used without further purification for the next step.

The cyanohydrin product (2 g, 0.018 mol) was added dropwise to 8 mL of NH_4OH and stirred overnight at room temperature. The reaction mixture was then extracted with CH_2Cl_2 (5 × 10 mL). The CH_2Cl_2 extracts were combined and extracted with 10% aqueous HCl (5 × 10 mL). The aqueous extracts were combined and rotary evaporated to give 2.7 g (0.018 mol, 100%) of [1-^{13}C]-DL-norleucine nitrile hydrochloride as a light yellow solid.

The ^{13}C-labeled DL-norleucine nitrile was coupled to *N*-(methyloxycarbonyl)-L-phenylalanine and subsequently used to generate the methyl thiono esters and ethyl dithio esters as described previously.[10] Analyses of the final products agreed with calculated values within ±0.4%.

Resonance Raman (RR) Instrumentation. RR spectra were obtained with 324-nm Kr^+ laser excitation, a Spex Triplemate, and a Tracor Northern UV-enhanced diode array.[13] In previous experiments RR

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Table I. Kinetic Parameters for *N*-(Methyloxycarbonyl)-L-phenylalanyl-L-amino Acid Methyl Thiono Esters[a]

	methyl thiono ester	
P_1 amino acid	pH stat[b] k_{cat}, s^{-1}	stopped flow[c] k_3, s^{-1}
glycine	0.61 ± 0.02 (0–0.2 mM)	0.64 ± 0.01
alanine	0.85 ± 0.08 (0–2.0 mM)	0.85 ± 0.04
ethylglycine	2.2 ± 0.2 (0–1.5 mM)	1.93 ± 0.01
norvaline	1.7 ± 0.2 (0–0.75 mM)	1.90 ± 0.02
norleucine	2.2 ± 0.1 (0–0.75 mM)	1.50 ± 0.02

[a] See Experimental Procedures for conditions. [b] Substrate concentrations given in parentheses. [c] Comparison of k_3 from stopped-flow ([E] ≫ [S]) with k_{cat} from pH stat ([S] ≫ [E]) indicates that nonproductive binding did not occur during the pH stat experiments and also confirms that deacylation is rate limiting.

spectra of dithioacyl papains have been collected from a reaction mixture of enzyme and methyl thiono ester contained in a cuvette. However, with the substrates used in this work precipitation of substrate and/or product occurred ca. 20–30 s after mixing enzyme and substrate. Thus RR data of the dithioacyl papains described in this work were obtained with use of a flow system, the spectra being obtained ca. 10 s after mixing. The flow system used has been described before.[14] Commonly one syringe contained 0.2 mM papain in 50 mM sodium phosphate buffer pH 6.5, 1 mM EDTA, and 20% acetonitrile while the second contained 2 mM substrate in 50 mM sodium phosphate buffer pH 6.5, 1 mM EDTA, and 20% acetonitrile.

Kinetics. Steady-state deacylation rates (k_{cat}) were obtained with use of a Radiometer RTS 822 pH-stat. Product formation was titrated with NaOH solutions prepared daily and standardized against a standard HCl solution. Reactions were performed at pH 6.5, 25.0 ± 0.1 °C, and the reaction mixture contained 0.3 M NaCl, 1.0 mM EDTA, and 20% acetonitrile. For the methyl thiono ester substrates the papain concentration was 1.0 μM. Substrate concentrations ([S]) are given in Table I. Initial rates (v) were obtained directly from the recorder trace and kinetic parameters were calculated by linear regression of plots of $[S]/v$ against [S]. Values of k_{cat} are an average of at least two determinations.

In order to ensure that the steady-state kinetic parameters were not subject to nonproductive substrate binding, deacylation rates (k_3) were also determined under conditions of [E] ≫ [S] with a Cantech Scientific Ltd. (Winnipeg, Canada) stopped-flow instrument interfaced to a series 300 Hewlett-Packard computer. Reactions were performed at 25.0 ± 0.1 °C with 300 μM papain and 10 μM substrate in 50 mM sodium phosphate buffer, 0.2 M NaCl, 5.0 mM EDTA, and 20% acetonitrile. Rapid acylation followed by slower deacylation of the enzyme was monitored at 315 nm. Deacylation rate constants (k_3) were obtained by fitting a single exponential function to the observed deacylation curves. Each k_3 is the average of at least four reactions.

Results and Discussion

In the present work a series of *N*-(methyloxycarbonyl)-L-phenylalanyl-L-amino acid ethyl dithio esters and the corresponding dithioacyl papains have been studied in which the side chain of the P_1 amino acid has been linearly extended from two carbon atoms (ethylglycine, CH_3CH_2–) to three carbon atoms (norvaline, $CH_3CH_2CH_2$–), and to four carbon atoms (norleucine, $CH_3CH_2CH_2CH_2$–). While no crystallographic studies have yet been undertaken for these dithio esters, the RR data can be explained by reference to the existing RR spectra–structure data base for the *N*-acylalanine and, to a lesser extent, *N*-acylglycine ethyl dithio esters, wherein examples already exist for both A-type and B-type structures in the crystalline state. The present studies reveal that, in contrast to *N*-acylalanine and *N*-acylglycine ethyl dithio esters, the dithio esters based on ethylglycine, norvaline, and norleucine exist in aqueous solution in only one main structure. The evidence strongly suggests that this structure is an A-type

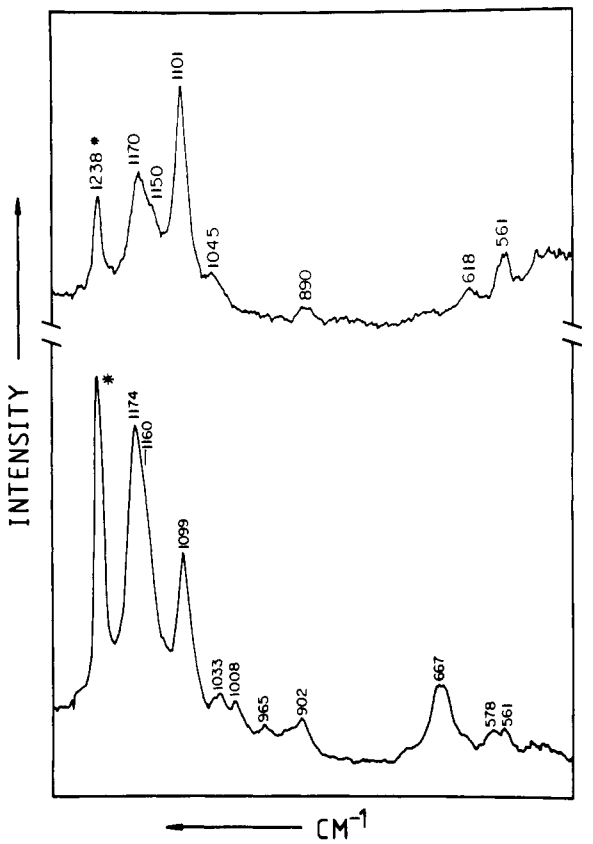

Figure 2. Resonance Raman (RR) spectra of (top) *N*-(β-phenylpropionyl)-DL-alanine ethyl dithio ester and (bottom) *N*-(methyloxycarbonyl)-L-phenylalanyl-DL-alanine ethyl dithio ester in the solid state. The spectra are reproduced from ref 5 and represent the characteristic RR signatures of conformer B and conformer A alanine-based dithio esters, respectively. The asterisks indicate a laser plasma line. The spectra are plotted on different abscissa scales.

conformation. Furthermore RR studies on the corresponding dithioacyl papains indicate that the acyl groups also adopt this structure in the active site of the enzyme.

RR Band Signatures for A, B, and C_5 Conformers of Glycine and Alanine-Based Dithio Esters. Combined RR and FTIR studies have shown that in CCl_4 *N*-acylglycine[1] and *N*-acylalanine[5] dithio esters exist as an intramolecularly hydrogen bonded C_5 conformation in which the NH group of the dithio ester amino acid is hydrogen bonded to the dithio ester thiono (C=S) atom (Figure 1). This conformation is expected to have ψ' similar to that found for A-type conformers (ψ' near ±160°) but to differ from the conformer A structure via a rotation about the NH–CHR'CS bond (ϕ') which allows the NH proton to hydrogen bond to the thiono sulfur atom. The RR spectra for the alanine and glycine dithio esters are very similar and are characterized by a single intense band at ca. 1170–1180 cm^{-1} (referred to previously as band I) in the 1000–1200-cm^{-1} region of the spectrum. There are also minor features at ca. 1100 and 1040 cm^{-1} as well as in the 500–800-cm^{-1} region.

In aqueous/acetonitrile solution *N*-acylglycine[1] and *N*-acylalanine[5] ethyl dithio esters exist as a mixture of two conformational populations, conformers A and B. These structures differ from each other via a rotation of ca. 150–180° about the amino acid dithio ester NHCHR'–CS bond (ψ') (Figure 1). For B-type conformers ψ' is near ±15° and this structure is characterized by the close approach of the nitrogen and thiol sulfur atoms.[2,3,6] In contrast for A-type dithio esters ψ' is near ±160° and the nitrogen atom is cis to the thiono (C=S) sulfur atom.[2,4,5] Each conformer is associated with a characteristic set of bands in the RR spectrum which have been identified following combined spectroscopic and structural studies on crystalline dithio esters. For the alanine-based dithio esters the RR signature for conformer B comes from *N*-benzoyl-DL-alanine[3] and *N*-(*p*-nitrobenzoyl)-

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DL-alanine[5] dithio esters which crystallize in B-type conformations and is characterized by an intense band at ca. 1100 cm^{-1} (referred to in previous publications as band II), a medium intensity broad composite feature at 1150–1170 cm^{-1} (referred to in previous publications as band I), and a band at 560 cm^{-1} (Figure 2). Similarly, the RR signature for conformer A comes from *N*-(methyloxycarbonyl)-L-phenylalanyl-DL-alanine ethyl dithio ester[4] which crystallizes in an A-type conformation and is characterized by an intense band near 1170 cm^{-1} (band I), a medium intensity narrow band near 1100 cm^{-1} (band II), and intensity around 660–680 cm^{-1} (Figure 2). Thus in aqueous solution conformers A and B both make differential contributions to band intensity in the 1150–1170- and 1100-cm^{-1} regions. A major piece of evidence for the coexistence of two conformational populations in solution stems from the temperature dependence of the intensity ratio of band I to band II. These studies have revealed that for both glycine[15] and alanine[5] dithio esters conformer B is the thermodynamically favored structure (for glycine dithio esters ΔH for A–B is ca. 2–4 kJ mol^{-1}).

N-(Methyloxycarbonyl)-L-phenylalanyl-L-ethylglycine, -L-norvaline, and -L-norleucine Ethyl Dithio Esters. (a) Evidence for a C_5 Structure in CCl_4. Figure 3 compares the RR spectra obtained for *N*-(methyloxycarbonyl)-L-phenylalanyl-L-ethylglycine (MeO-L-Phe-L-Ethylgly), *N*-(methyloxycarbonyl)-L-phenylalanyl-L-norvaline (MeO-L-Phe-L-Norval), and *N*-(methyloxycarbonyl)-L-phenylalanyl-L-norleucine (MeO-L-Phe-L-Norleu) ethyl dithio ester in CCl_4. All three spectra are very similar to the corresponding spectrum of *N*-(methyloxycarbonyl)-L-phenylalanyl-L-alanine (MeO-L-Phe-L-Ala) ethyl dithio ester in CCl_4 (Figure 3).[5] As the side chain is extended through the series band I is observed to progressively decrease in frequency. Thus band I is at 1183 cm^{-1} for MeO-L-Phe-L-Ala ethyl dithio ester, 1173 cm^{-1} for MeO-L-Phe-L-Ethylgly ethyl dithio ester, 1169 cm^{-1} for MeO-L-Phe-L-Norval ethyl dithio ester, and 1168 cm^{-1} for MeO-L-Phe-L-Norleu ethyl dithio ester. The ethylglycine, norvaline, and norleucine ethyl dithio esters also have weak features at ca. 1100, 1035, and 720 cm^{-1} and lack the characteristic alanine marker at 1000 cm^{-1}. Figure 3 also shows the effect of ^{13}C substitution on the spectrum of MeO-L-Phe-L-Norleu ethyl dithio ester, wherein band I at 1168 cm^{-1} in the unlabeled spectrum is shifted to 1153 cm^{-1}. Additionally ^{13}C substitution also causes an intensification and decrease in frequency of the band at 1102 to 1091 cm^{-1}. The effect of isotopic substitution is consistent with the presence of only one conformational population in solution. From the overall similarities in the RR spectra of the unlabeled compounds, we may conclude that the MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu ethyl dithio esters are present as C_5 structures in CCl_4.

(b) Evidence for a Single Major Population in H_2O/Acetonitrile Solution. The RR spectra of MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu ethyl dithio ester in H_2O/acetonitrile solution are shown in Figure 4. Comparison with the RR spectra obtained in CCl_4 shows that upon solvation in H_2O/acetonitrile there is a significant intensity increase in the bands at ca. 1100 (band II) and 1040 cm^{-1}. There is also probably an intensity increase in the feature around 720 cm^{-1} and the associated shoulder at 650 cm^{-1}. However peak positions in the two solvent systems are very similar. The C_5 conformer observed in CCl_4 cannot exist in H_2O/acetonitrile due to intermolecular hydrogen bonding between the NH group and solvent acceptor molecules. Thus there is an alteration in ϕ' away from that required for intramolecular hydrogen bonding toward the value observed for an A-type conformer (ϕ' ca. −80°). For alanine[5] and glycine[1] dithio esters an analogous increase in band II intensity is observed upon transfer from CCl_4 to H_2O/acetonitrile, which is explained by the formation, via rotation about the NHCHR′–CS bond (ψ'), of a B conformer population in solution. However in H_2O/acetonitrile solution the band I/band II ratio for MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu ethyl dithio esters is observed to be temperature independent from +25 to −30 °C (data not shown). Providing, as is the case for alanine-based dithio esters, that conformers A and B for MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu ethyl dithio ester contribute disparately to the RR intensity observed at ca. 1165 (band I) and 1100 (band II) cm^{-1} (Figure 4), this strongly suggests that only one conformational structure is present in solution.

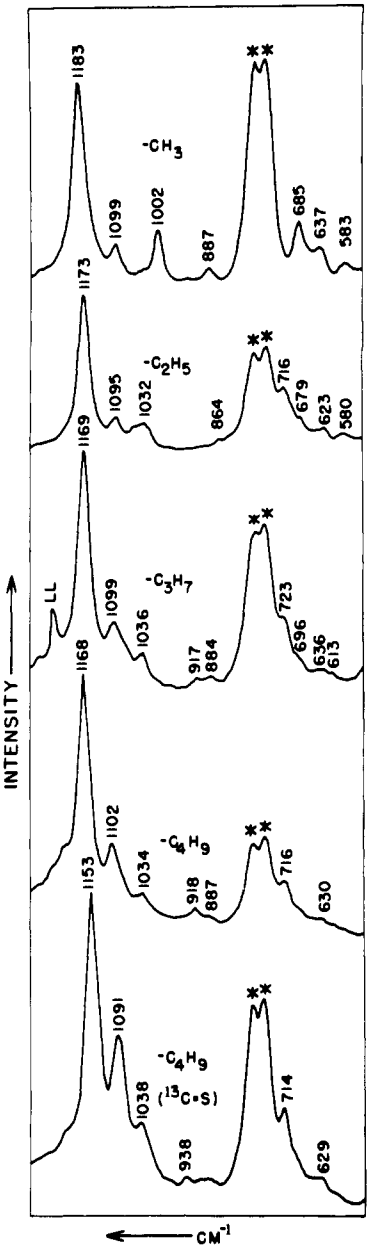

Figure 3. Resonance Raman (RR) spectra of *N*-(methyloxycarbonyl)-L-phenylalanyl-L-amino acid ethyl dithio esters 2 mM in CCl_4, where the amino acid is alanine ($-CH_3$), ethylglycine ($-C_2H_5$), norvaline ($-C_3H_7$), and norleucine ($-C_4H_9$) and the (bottom) spectrum of the $^{13}C{=}S$ derivative of *N*-(methyloxycarbonyl)-L-phenylalanyl-L-norleucine ethyl dithio ester ($-C_4H_9$, $^{13}C{=}S$): 324-nm laser excitation, 100-mW laser power, 12-cm^{-1} experimental resolution, acquisition time 10 × 1 s. Sample assembly: stirred cuvette. The asterisks indicate features due to solvent.

This conclusion is supported by comparison of the fwhm (full width at half maximum, cm^{-1}) values of band I for MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu ethyl dithio esters in solution with fwhm values for MeO-L-Phe-L-Ala ethyl dithio ester in solution and in the solid state. Thus fwhm is 31 cm^{-1} for MeO-L-Phe-L-Ala ethyl dithio ester in the solid state, wherein only conformer A is expected to occur, while it is 45 cm^{-1} for MeO-L-Phe-L-Ala ethyl dithio ester in aqueous

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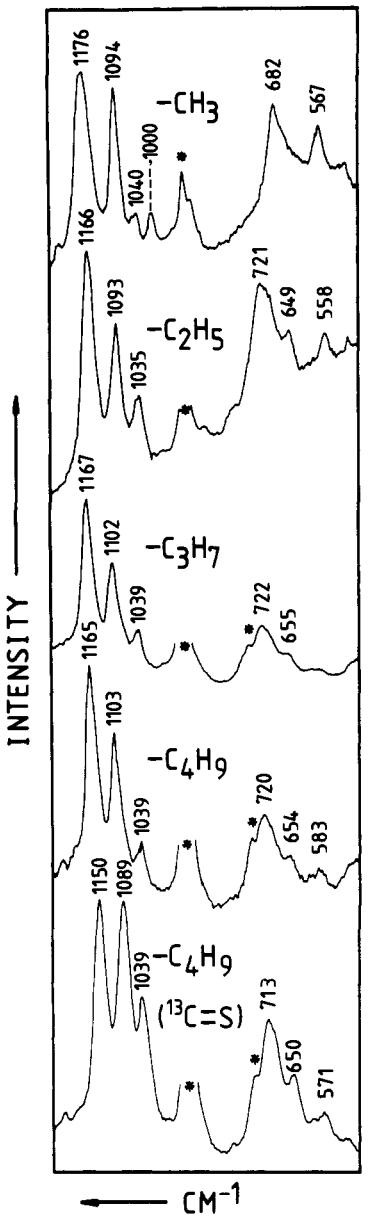

Figure 4. Resonance Raman (RR) spectra of *N*-(methyloxycarbonyl)-L-phenylalanyl-L-amino acid ethyl dithio esters in H_2O/acetonitrile, where the amino acid is alanine ($-CH_3$), ethylglycine ($-C_2H_5$), norvaline ($-C_3H_7$), and norleucine ($-C_4H_9$) and (bottom) spectrum of the $^{13}C{=}S$ derivative of *N*-(methyloxycarbonyl)-L-phenylalanyl-L-norleucine ethyl dithio ester ($-C_4H_9$, $^{13}C{=}S$). All ethyl dithio esters are 0.3 mM in 30% acetonitrile/H_2O except *N*-(methyloxycarbonyl)-L-phenylalanyl-L-alanine ethyl dithio ester which is 0.25 mM in 2.5% acetonitrile/H_2O. Spectral conditions are the same as in the legend to Figure 3. The asterisks indicate features due to solvent.

solution where both conformers A and B are expected to occur.[5] In contrast fwhm is 30–34 cm^{-1} for MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu ethyl dithio esters in solution, supporting the presence of only one conformer for these dithio esters in solution.

Finally the existence of only a single major conformation in solution for MeO-L-Phe-L-Norleu ethyl dithio ester is supported by the effect of ^{13}C substitution on the RR spectrum of this compound. Thus ^{13}C labeling of the C=S group causes band I at 1165 cm^{-1} in the unlabeled compound to shift to 1150 cm^{-1} and causes band II at 1103 cm^{-1} in the unlabeled spectrum to increase in intensity and shift to 1089 cm^{-1}. There is also an intensity increase in the 1039-cm^{-1} band. Additionally isotopic substitution results in intensification and a shift to lower frequency of the 720-cm^{-1} feature. In the 1000–1200-cm^{-1} region there is thus no band splitting upon ^{13}C substitution as might be expected to occur if the 1165- or 1103-cm^{-1} bands (^{12}C) contained contributions from A and B conformer modes. Moreover the ca. 15-cm^{-1} decrease observed for both bands I and II suggests that the effect of isotopic substitution is being distributed over two modes arising from the same conformer.

The Single Conformation Observed in H_2O/Acetonitrile Solution Is an A-Type Conformer. The RR spectra of MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu ethyl dithio esters in H_2O/acetonitrile solution (Figure 4) have very similar band profiles in the 1000–1200-cm^{-1} region compared to MeO-L-Phe-L-Ala ethyl dithio ester in the solid state wherein only a single A-type conformation is found[5] (Figure 2). Thus for each dithio ester band I is narrow (relative to the breadth of band I observed for crystalline B conformer alanine dithio esters, Figure 2)[3,5] and more intense than band II at ca. 1090–1100 cm^{-1}. This suggests that in solution the ethylglycine, norvaline and norleucine dithio esters are present as A-like conformers. Additionally there is very marked similarity between the peak positions observed in H_2O/acetonitrile solution and in CCl_4 for the ethylglycine, norvaline, and norleucine dithio esters. The main difference between the RR spectra obtained from the two solvents is the decreased band intensity for the bands at 1090–1100 and 1035–1040 cm^{-1} for the dithio esters in CCl_4. This suggests that the conformer found in H_2O/acetonitrile solution is C_5-like. As the main difference between the C_5 and A conformer structures is rotation about the NH–CHR'CS dithio ester bond (ϕ') the results are consistent with rotation about this bond influencing the relative intensities of the bands observed at 1090–1100 and 1035–1040 cm^{-1}. It is certain however that the RR spectra of MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu ethyl dithio esters in H_2O/acetonitrile solution do not resemble the RR spectra of B conformer alanine dithio esters wherein a broad composite band I with intensity at ca. 1150–1170 cm^{-1} is expected[3,5] (Figure 2). While no crystallographic studies have been done on the ethylglycine, norvaline, and norleucine dithio esters, RR spectra of these three compounds have been obtained in the solid state (data not shown). The rational behind this approach is that previous studies have shown that many glycine[8] and alanine[5] dithio esters solidify in a single conformation. Interestingly the RR spectra of solid MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu ethyl dithio esters show maximum RR intensity in band II at 1090–1100 cm^{-1} and a broad band at 1150–1170 cm^{-1}. These spectra are similar to RR spectra of B conformer alanine dithio esters[3,5] (Figure 2), and it is concluded that the ethylglycine, norvaline, and norleucine dithio esters solidify in a B-type conformation. Comparison of these spectra with that obtained in H_2O/acetonitrile solution strengthens our hypothesis that the single dithio ester conformation observed in solution is A-like.

The Acyl Group Conformation in the Dithioacyl Papains Is A-like. The RR spectra of MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu dithioacyl papains are shown in Figure 5. These spectra are very similar to the corresponding spectra of the model ethyl dithio esters in H_2O/acetonitrile solution (Figure 4). For all three dithio esters there is close similarity between the positions and relative intensities of bands I and II for the model and acyl–enzyme spectra, although the fwhm of the enzyme-bound dithio ester bands is somewhat reduced. In addition the band observed in the model spectra at ca. 720 cm^{-1} has shifted to ca. 695–700 cm^{-1} in the acyl–enzyme spectra. Finally the band at ca. 1040 cm^{-1} for the ethyl dithio esters is absent in the dithioacyl papain spectra. The disappearance of a feature near 1040 cm^{-1} on binding to the enzyme is also observed for glycine-based dithio esters.[7,8] Finally there is very good correspondence between the $^{13}C{=}S$-substituted norleucine ethyl dithio ester RR spectrum and that for the norleucine dithioacyl papain.

On the basis of the similarity in the RR spectra of the MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu ethyl dithio esters in solution and the corresponding dithioacyl papains, we conclude that the conformation of the acyl group in the dithioacyl enzymes is very similar to that observed

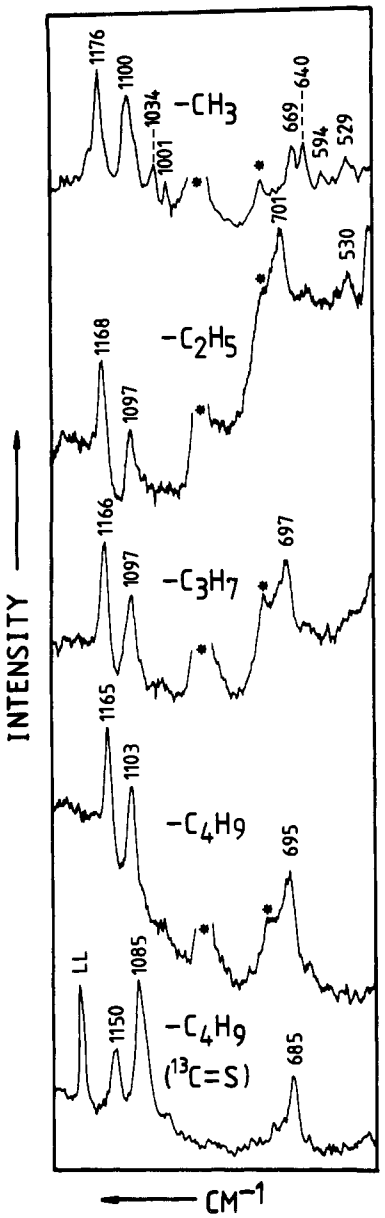

Figure 5. Resonance Raman (RR) spectra of *N*-(methyloxycarbonyl)-L-phenylalanyl-L-amino acid dithioacyl papains, where the amino acid is alanine ($-CH_3$), ethylglycine ($-C_2H_5$), norvaline ($-C_3H_7$), and norleucine ($-C_4H_9$) and (bottom) spectrum of the $^{13}C{=}S$ derivative of *N*-(methyloxycarbonyl)-L-phenylalanyl-L-norleucine dithioacyl papain ($-C_4H_9$, $^{13}C{=}S$). For the ethylglycine, norvaline, and norleucine dithioacyl papains spectra were obtained in a flow system. Conditions after mixing were as follows: 0.1 mM papain, 1 mM substrate, 20% acetonitrile, 50 mM phosphate buffer, 1 mm EDTA, pH 6.5. Spectral conditions were as in the legend to Figure 3 except with an acquisition time 20 × 3 s. The spectrum of *N*-(methyloxycarbonyl)-L-phenylalanyl-L-alanine dithioacyl papain is reproduced from 9. The asterisks indicate features due to solvent.

in solution and is a conformer A structure.

Kinetics: Acyl Enzymes with Markedly Different Structures Deacylate with Similar Rates. Within experimental error the deacylation kinetic rates (k_{cat}) for MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu dithioacyl papains are identical being ca. 2.0 s^{-1} (Table I; k_{cat} determined by pH state for the methyl thiono ester substrates). By comparison k_{cat} is 0.85 s^{-1} for MeO-L-Phe-L-Ala dithio acyl papain and 0.61 s^{-1} for *N*-(methyloxycarbonyl)-L-phenylalanylglycine (MeO-L-Phe-Gly) dithioacyl papain (Table I). Thus the substrates having extended side chains on the P_1 amino acid and which adopt an A-like conformation in the active site only deacylate ca. 3-fold faster than MeO-L-Phe-Gly dithioacyl papain, the acyl group of which is known to be bound in a B-type conformation in the active

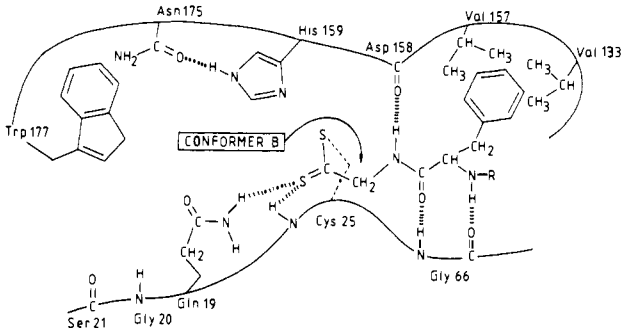

Figure 6. Schematic representation of the binding of a Phe-Gly dithioacyl group in the active site of papain as a B conformer. Note that the Phe side chain is bound in the S_2 binding site (comprising in part the enzyme side chains of Val 133 and Val 157) and that the dithio ester C=S group is hydrogen bonded in the oxyanion hole (backbone NH of Cys-25 and side chain NH of Gln-19).

site.[8] This is a remarkable observation given that for the model ethyl dithio esters the A and B conformers differ by a 150° to 180° rotation about the NHCHR′–CS bond and suggests that a major change in acyl group conformation can occur in the active site with only a small kinetic penalty.

Indeed one explanation for the ca. 3-fold lower deacylation rate for MeO-L-Phe-Gly dithioacyl papain is the presence of the nitrogen–sulfur interaction in the B conformer structure of this acyl enzyme[6,8] and which is absent in the conformer A dithio ester structure postulated to occur in MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu dithioacyl papains. Studies on a series of para-substituted *N*-benzoylglycine dithioacyl papains have shown that an increase in strength of the nitrogen–sulfur interaction can be related to a decrease in k_{cat} for these acyl enzymes.[16] Thus the absence of this contact in the A-type conformer acyl enzymes could account for the slight increase in k_{cat} discussed above. However, given the radically different stereochemistries of conformers A and B this explanation is only one of a number which could be put forward.

Modeling an A-Type Conformer into the Active Site of Papain. The B conformer structure observed with RR spectroscopy for all glycine-based dithioacyl papains is the generally accepted "normal" acyl enzyme structure. This statement is based mainly on the X-ray crystallographic studies of Drenth and co-workers, who determined the structure of a number of peptide–chloromethyl ketone papain complexes.[17] The acyl enzyme structure modeled by Drenth from the inhibitor complexes is characterized by a B-type conformation about the NHCHR′–CO bond of the P_1 acyl amino acid, where the P_1 amino acid is identified by using the nomenclature established by Berger and Schecter.[18] Additionally the acyl carbonyl group is hydrogen bonded in the oxyanion hole, comprising the backbone NH group of Cys-25 and the side chain NH group of Gln-19, and the P_2 L-phenylalanine residue is bound in the enzyme's S_2 specificity pocket, comprising in part the side chains of Val-133 and Val-157. This structure is shown schematically in Figure 6.

The similar rates of deacylation of the A- and B-type dithioacyl papains suggest that the thiono C=S group in both structures is bound similarly in the active site relative to the oxyanion hole, which provides transition-state stabilization during the reaction, and to the imidazole side chain of His-57, which acts as a general base catalyst in the deacylation reaction. If the C=S group is maintained in the position observed for the B conformer, molecular graphics analysis suggests that rotation about the NHCHR′–CS bond, required to generate an A-type conformer, will result in unfavorable interactions between the acyl Phe residue and enzyme groups. Thus in order to accommodate an A-type conformer in

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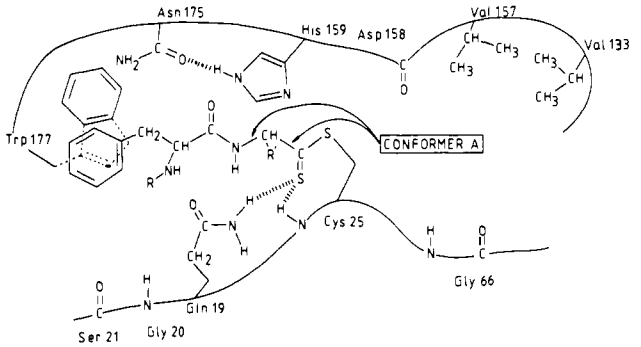

Figure 7. Schematic representation of the binding of a Phe-NH-CHR'-C=S dithioacyl group in the active site of papain as an A-type conformer. Note that while it is proposed that the dithio ester C=S group is bound in the oxyanion hole, the acyl group is bound backwards in the active site and occupies the S_1' and S_2' enzyme subsites.

the active site there must be a major relocation of the acyl group.

Recent molecular graphics analysis (unpublished work in this laboratory) of the X-ray structure of tosyl-L-lysine chloromethyl ketone papain[19] has revealed that the lysine residue in this structure is bound in the active site as an A-type conformer. Additionally, the acyl carbonyl group is bound in the oxyanion hole. However the acyl group is not occupying the enzyme's S_1 and S_2 binding sites but instead is bound in the S_1' and S_2' binding sites (nomenclature of Berger and Schecter[18]). Thus this A-type conformer acyl enzyme is bound backwards in the active site relative to the known mode of binding of the B conformer acyl enzymes. Significantly the tosyl-L-lysine chloromethyl ketone inhibitor is based on an amino acid with a long side chain compared to the other B conformer chloromethyl ketone inhibitors which have glycine or alanine as the P_1 amino acid. This suggests one possible way in which the acyl groups of MeO-L-Phe-L-Ethylgly, MeO-L-Phe-L-Norval, and MeO-L-Phe-L-Norleu dithioacyl papains could be accommodated in the active site as conformer A structures. A schematic representation of this mode of binding is shown in Figure 7. Obviously binding of the acyl fragment backwards in the active site precludes interaction of the acyl groups P_2 residue, in this case L-phenylalanine, with the enzyme's S_2 specificity pocket. That this may be the case is supported by the observation that the identity of the P_2 residue has little or no affect on the rate of deacylation. This is demonstrated by comparison of the observed deacylation rates for *N*-acetylglycine *p*-nitrophenyl ester and *N*-acetyl-L-phenylalanineglycine *p*-nitrophenyl ester, wherein k_{cat} is 2.0 and 6.6 s^{-1}, respectively.[20]

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Photogeneration of Organic Bases from *o*-Nitrobenzyl-Derived Carbamates

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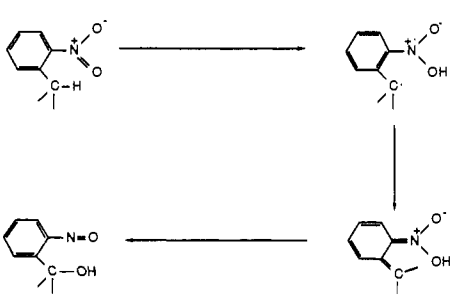
Abstract: The design of novel photoprecursors of organic bases and the steric and electronic factors that control their quantum-efficient transformation into free amines or diamines have been investigated. The basic design involves the protection of amines with photolabile [(*o*-nitrobenzyl)oxy]carbonyl groups or α-substituted analogues. The resulting protected amines owe their light sensitivity to the classical *o*-nitrobenzyl photorearrangement and cleanly liberate free amine in both the solid state and in solution upon irradiation with UV light below 400 nm. Several designs were explored in which the structure of the photoactive center was varied systematically to investigate the influence of various steric and electronic effects. In all cases, the practical potential of these photoactive carbamates as organic sources of photogenerated base was evaluated by product analysis of solution photolysates, by determination of their solid-state quantum efficiencies, and by measurement of their thermal properties. For example, the quantum efficiency of various carbamates for cyclohexylamine photogeneration at 254 nm ranged from 0.11 to 0.62 depending on both α-substituent and *o*-nitro substitution patterns, confirming the importance of both steric and electronic considerations. Similar results were obtained with other base photoprecursors and all showed good thermal stabilities.

Introduction

The in situ generation of active species capable of catalytic action by external means such as photoirradiation, thermal activation, or other processes has stimulated research in areas as varied as catalysis, microlithography, and biosensors. In particular, the development of compounds that act as efficient sources of acid upon irradiation has led to elegant new developments in the chemistry of radiation-sensitive materials for microelectronics[1] and in the coatings industry.[2] While numerous novel systems have resulted from a variety of photoprecursors of acid, the same has not yet been realized for base-catalyzed or base-promoted processes, as the concept of photogenerated base remains largely unexplored.

Scheme I



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