Rate-Structure Correlation for Enzyme-Substrate Intermediates: Resonance Raman and Kinetic Studies on Some *N*-Benzoylglycine (Dithioacyl)papains

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Abstract: By means of a multichannel-based detection system, complete resonance Raman (RR) spectra have been recorded for a series of *N*-benzoylglycine (dithioacyl)papains in which the *N*-benzoyl group is substituted in the para position by H, OCH_3, CH_3, Cl, or NO_2. The RR data show that each dithioacyl enzyme forms a single conformational population and that throughout the series the torsional angles about the glycinic —NH—CH_2—C(=S) bonds of the bound acyl groups are invariant. The angles are such that the glycinic nitrogen comes into close contact with the sulfur atom of cysteine 25, giving rise to a geometry known as conformer B. In solution, each of the corresponding *N*-benzoylglycine ethyl dithio esters forms a population of B conformers having a very similar geometry, and N···S(thiol) interaction, to that selected by the active site. In addition, in solution the conformer B population is in equilibrium with other conformers, and the RR spectra show that the relative population of conformer B in the series of para-substituted *N*-benzoylglycine ethyl dithio esters correlates with the electronic nature of the para group. Increasing electron-donating ability of the para substituent increases the basicity of the glycinic nitrogen which, in turn, leads to an increase in the strength of the N···S(thiol) interaction and an increase in the relative population of conformer B. For the (dithioacyl)papains, stopped-flow kinetic measurements reveal that k_3, the rate constant for deacylation, also correlates with the nature of the benzoyl para substituent; an increase in the electron-donating power of the para group leads to a decrease in k_3. Thus, it appears that the para substituent also modulates the strength of the N···S(thiol) interaction in the active site and, since this change of strength is expressed in k_3, that the rate-limiting step in deacylation involves breaking the N···S contact.

A classical physical-organic approach to the study of chemical reaction mechanisms involves systematically varying substituents in one or more of the reactants. By noting the dependence of reactivity on the steric and electronic properties of the substituents a great deal can be learned concerning the structures of transition states and the factors that control the mechanism. While it would be highly desirable, such an approach usually cannot be pursued in the study of enzyme mechanisms.[1] A major reason for this limitation is that structure-reactivity studies with enzymes tend to measure the combined effects of changes in the structure of the substrate on its interactions with the enzyme and the effects of the electronic changes in the transition state. However, recent studies on (dithioacyl)papains offer the potential for overcoming the limitations usually encountered in enzyme studies.[2] The reaction under consideration, esterolysis by cysteine proteases, has a fairly well-understood pathway containing an acyl enzyme intermediate, and for a series of acyl groups we can monitor the conformation of each intermediate by means of its resonance Raman (RR) spectrum. Thus, the effects, if any, of varying the substituent on the substrate can be measured. Finally, the reactivity of each dithioacyl enzyme intermediate can be assessed by using stopped-flow kinetics to measure the deacylation rate constant, k_3. Thus, with a series of known intermediates of known structure and reactivity the potential exists for establishing meaningful structure-reactivity relationships.

The means by which thiono ester substrates are used to form dithioacyl enzymes with cysteine proteases and thence to generate the RR spectrum of the group undergoing transformation in the active site is outlined in the preceding[3] and in other publications.[2,4,5] Many of the properties of the enzyme-bound acyl groups can be understood by referring to model compounds based on *N*-acylglycine dithio esters. In solution the latter compounds exist as a mixture of three major rotamers. In non-hydrogen bonding solvents the mixture consists of conformer B (Figure 1) in which the glycine N atom comes into close contact with the thiol S atom and a conformer known as C_5 (Figure 1) wherein the NH group H bonds to the thiono S and the NH—CH_2—C(=S) fragment forms a planar five-membered ring.[6] In hydrogen-bonding solvents a population of conformer B exists in equilibrium with a second rotamer known as conformer A.[7] The latter is closely related to C_5 but participates in H bonding to solvent rather than forming an intramolecular H bond. The RR spectrum of the dithio ester is very sensitive to changes in vibrational coupling caused by going from one rotamer to another and, in consequence, the RR signature of each rotamer is characteristic. In the present study, the conformational signatures allow us to monitor the changes in relative solution populations with variation of para substituent in a series of *N*-benzoylglycine ethyl dithio esters. The changes in population are accounted for in terms of changing strength of the N···S contact in conformer B. The RR signatures also allow us to monitor conformational events in the active site, and it is found that the enzyme exerts rotamer selection with every *N*-acylglycine substrate studied thus far binding solely in a B-type

Table I. Absorption Maxima of Para-Substituted *N*-Benzoylglycine Dithio Esters, $XC_6H_4C(=O)NHCH_2CSS—Y$

X	Y = C_2H_5,[a] nm	Y = papain,[b] nm
H	308[c] ($\epsilon = 1.11 \times 10^4$)	315
OCH_3	307	314
CH_3	307 ($\epsilon = 1.07 \times 10^4$)	314
Cl	306.5 ($\epsilon = 1.17 \times 10^4$)	314
NO_2	305	*d*

[a] In CH_3CN. [b] Conditions given in ref 4. [c] For the ethyl ester the extinction coefficients in CH_3CN and CCl_4, and thus of the B and C_5 forms, are very similar.[8] [d] Obscured by excess substrate.

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(1) Fersht, A. "Enzyme Structure and Mechanism"; W. H. Freeman: Reading, UK, 1977; p 59.
(2) Carey, P. R.; Storer, A. C. *Acc. Chem. Res.* **1983**, *16*, 455-460.
(3) Varughese, K. I.; Storer, A. C.; Carey, P. R. *J. Am. Chem. Soc.*, preceding paper in this issue.
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(5) Storer, A. C.; Lee, H.; Carey, P. R. *Biochemistry* **1983**, *22*, 4789-4796.
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(7) Huber, C. P.; Ozaki, Y.; Pliura, D. H.; Storer, A. C.; Carey, P. R. *Biochemistry* **1982**, *21*, 3109-3115.
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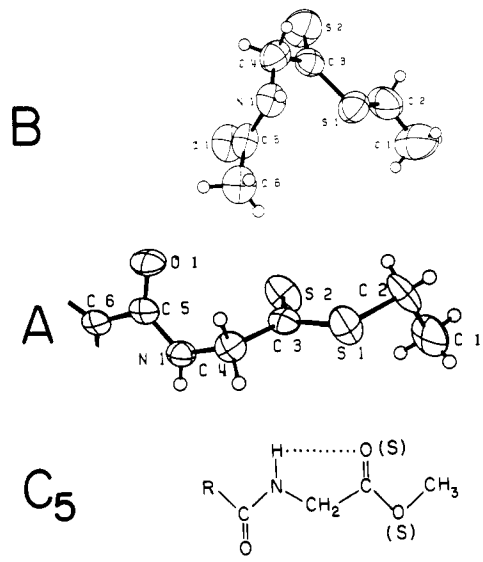

Figure 1. Conformers B, A, and C_5. Structures B and A are reproduced from ref 7 with permission.

conformation. In the present series of para-substituted *N*-benzoylglycine (dithioacyl)papains the variation of the strength of the N···S contact, in the framework of conformer B, correlates with the change in the deacylation rate constant. Thus, we can relate the variation of a single rate constant to the variation of a single interatomic enzyme–substrate contact in the active site.

Experimental Section

Materials. Papain was purchased from the Sigma Chemical Co. as a suspension in sodium acetate. The papain was further purified by affinity chromatography, stored as the inactive mercuriopapain, concentrated, and reactivated as described in ref 5. The enzyme prepared in this way was found to contain >70% of active cysteine per mol of protein, using 5,5′-dithiobis(2-nitrobenzoic acid).

The substituted *N*-benzoylglycine thiono ester substrates and dithio ester model compounds were synthesized by the procedures outlined in ref 4 and 8, respectively. The compounds were purified by crystallization and column chromatography on silica gel with acetonitrile–ether mixture (1:9) as eluant. The results of elemental analysis of all the compounds synthesized were within acceptable limits (i.e., 0.02 times the theoretical values), and the purity of the samples was checked by NMR.

Methods. UV absorption spectra were obtained with Cary 118 and 219 spectrophotometers, and the near-UV absorption maxima (recorded using Yankeelov cells for the enzyme intermediates) for the dithio esters and dithioacyl enzymes are given in Table I. The RR spectra of the dithioesters shown in Figures 2 and 3 were recorded by using a scanning system based on a Spex 0.5m double monochromator.[4] A horizontal quartz capillary flow cell was used to obviate photodecomposition. The RR spectra of the (dithioacyl)papains shown in Figure 5 were obtained by using a multichannel system based on a Spex triplemate spectrometer equipped with a Tracor-Northern TN-6132 intensified linear diode array. The multichannel instrumentation is described in detail in ref 9. Typically, the reaction mixtures used to generate the RR spectra contained 1.5×10^{-4} M papain, 5×10^{-3} M substrate, 20% CH_3CN, and phosphate buffer pH 5.5. The sample consisted of approximately 1 mL of reaction mixture contained in a quartz cuvette. The solution was stirred by a magnetic "pip" to obviate photodecomposition or photoisomerization. The (dithioacyl)papain RR spectra were obtained by using ~100-mW 324-nm Kr^+ excitation. The spectra were recorded approximately 30 s after starting the reaction with a total acquisition time of 20 s (20 × 1 s). Raman frequencies were calibrated by two emission lines from an argon lamp which correspond to Raman shifts of 742.5 and 1225.9 cm^{-1} with 324-nm excitation. Absolute accuracy of the Raman peak positions is believed to be ±2 cm^{-1} with reproducibility of better than ±1 cm^{-1} for sequential spectra.

Kinetic data were obtained using a stopped-flow spectrometer (Cantech Scientific Ltd., Winnipeg, Manitoba) coupled via a transient recorder to a Commodore Pet microcomputer. The data (256 points, 8-bit resolution) were fitted to a single exponential function using a program based on Marquardt's nonlinear least-squares curved fitting procedure.[10]

(10) Marquardt, D. W. *J. Soc. Ind. Appl. Math* **1963**, *11*, 431–441.

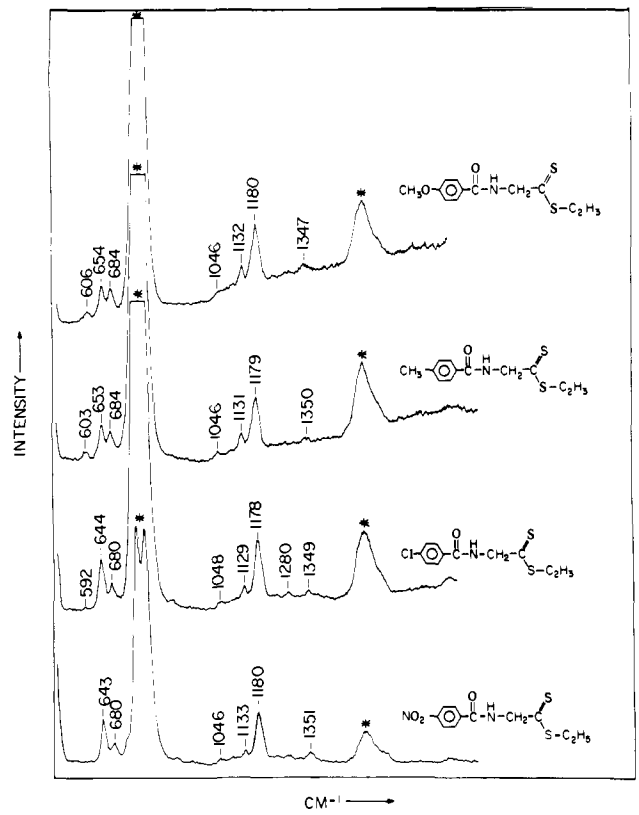

Figure 2. RR spectra of the model compounds (≈4×10^{-4} M) in CCl_4 solution. Spectral conditions: Spex 0.5m scanning instrument, flow cell, ≈20 mW, 324-nm excitation, 8-cm^{-1} spectral slit. Asterisk denotes solvent feature.

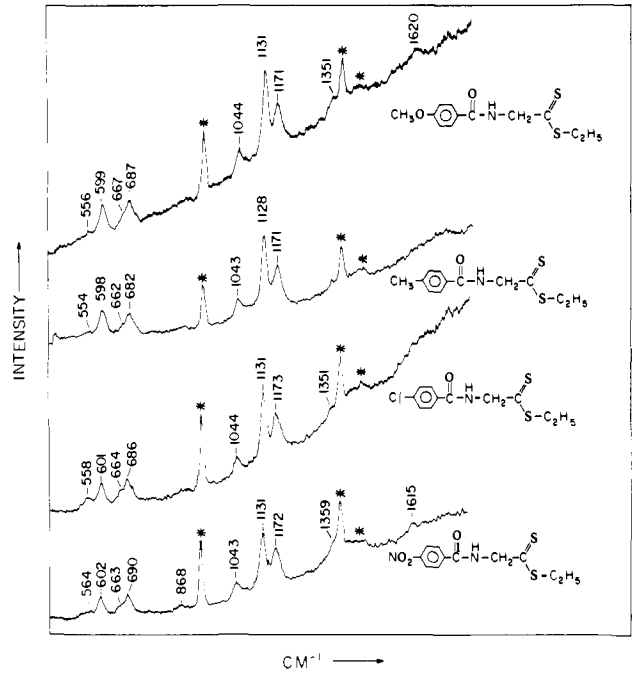

Figure 3. RR spectra of the model compounds (≈4×10^{-4} M) in 80% H_2O/20% CH_3CN solution. Spectral conditions: Spex 0.5m scanning instrument, flow cell, ≈20 mW, 324-nm excitation, 8-cm^{-1} spectral slit. Asterisk denotes solvent feature.

Measurements were made at 20 °C, pH 6.5 in a buffer containing 50 mM Na_2HPO_4, 0.2 M NaCl, 5 mM EDTA (pH'ed using HCl), and 20% CH_3CN. The enzyme concentration used was 5 μM, and the reactions were monitored at 315 nm. The substrate concentration ranges and number of determinations at each concentration were the following: *p*-CH_3O, 0.2–8 mM, 3–5 determinations; *p*-CH_3, 0.2–4 mM, 2–6 de-

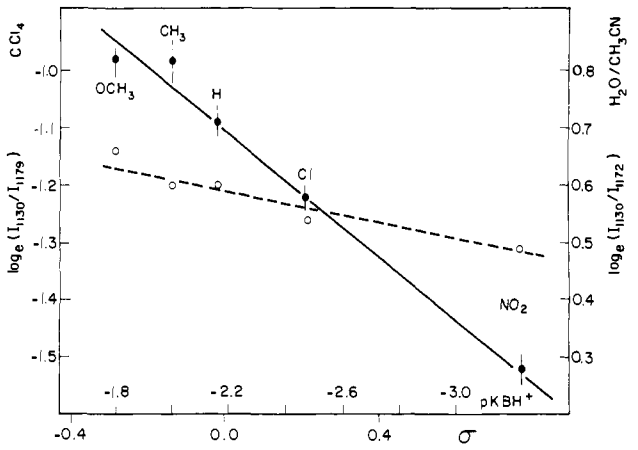

Figure 4. Logarithm of the ratio of RR peak heights at 1130 and near 1175 cm^{-1}, $\log_e [I_{1130}/I_{1175}]$ vs. pK_A and Hammett σ constants for the para substituents in a series of *N*-benzoylglycine ethyl dithio esters. The solid line is drawn through the values obtained in CCl_4. The open circles refer to the measurements in CH_3CN/H_2O. The error bars are estimated from the uncertainty in taking base lines for intensity measurements.

terminations; *p*-H, 0.4–10 mM, 2–5 determinations; *p*-Cl, 0.2–2 mM, 2–5 determinations; *p*-NO_2, 0.4–1 mM, 5–15 determinations. A combination of poor solubility and absorbance of the *p*-NO_2 substrate severely restricted the usable concentration range of this substrate.

Results and Discussion

Control of the Rotamer Population via the Para Substituents on the Benzoyl Group. The RR signatures of conformers A, B, and C_5 are dealt with in detail elsewhere.[3,6,8] For the present discussion it need only be reiterated that in H_2O/CH_3CN or CCl_4 solution the peak near 1130 cm^{-1} is due to the population of conformer B; in H_2O/CH_3CN solution the peak near 1172 cm^{-1} is due mainly to conformer A, while in CCl_4 the peak near 1178 cm^{-1} is due mainly to C_5.

The RR spectra seen in Figure 2 show that in CCl_4 the relative population of conformer B changes upon varying the benzoyl para substituent. By taking the height of the 1130-cm^{-1} peak (I_{1130}) as a measure of the concentration of conformer B and the height of the 1179-cm^{-1} peak as a measure of conformer C_5 (with the possibility that a small amount of conformer A may also contribute to the 1179-cm^{-1} peak) it is seen that the population ratio depends markedly on the nature of the para substituent.[11] For example, with the electron-donating OCH_3 in the para position I_{1130}/I_{1179} = 0.38, while with the electron-withdrawing NO_2 group in the para position I_{1130}/I_{1179} = 0.22. Figure 4 demonstrates that there is a good correlation between the intensity ratio, and therefore the relative populations, and the electronic nature of the para substituents. A plot of $\log_e [I_{1130}/I_{1179}]$ vs. pK_A (of the corresponding benzamides[12]) yields a straight line. Since pK_A is strongly correlated with Hammett σ constants,[12] $\log_e [I_{1130}/I_{1179}]$ also correlates with the σ values (Figure 4). A similar correlation for the populations in H_2O/CH_3CN (Figure 3), is seen in Figure 4. These results show that as the para substituent in the phenyl ring increases the basicity of the amide group there is a concomitant increase in the population of conformer B.[13] Taken with the findings of the preceding publication,[3] the results provide further insight into the N···S contact characteristic of conformer B. In the preceding paper[3] the N···S contact is described as a N(HOMO)···S(LUMO) overlap in which the N HOMO is essentially a p orbital occupied by a lone pair of electrons and the unoccupied orbital is based on a S–C σ^* or a S d orbital. Thus, as the para substituent on the benzoyl moiety increases the electron-donating ability of the N lone pair;[14] the strength of the N···S (thiol) interaction increases with a resultant increase in the relative amount of conformer B. The linear nature of the plot in Figure 4 for both CCl_4 and H_2O/CH_3CN solutions shows that the electron-donating ability of the N atom, controlled by the para substituents, correlates with the free energy difference between the B and A or C_5 conformers. The governing factor in this correlation may be the effect of the changes in the strength of the N···S interaction on enthalpy differences between rotamers. It is apparent that there is a larger variation in values ΔG for the rotamers in CCl_4 compared to H_2O/CC_3CN since larger changes are observed in $\log_e [I_{1130}/I_{1179}]$ for the former solvent. In conformer A, the non-B conformer in H_2O/CH_3CN, there is a close contact between the amide N atom and the thione S atom[7] and this may also involve an interaction that can be modulated by the change in basicity of the amide group. At present there is no information on N···S(thione) contacts analogous to that on N···S(thiol) contacts.

The stereochemistry of the N···S(thiol) attraction appears to be unchanged throughout the series of dithio esters with differing para substituents since the RR peak positions in Figure 2 do not vary. This finding is consonant with the conclusion reached in the foregoing and accompanying publication[3] that the ϕ' and ψ' torsional angles take on values permitting maximum overlap of the N lone pair of electrons with an unoccupied orbital based on S. The invariance of the position of the band near 1130 cm^{-1} in Figures 2 and 3 taken with the X-ray analyses[3] of *N*-benzoylglycine ethyl dithio ester and *N*-(*p*-chlorobenzoyl)glycine ethyl dithio ester suggests that ϕ' and ψ' are constant to within a few degrees for the para-substituted series in solution. Thus, the N···S interaction requires a well-defined stereochemistry, and changes in the strength of the interaction are expressed within that same stereochemistry.

Conformation of the Enzyme-Bound Acyl Groups. The RR spectra of the dithioacyl enzymes complexes seen in Figure 5, taken with our earlier studies on dithioacyl enzyme and ester systems,[3-8] allow us to describe the conformation of the acyl group in the active site. It has been established by isotopic substitution[5,6] and combined X-ray crystallographic–spectroscopic analyses[3,7] that a *N*-benzoylglycine dithio ester compound in a B-type conformation (Figure 1) gives rise to marker bands near 1170 (medium intensity), 1130 (intense), and 595 cm^{-1} (medium intensity). These marker bands are seen in the RR spectrum of unsubstituted *N*-benzoylglycine (dithioacyl)papain (Figure 5, top) and in each of the corresponding para-substituted derivatives (Figure 5). Moreover, the positions of the marker bands are the same within the limits of experimental error. It follows that each of the para-substituted *N*-benzoylglycine dithioacyl groups assumes a

(11) Peak intensity of a mode due to a given conformer is proportional to the conformer population multiplied by a resonance Raman intensity factor. The latter is a complex function of parameters such as the absorption transitions, extinction coefficient, and the wavelength of the light used to obtain the RR spectrum. We assume that the dithio ester chromophores' extinction coefficients are very similar throughout the series of para-substituted *N*-benzoylglycine ethyl dithio esters—the data in Table I provide some justification for this assumption and indicate that the extinction coefficients for the C_5 and B forms of each molecule are very similar. Thus the ratio of two bands due to conformer B and, e.g., C_5 is equal to a constant × (population B ÷ population C_5) throughout the series.

(12) Edward, J. T.; Chang, H. S.; Yates, K.; Stewart, R. *Can. J. Chem.* **1960**, *38*, 1518–1525.

(13) Since the proton-donating ability of the amide should decrease with increased basicity, the stability of the H-bonded C_5 conformer in CCl_4 might be expected to decrease through the series from *p*-NO_2 to *p*-OCH_3. However, this does not account for the osbserved population variation since the effect on the stabillty of the C_5 conformer is probably negligible. This conclusion follows from the observation that for the corresponding series of *N*-benzoylglycine dioxygen esters in which no N···S interaction is possible, there is no population variation in CCl_4 although both C_5 and non-C_5 conformers are present (unpublished work, this laboratory).

(14) Although the basicity of amides is generally expressed through the oxygen atom rather than the nitrogen atom,[15,16] i.e., O-protonation predominates over N-protonation, there is evidence in the literature that electrophiles can complex with the amide nitrogen, e.g., in iodine–amide complexes[17] and in some metal-catalyzed amide hydrolyses.[18]

(15) Sigal, H.; Martin, R. B. *Chem. Rev.* **1982**, *82*, 385–426.

(16) Homer, R. B.; Johnson, C. D. In "The Chemistry of Amides"; Zabicky, J., Ed.; Interscience: New York, 1970; pp 187–243.

(17) Drago, R. S.; Bafus, D. *J. Phys. Chem.* **1961**, *65*, 1066–1067.

(18) Houghton, R. P.; Puttner, R. R. *J. Chem. Soc. D* **1970**, 1270–1271.

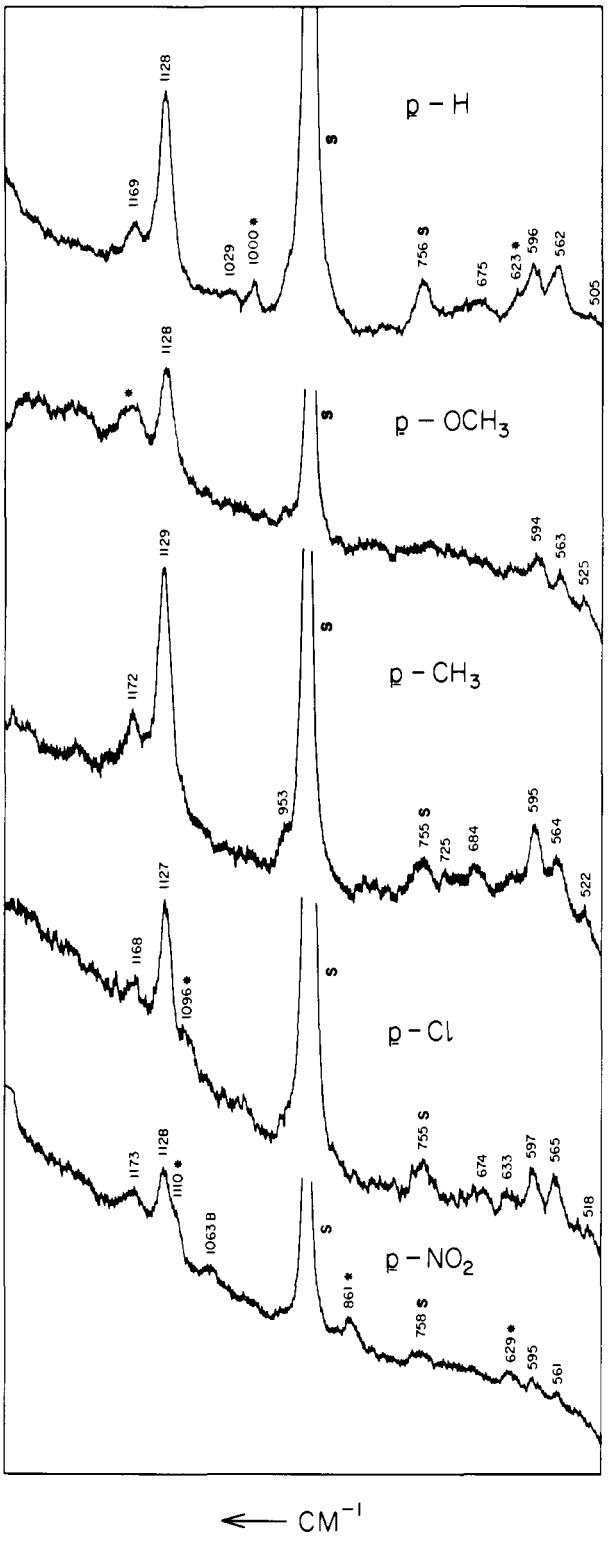

Figure 5. RR spectra of *N*-benzoylglycine (dithioacyl)papains with para substituents (denoted on each trace) on the benzoyl group. Spectral conditions: multichannel detection; 100 mW, 324-nm excitation; 12-cm^{-1} spectral slit, 20-s acquisition time (1 s × 20 accumulations). Substrate/product features are marked with an asterisk. S = CH_3CN feature, B = phosphate buffer.

B-type conformation in papain's active site and that there is little or no variation in conformation about the ϕ',ψ' angles.

Several of the reaction mixtures give rise to features that are due to preresonance bands from the *p*-benzamide portion. Since this portion is present in the substrate and product it is at high concentration compared to the dithioacyl group. The *p*-benzamide bands are marked on the spectra in Figure 5 by an asterisk.

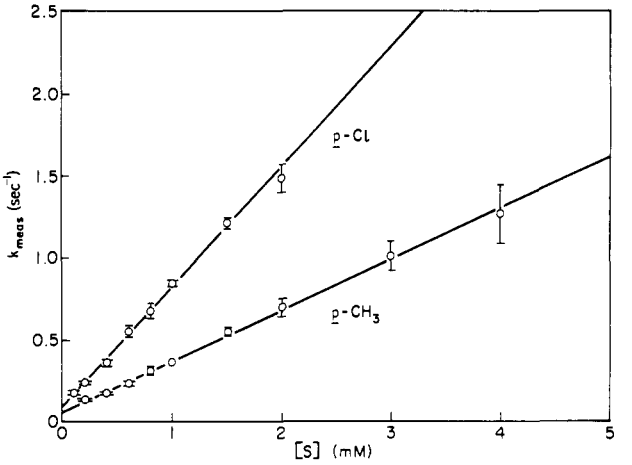

Figure 6. Typical plot of the measured exponential rate constants (k_{meas}) against substrate concentrations (s) for two of the para-substituted *N*-benzoylglycine thiono esters reacting with papain. The error bars represent the standard deviations for each data set.

Contributions from the CH_3CN, present at ≈20% concentration to keep the substrates in solution, are observed near 920 and 750 cm^{-1}. The CH_3CN peak at 920 cm^{-1} partially or totally obscures the characteristic *N*-benzoylglycine (dithioacyl)papain peak which occurs near 950 cm^{-1}. (Dithioacyl)papains also give rise to a weak peak near 675 cm^{-1}, assigned to the stretching motion of the S–C bond of cysteine 25,[5] and evidence for this is seen in the better quality spectra of Figure 5. Since the weak band near 520 and the medium-intensity feature near 560 cm^{-1} are both assigned to a B-type conformer, we have accounted for every peak seen in the spectra—either in terms of a conformer B mode or a solvent or a substrate peak. Therefore, at the present level of signal-to-noise ratio we have no evidence for any conformer, apart from a B type, in the active site.

Correlation between the Strength of the N···S Interaction and the Deacylation Rate Constant. Figure 6 shows some results of the stopped-flow study. The measured exponential rates are plotted against the substrate concentrations used for each substrate. The experimental points are the averages of from 3–15 determinations (see Experimental Section for details). The error bars represent the standard deviations for each data set. The straight lines through the points were obtained using a linear least-squares procedure weighted according to the various standard deviations.

A minimal reaction scheme[19] for the papain-catalyzed hydrolysis of ester (or thiono ester) substrates is

$$\mathrm{RC(=X)OR' + HS\text{-}papain} \overset{K_s}{\rightleftharpoons} \mathrm{RC(X)OR'\cdot HS\text{-}papain\ (Michaelis\ complex)} \xrightarrow{k_2} \mathrm{RC(=X)S\text{-}papain\ (acyl\ enzyme) + R'OH} \xrightarrow[+H_2O]{k_3} \mathrm{RC(=X)OH + HS\text{-}papain}$$

where X = O, esters, or X = S, thiono esters. The reaction proceeds through the formation of a Michaelis complex and an acyl enzyme intermediate. K_s represents the dissociation constant for the enzyme–substrate complex, and k_2 and k_3 represent the rate constants for the rate-limiting steps in the acylation and deacylation processes, respectively. These rate constants do not necessarily represent the bond-forming and bond-breaking steps in the two processes. They are defined by the slowest steps in the conversion of the Michaelis complex to the acyl enzyme and of the acyl enzyme to the free enzyme, respectively. Both conversions have been suggested to involve tetrahedral intermediates.[19]

The measured exponential rates (k_{meas}) are related to the constants in the reaction scheme above by eq 1,[20] where s =

$$k_{meas} = \frac{k_2 s}{K_s + s} + k_3 \qquad (1)$$

(19) Lowe, G. *Tetrahedron* **1976**, *32*, 291–302.

Table II. Stopped-Flow Kinetic Data for the Reaction of Para-Substituted *N*-Benzoylglycine Thiono Esters with Papain

para group	pK_A[12]	k_3, s^{-1}	k_2/K_s, s^{-1} M^{-1}
CH_3O	−1.8	0.030 ± 0.014	360 ± 19
CH_3	−2.01	0.061 ± 0.006	303 ± 6
H	−2.16	0.082 ± 0.011	224 ± 9
Cl	−2.47	0.093 ± 0.007	738 ± 15
NO_2	−3.23	0.178 ± 0.024	377 ± 43

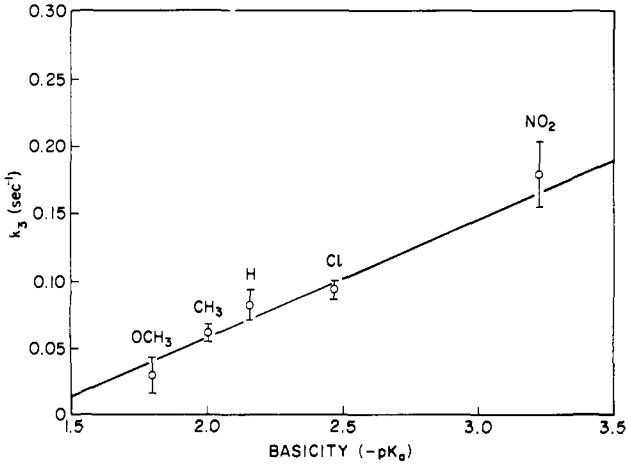

Figure 7. Correlation between the k_3, for the dithioacyl enzyme, and the basicity (pK_A) of the corresponding benzamide group in a series of substituted *N*-benzoylglycine (dithioacyl)papains. The para substituents are denoted on the figure.

substrate concentration. From plots of k_{meas} against s it should be possible to obtain values for k_3 (intercept when $s = 0$), k_2/K_s (slope at $s = 0$), and k_2 (from the limiting value of k_{meas} at high s). The concentration ranges used in this study were limited by the solubility of the substrates, and over the available concentration ranges no limiting values for k_{meas} were detectable. This indicates that for these substrates under the experimental conditions used $K_s \gg s$ and so eq 1 becomes

$$k_{meas} \simeq (k_2/K_s)s + k_3$$

Thus plots of k_{meas} against s yield straight lines from which values for k_2/K_s and k_3 can be obtained (Figure 6). The results of such analyses are given in Table II along with the basicities of the benzamide functions. From Table II it can be seen that there is no discernable trend in k_2/K_s, whereas k_3 appears to be strongly correlated with the basicity of the benzamide portion of the substrates. The correlation is demonstrated in Figure 7 where k_3 is plotted against pK_A. As the amide group becomes less basic, in the extreme case with the electron-withdrawing NO_2 group as the para substituent, k_3 increases. As shown above, decreasing basicity leads to a weaker N···S interaction in conformer B for the model compounds. The RR data show conformer B is the single invariant form found in the active site, and it is proposed that, as for the model compounds (which also have identical B conformations), the strength of the N···S contact in the active site is modulated by the para substituent. Thus, for the dithioacyl enzymes the strength of the N···S interaction correlates with the rate of deacylation; a stronger interaction decreases the rate and vice versa. The most likely explanation of this observation is that the rate-limiting step in the deacylation process involves the breaking of the N···S interaction either in the dithioacyl enzyme or in another intermediate further along the reaction pathway, i.e., a tetrahedral intermediate. Since it is the dithioacyl enzyme that accumulates during the steady-state turnover of thiono ester substrates, the favored explanation is that the rate-limiting breaking of the interaction occurs in the dithioacyl enzyme. Moreover, it seems probable that the N···S interaction is broken in order to form the tetrahedral intermediate since preliminary hard-sphere calculations (unpublished work, this laboratory) show that the most likely conformation of this intermediate is one in which the bonds on the C_α carbon and tetrahedral "carbonyl" carbon are arranged gauche to one another, i.e., that the N–C_α–C–S torsional angle would be approximately ±60° whereas in the dithioacyl enzyme it is approximately 0°. This increase in the torsional angle would effectively break the N···S interaction.

Another possible explanation of the correlation between the k_3 values and the basicities of the benzamide functions (Figure 7) is that, since basicity is proportional to the Hammett σ constants of the substituents, the correlation with k_3 arises from a charge-transfer interaction between the benzamide portion of the acyl group and the enzyme. However, on the basis of extensive nitrile inhibitor and ester substrate studies, Williams et al.[21,22] concluded that their results were inconsistent with charge-transfer binding of *N*-benzoylglycine-type acyl groups to the enzyme. Also a variation in a strong charge-transfer interaction of the acyl group with the enzyme might result in a minor variation in the conformations of the bound acyl groups, and no such variation was detected in the RR studies detailed above. In conclusion, therefore, the weight of the evidence favors the breaking of the N···S contact in the rate-determining step for deacylation, and in all likelihood, this occurs between the acyl enzyme and the tetrahedral intermediate.

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