Comparison of the Kinetics and Mechanism of the Papain-Catalyzed Hydrolysis of Esters and Thiono Esters†

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ABSTRACT: The kinetic constants for the papain-catalyzed hydrolysis of the methyl thiono esters of *N*-benzoylglycine and *N*-(β-phenylpropionyl)glycine are compared with those for the corresponding methyl ester substrates. The k_2/K_s values for the thiono esters are 2–3 times higher than those for the esters, and both show bell-shaped pH dependencies with similar pK_a's (approximately 4 and 9). The k_3 values for the thiono esters are 30–60 times less than those for the esters and do not exhibit a pH dependency. Solvent deuterium isotope effects on k_2/K_s and k_3 were measured for the ester and thiono ester substrates of both glycine derivatives. Each thiono ester substrate showed an isotope effect similar to that for the corresponding ester substrate. Moreover, use of the proton inventory technique indicated that, as for esters, one proton is transferred in the transition state for deacylation during reactions involving thiono esters and the degree of heavy atom reorganization in the transition state is very similar in both cases. The k_3 values for the hydrolysis of a series of para-substituted *N*-benzoylglycine esters were found to correlate with the k_3 values for the corresponding para-substituted thiono esters [Carey, P. R., Lee, H., Ozaki, Y., & Storer, A. C. (1984) *J. Am. Chem. Soc. 106*, 8258–8262], showing that the rate-determining step for the deacylation of both thiolacyl and dithioacyl enzymes probably involves the disruption of a contact between the substrate's glycinic nitrogen atom and the sulfur of cysteine-25. It is concluded that the hydrolysis of esters and thiono esters proceeds by essentially the same reaction pathway. Due to an oxygen–sulfur exchange process the product released in the case of the *N*-(β-phenylpropionyl)glycine thiono ester substrate is the dioxygen acid; however, for the *N*-benzoylglycine thiono ester substrate, the thiol acid is the initial product. This thiol acid then acts as a substrate for papain and reacylates the enzyme to eventually give the dioxygen acid product. It is shown that thiol acids are excellent substrates for papain.

The impetus for a comparative kinetic study of the papain-catalyzed hydrolysis of *N*-acylglycine esters and thiono esters originates in our use of thiono esters to produce chromophoric dithioacyl enzymes for spectroscopic investigations. The sulfur-for-oxygen substitution in ester substrates results in the formation of dithioacyl enzymes that are chromophoric (λ_{max} near 315 nm) (Lowe & Williams, 1965a) and that are ideally suited for the application of resonance Raman spectroscopy (Storer et al., 1979). The spectroscopic data demonstrate that all glycine-based thiono ester substrates tested with a wide variety of cysteine proteinases form a single homogeneous conformational population of active-site-bound dithioacyl groups, designated conformer B, in which the glycine's amide N atom comes into close contact with the thiol S atom of cysteine-25 (Ozaki et al., 1982; Huber et al., 1982; Storer et al., 1983; Carey et al., 1983, 1984b; Carey & Storer, 1983, 1984; Brocklehurst et al., 1984; Varughese et al., 1984). There is evidence from some of these studies that the strength of the N···S interaction can be modified by varying the basicity of the amide group containing the nitrogen atom (Carey et al., 1984b). Moreover, it has been shown that the rate constant for the rate-limiting step for deacylation (k_3) of dithioacyl papains is influenced by the strength of the interaction, indicating that this step for thiono ester substrates involves breaking or weakening of the N···S contact. In order to investigate if the detailed mechanistic information elicited for dithioacyl papains might also provide insight into the behavior of thiolacyl enzymes, a comparison of the kinetics of ester and thiono ester hydrolysis by papain has been undertaken.

Papain, and the other cysteine proteinases, catalyzes the hydrolysis of *N*-acyl-L-amino acid esters and amides by the well-known acyl enzyme mechanism (Scheme I). This mechanism comprises at least three steps: binding (binding constant $K_s = k_{-1}/k_1$), acylation (rate constant k_2), and deacylation (rate constant k_3) (Lowe, 1976; Polgár, 1977; Brocklehurst et al., 1981). In addition, there is little doubt that transient tetrahedral intermediates are involved in both the acylation and deacylation steps (Westerik & Wolfenden, 1972; Clark et al., 1977; Gamcsik et al., 1983). All substrates tested with papain have shown a bell-shaped pH dependence for k_{cat}/K_m characterized by pK_a's of approximately 4.2 and 8.5. This indicates that the ionizations of at least two enzyme groups are involved in the acylation process (Lowe, 1976; Polgár, 1977; Brocklehurst et al., 1981). It is generally agreed that the thiol of cysteine-25 and the imidazole of histidine-159 are the groups in question. In the native enzyme these groups are thought to interact and to exist predominantly as an ion pair, with the thiolate anion acting as the nucleophile in the acylation process. For most ester substrates deacylation is rate-limiting and k_{cat} exhibits a sigmoidal pH dependence with an apparent pK_a that depends on the nature of the acyl group but that is generally near 4. The pK_a has been assigned to the imidazole group of histidine-159, which is thought to act as a general base in the deacylation process.

For ester substrates, Szawelski & Wharton (1981) concluded that in the deacylation process the general-base-catalyzed formation of the tetrahedral intermediate is rate-limiting. Using the results from a proton inventory study, in conjunction with other available evidence and theoretical considerations, Szawelski & Wharton (1981) also proposed a structure for

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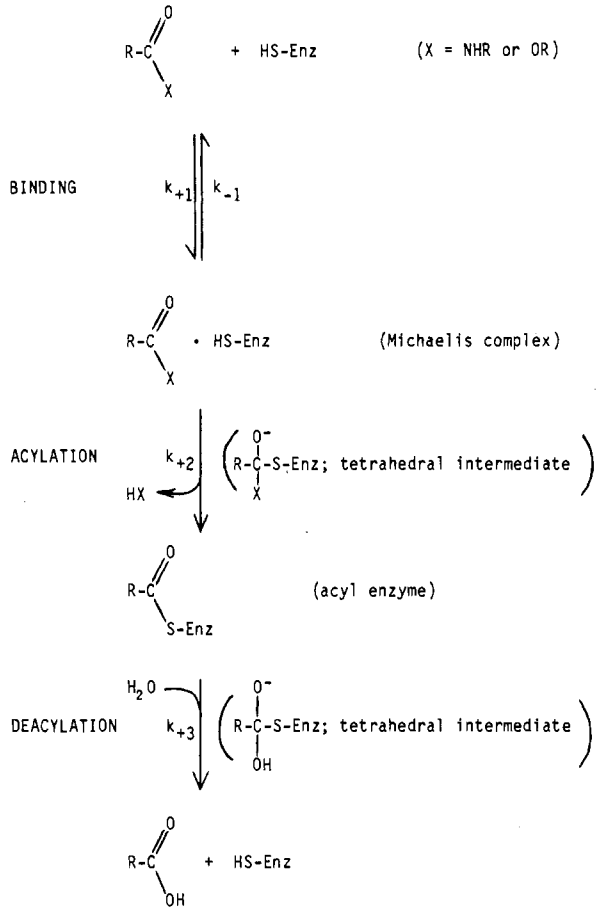
Scheme I



the transition state of the rate-limiting step in deacylation. A similar approach has been used in this work for the thiono esters, and possible similarities and differences in the structure of the transition states are discussed below. Moreover, a correlation observed for k_3 values permits a comparison of the structural changes occurring between the acyl enzyme and the tetrahedral intermediate for deacylation for ester and thiono ester substrates. A detailed comparison of the pH dependence of the kinetic rate constants is also made, and some comparisons are drawn with the earlier study of Asbóth & Polgár (1983).

Experimental Procedures

Materials. Papain was purchased from Sigma Chemical Co. as a suspension in sodium acetate. Papain was further purified according to the mercurial agarose column method described by Sluyterman & Wijdenes (1970). The purified enzyme was concentrated by using an Amicon concentrator and stored in its inactive mercurial form. The enzyme was activated as required by addition of 3 μL of β-mercaptoethanol per milliliter of enzyme solution and by stirring for 30 min. β-Mercaptoethanol was then removed by passage of the enzyme mixture down a Sephadex G-15 column. The thiol content of the enzyme was determined with 5,5′-dithiobis(2-nitrobenzoic acid) as described by Ellman (1959). The enzyme prepared in this way was found to contain >0.8 mol of active cysteine per mole of protein (on the basis of an $A_{280}^{1\%}$ of 25 and a molecular weight of 23 000).

The thiono ester substrates [*N*-benzoylglycine methyl thiono ester and *N*-(β-phenylpropionyl)glycine methyl thiono ester] were synthesized as described by Ozaki et al. (1982). The thiol acids were synthesized as described by Cronyn & Jiu (1952), and the oxygen acids and esters were prepared from the acid chlorides and glycine or glycine methyl ester, respectively. The results of elemental analysis of all compounds synthesized agreed within acceptable limits (±0.02 × calculated percentage) with the theoretical values.

Methods. All kinetic measurements were made at 20 °C. Steady-state kinetic measurements were made with a Radiometer RTS822 recording titration system. The acid product of the hydrolysis reaction was titrated by using a 20 mM NaOH solution that was freshly prepared daily and standardized against a standard HCl solution. Reaction mixtures typically consisted of 0.3 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 20% v/v CH_3CN, 5 (for the thiono esters) or 0.5–1 μM (for the esters) papain, and substrate. Substrates and their concentrations were as follows: *N*-benzoylglycine methyl thiono ester, 0–2.0 mM; *N*-(β-phenylpropionyl)glycine methyl thiono ester, 0–1.0 mM; *N*-benzoylglycine methyl ester, 0–40 mM (*p*-CH_3O derivative, 0–40 mM; *p*-CH_3 derivative, 0–40 mM; *p*-Cl derivative, 0–10 mM; *p*-NO_2 derivative, 0–10 mM); *N*-(β-phenylpropionyl)-glycine methyl ester, 0–20 mM. At the lower pHs, where the K_m values for these substrates increased, the upper limit of substrate concentrations was increased proportionately. Autodigestion of the enzyme was negligible under the conditions used, and the base-catalyzed hydrolysis of the substrate only became significant above pH 9. Few pH-stat results were obtained for the thiono esters above pH 6, since above this pH a slow negative rate, due to the CH_3CN cosolvent, was observed. This effect was negligible for the ester substrates. All reaction mixtures were titrated for several minutes, and the recorded slopes were linear for sufficient time to enable the initial rates to be determined accurately by using a ruler. The rates were corrected for the degree of protonation of the acid product at low pHs by allowing the reactions to go to completion and calculating proton stoichiometries from the total volume of base consumed. For the solvent isotope studies 99.7 atom % D deuterium oxide was used in the preparation of all solutions. The enzyme was prepared in a concentrated form in H_2O such that the final % D of the assay mixtures was never less than 98. For the proton inventory measurements a pH of 5.6 was chosen since it was shown that k_{cat} (k_3) is constant for all the substrates above pH 5 and also that the ratio $k_3(H_2O)/k_3(D_2O)$ remains constant over the pH range 5.0–6.0 (data not shown). Substrate concentrations were chosen such that $S \geq 10 \times K_m$ in order that the relationship $v_i \simeq k_3 e_0$ could be used in measuring k_3.

The data sets obtained with the pH stat were fitted to the Michaelis–Menten equation by using weights proportional to the initial velocities. The equations used are those given by Cornish-Bowden (1976), and the validity of the weighting system was tested experimentally. Six initial velocity measurements were made at each of eight substrate concentrations that were chosen to give initial velocities evenly spaced across the range of velocities used in this study. The substrate used was *N*-benzoylglycine methyl ester. It was found that the standard deviations of the data sets are proportional to the magnitude of the mean velocities (correlation coefficient = 0.877).

Transient-state kinetic data were obtained by using a stopped-flow spectrometer (Cantech Scientific Ltd., Winnipeg, Manitoba, Canada) coupled via a transient recorder to a Commodore Pet microcomputer. The data (256 points, 8-bit resolution) were fitted to a single exponential function in the case of *N*-benzoylglycine methyl thiono ester and to a single exponential plus a constant rate in the case of *N*-(β-phenylpropionyl)glycine methyl thiono ester. The constant rate in-

troduced for the latter substrate is thought to be due to a slow reaction involving the hydrogen sulfide produced as a primary product in the reaction (see below). The absence of the constant rate in the case of the *N*-benzoylglycine substrate is due to the fact that for this substrate H_2S is produced only after the substrate is significantly depleted and so no side reaction is observed on the time scale of the stopped-flow experiments. Since all observations involved transmission changes of $\leq 10\%$, it was assumed that concentration is proportional to transmission change (Hiromi, 1979). The curve fitting was carried out according to the nonlinear least-squares procedure of Marquardt (1963). All stopped-flow measurements were made at 20 °C in 20% (v/v) aqueous acetonitrile solutions (to increase substrate solubility) buffered according to the pH range: pH 3.5–4.9, 50 mM sodium citrate; pH 4.6–5.4, 50 mM sodium acetate; pH 5.9–8.05, 50 mM disodium hydrogen phosphate; pH 8.8–9.1, sodium borate. In an attempt to maintain a constant ionic strength all solutions were made up to 0.3 M in Na^+ by the addition of NaCl. All buffers contained 5 mM EDTA. The rates of hydrolysis of substrates by papain have been shown to be independent of the buffer components for acetate, phosphate, and borate (Whitaker & Bender, 1965), and the overlap between the citrate and acetate buffers in the present study extends this conclusion to the citrate buffer. Lucas & Williams (1969) have shown that in 20% (v/v) acetonitrile–water there is little change in the kinetic parameters for the papain-catalyzed hydrolysis of *N*-benzoylglycine methyl ester compared with water alone. For the stopped-flow study 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 as follows: for *N*-benzoylglycine methyl thiono esters, 0.4–10 mM and 2–7; for *N*-(β-phenylpropionyl)glycine methyl thiono ester, 0.1–5 mM and 2–9.

The exponential rates (k_{meas}) obtained with the stopped-flow apparatus are related to the rate constants shown in Scheme I by the equation:

$$k_{meas} = k_2S/(K_s + S) + k_3 \quad (1)$$

where $K_s = k_{-1}/k_1$. The derivation of this equation (Holloway et al., 1971) assumes that $k_{-1} \gg k_2$, i.e., that the binding of the substrate to the enzyme is in pseudoequilibrium. Thus, from plots of k_{meas} against S (the substrate concentration) it should be possible to obtain the values of k_2, k_3, and K_s. The substrate concentration ranges used in this study were limited by the solubility of the substrates, and all the plots of k_{meas} against S gave straight lines, indicating that in all cases $K_s \gg S$ and that eq 1 can be simplified to

$$k_{meas} = k_2S/K_s + k_3 \quad (2)$$

The constants obtained from the plots are therefore k_2/K_s and k_3. The values of these constants are obtained by a linear least-squares fit of the data to eq 2 using the standard deviations of the sets of k_{meas} values as weights.

Results and Discussion

Kinetics of Papain-Catalyzed Thiono Ester Hydrolysis. The Michaelis–Menten parameters k_{cat} and K_m obtained from the steady-state reaction rates are related to the rate constants in Scheme I by the equations (Lucas & Williams, 1969):

$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (3)$$

$$k_{cat}/K_m = k_2k_1/(k_{-1} + k_2) \quad (4)$$

if $k_{-1} \gg k_2$ (as is assumed in the derivation of eq 1 for the transient-phase kinetics), and since $k_2 \gg k_3$ (see below), it then follows that $k_{cat} \simeq k_3$ and $k_{cat}/K_m \simeq k_2/K_s$.

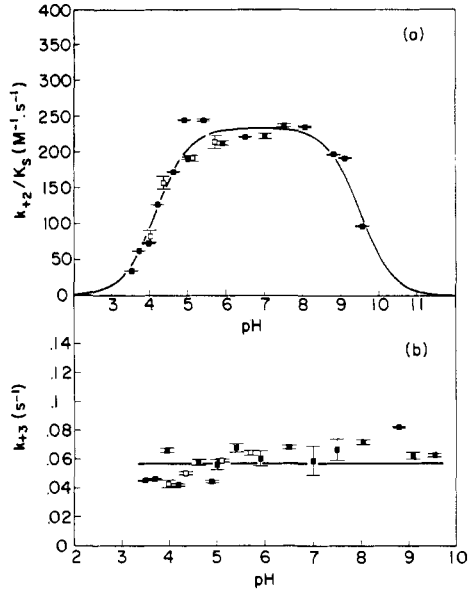


FIGURE 1: Plots of (a) k_2/K_s and (b) k_3 against pH for *N*-benzoylglycine methyl thiono ester. The data points were obtained by using either the pH-stat (□) or the stopped-flow spectrometer (■). The error bars represent the standard deviations of the data points. For the k_2/K_s plot the solid line is a theoretical one calculated by using pK_a's of 4.15 and 9.5 and a limiting value of 235 M^{-1} s^{-1} for k_2/K_s. The solid line in the k_3 plot is the weighted average of the k_3 values (0.056 ± 0.011 s^{-1}).

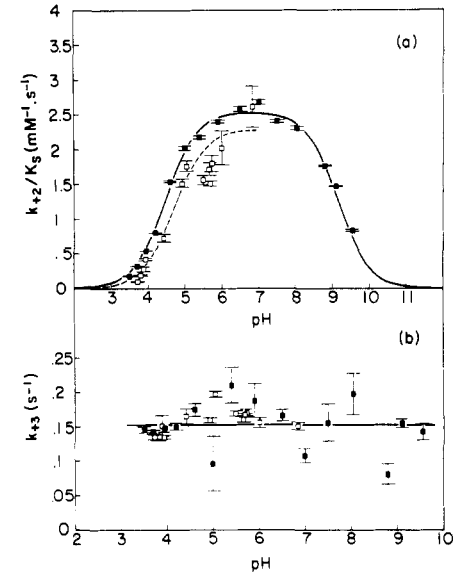


FIGURE 2: Plots of (a) k_2/K_s and (b) k_3 against pH for *N*-(β-phenylpropionyl)glycine methyl thiono ester. The data points were obtained by using either the pH-stat (□) or the stopped-flow spectrometer (■). The error bars represent the standard deviations of the data points. For the k_2/K_s plot the solid line is a theoretical one calculated by using pK_a's of 4.47 and 9.15 and a limiting value of 2550 M^{-1} s^{-1} for k_2/K_s. The dashed line was calculated by using a pK_a of 4.7 and a limiting value of 2300 M^{-1} s^{-1} for k_2/K_s. The solid line in the k_3 plot is the weighted average of the k_3 values (0.153 ± 0.28 s^{-1}).

The pH dependencies of k_2/K_s and k_3 obtained for *N*-benzoylglycine and *N*-(β-phenylpropionyl)glycine methyl thiono esters by the pH-stat and stopped-flow spectrometer methods are shown in Figures 1 and 2. The limiting values for k_2/K_s and k_3 are 235 M^{-1} s^{-1} and 0.056 ± 0.011 s^{-1}, respectively, for the *N*-benzoylglycine substrate and 2550 M^{-1} s^{-1} and 0.153 ± 0.028 s^{-1}, respectively, for the *N*-(β-phenyl-

Table I: Kinetic Constants Obtained for the Papain-Catalyzed Hydrolysis of Ester and Thiono Ester Substrates at pH 6.5 and 20 °C

	ester[a]		thiono ester[b]	
acyl group	k_2/K_s (M^{-1} s^{-1})	k_3 (s^{-1})	k_2/K_s (M^{-1} s^{-1})	k_3 (s^{-1})
N-benzolyglycine	154 ± 5	3.53 ± 0.20	229 ± 1	0.052 ± 0.002
N-(*p*-methoxybenzoyl)glycine	52.6 ± 4.9	2.07 ± 0.39	360 ± 19	0.0297 ± 0.0135
N-(*p*-methylbenzoyl)glycine	115 ± 3	2.91 ± 0.13	303 ± 6	0.0612 ± 0.0064
N-(*p*-chlorobenzoyl)glycine	311 ± 18	6.41 ± 1.56	738 ± 15	0.0928 ± 0.0071
N-(*p*-nitrobenzoyl)glycine	196 ± 4	10.6 ± 3.5	377 ± 43	0.178 ± 0.024
N-(β-phenylpropionyl)glycine	714 ± 25[c]	4.73 ± 0.20	2590 ± 31	0.165 ± 0.008

[a] Obtained by using the pH stat. [b] Obtained by using the stopped-flow spectrometer. [c] Obtained at pH 6.

propionyl)glycine substrate. The limiting value of 235 M^{-1} s^{-1} obtained for k_2/K_s for *N*-benzoylglycine methyl thiono ester compares with a value of 540 M^{-1} s^{-1} reported by Asbóth & Polgár (1983). The difference of a factor of 2 can probably be accounted for by the different experimental conditions used. Both of these values are much higher than that of 22.8 M^{-1} s^{-1} reported by Lowe & Williams (1965a). For both thiono ester substrates used in this study k_2/K_s has a bell-shaped pH dependency with the acidic and basic limb pK_a's in the ranges of 4.0–4.5 and 9.0–9.5, respectively. There is, however, no apparent pH dependency of k_3 for either of the substrates.

Since the plots of k_{meas} against S obtained from the stopped-flow spectrometer are linear ($K_s \gg S$), it is possible to estimate lower limits for k_2 and K_s. For example, for *N*-(β-phenylpropionyl)glycine methyl thiono ester the maximum substrate concentration used is 5 mM and so $K_s \gg 5$ mM, and since $k_2/K_s = 2550$ M^{-1} s^{-1}, then $k_2 \gg 12.8$ s^{-1}; likewise, for *N*-benzoylglycine methyl thiono ester $K_s \gg 10$ mM and $k_2 \gg 2.35$ s^{-1}. Thus, clearly for the thiono esters deacylation is rate-limiting since for *N*-(β-phenylpropionyl)- and *N*-benzoylglycine methyl thiono esters the ratios of k_2/k_3 are ≫85 and ≫42, respectively.

From a kinetic study (data not shown) of the papain-catalyzed hydrolysis of *N*-benzoylglycine methyl ester and *N*-(β-phenylpropionyl)glycine methyl ester the limiting values of k_2/K_s were found to be 153 M^{-1} s^{-1} and 740 M^{-1} s^{-1}, respectively. As for the thiono esters, the ester k_2/K_s values exhibited bell-shaped pH dependencies with pK_a's of approximately 4.0 and 8.75. Similar bell-shaped pH dependencies of k_2/K_s have been obtained previously for *N*-benzoylglycine methyl ester (Lucas & Williams, 1969) and *N*-benzoylglycine ethyl ester (Sluyterman, 1964) and for numerous other ester substrates; e.g., see Whitaker & Bender (1965), Bender & Brubacher (1966), and Williams & Whitaker (1967). In all these studies, as well as the present study, only one acidic ionization and one basic ionization are required to fit the data to the bell-shaped profile. However, there are a few reports in the literature, based on more data points at lower pH values, that provide evidence that for papain-catalyzed ester hydrolysis the acidic limb of the k_2/K_s against pH plots is defined by two (Lewis et al., 1978) or three ionizations (Jarvis & Brocklehurst, 1982). Additional evidence for the involvement of more than one ionization comes from studies on papain using two-protonic-state reactivity probes (Brocklehurst & Little, 1972; Shipton & Brocklehurst, 1978). However, in the present study, for both esters and thiono esters, no evidence was found to suggest the involvement of more than one group with an acidic pK_a in the acylation process. This is possibly due to the fact that the most significant deviations from a simple sigmoidal curve, for k_2/K_s against pH plots in the acid region, occur below pH 3.5 (Lewis et al., 1978). In the present study no measurements were made below pH 3.5 due to the technical limitations of the method used.

The similarities in the k_2/K_s against pH profiles for the thiono esters and esters strongly suggest that the ionizing groups involved in the binding of substrate and the subsequent acylation of papain are the same in each case. The values of k_2/K_s for the thiono esters are 2–3-fold higher than the values for the corresponding esters, Table I. A similar result was obtained by Asbóth & Polgár (1983) for the methyl and thiono methyl esters of *N*-benzoylglycine. This difference in k_2/K_s can be explained by either a faster rate of acylation, a tighter binding of the thiono esters, or some combination of the two. There is insufficient information to decide between the possibilities although we note that the dissociation constant of *N*-benzoylglycine thioamide and papain is almost 100-fold less than that of *N*-benzoylglycine amide (Lowe & Yuthavong, 1971). Also, we note that thiono esters undergo base-catalyzed hydrolysis at a rate similar to that of esters (Asbóth & Polgár, 1983), suggesting that the inherent chemical reactivities of thiono esters and esters are quite similar.

The k_3 value for the methyl thiono ester of *N*-(β-phenylpropionyl)glycine is lower than that for the ester by a factor of 30 (Table I). Part of this difference could be explained by different reactivities of thiol esters and dithio esters; e.g., simple dialkyl dithioesters under base-catalyzed hydrolysis 6 times slower than the corresponding thiol esters (Storer, 1983). There is a marked difference in the pH dependence of the ester and thiono ester k_3 values, that for the *N*-(β-phenylpropionyl)glycine methyl thiono ester being pH-independent, whereas the k_3 for the methyl ester has a pK_a near 3.6 (data not shown). A pH-dependent k_3 is encountered for the majority of ester substrates [e.g., see Whitaker & Bender (1965), Bender & Brubacher (1966), Williams & Whitaker (1967), and Lucas & Williams (1969)] with the most notable exception being *N*-benzoylglycine substrates. Thus, k_3 values for both the methyl ester (data not shown; Lucas & Williams, 1969) and the methyl thiono ester of *N*-benzoylglycine reacting with papain are pH-independent. These k_3 values differ by a factor of approximately 60 (Table I), with the thiono ester rate being the slowest.

The generally observed pH dependence (p$K_a \simeq 4$) of k_3 values for ester hydrolysis by papain has been taken as evidence for the participation of histidine-159 as a general base in the deacylation process (see below). Although this process may in fact be occurring, the observed pH independence of k_3 values in some cases in the acidic pH region makes it difficult to cite the pH behavior of the k_3's as strong evidence for a general-base-catalyzed process.

Sulfur–Oxygen Exchange during Thiono Ester Hydrolysis. For the thiono esters the kinetic constants obtained by the two methods, i.e., pH-stat and stopped-flow spectrometer (Figures 1 and 2), are in good accord. However, for *N*-(β-phenylpropionyl)glycine methyl thiono ester there initially appeared to be a marked discrepancy in the values of k_2/K_s and k_3 obtained by the two methods. Due to the nature of the method the results obtained by using the pH-stat have to be corrected for the pK_a of the product, and it was assumed that the latter was the thiol acid with a pK_a of 3.34 (Table II). This assumption led to a pH-dependent k_3 with a pK_a near 4.

Scheme II

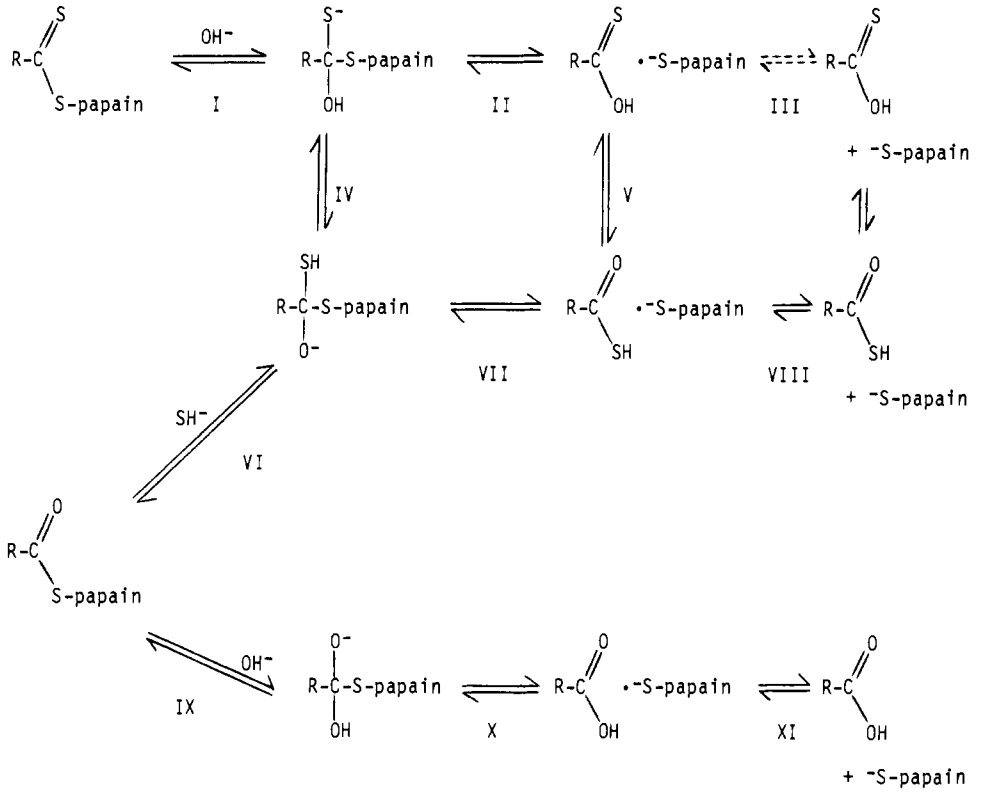


Table II: Acidic pK_a Values Determined by Titration under the Same Conditions Used for the Enzyme Assays

acid	pK_a
N-benzoylglycine	3.96
N-benzoylthioglycine	3.14
N-(β-phenylpropionyl)glycine	4.00
N-(β-phenylpropionyl)thioglycine	3.34

However, when the stopped-flow technique, which requires no correction for product pK_a, was used, the measured k_3's were independent of pH between pH 3.5 and 9.5. The anomaly was resolved when the proton stoichiometries for the reaction were measured by using the pH stat, and the product was found to have a pK_a of 4.01 (Figure 3). This corresponds to the pK_a of the corresponding carboxylic acid (Table II) and indicates that oxygen–sulfur exchange has occurred during the reaction and that the product released from the enzyme is the –COOH acid rather than the expected –COSH acid. The concept of desulfurization receives support from the observation that H_2S is given off from the reaction mixture. When a correction is made to the pH-stat results assuming a product pK_a of 4.00, good accord is seen between the pH-stat and stopped-flow results (Figure 2). A further observation is that the oxygen-for-sulfur exchange appears to be limited to acidic pHs. Above pH 7.0 one proton is released per molecule of product whereas a stoichiometry of two is expected, on the basis that H_2S, resulting from the sulfur–oxygen exchange (see below), has a pK_a near pH 7.0 and should contribute a proton in addition to the carboxylic acid product.

A comparison of the stopped-flow and pH-stat results for *N*-benzoylglycine methyl thiono ester contrasts sharply with the comparison of the stopped-flow and pH-stat results for *N*-(β-phenylpropionyl)glycine methyl thiono ester. For both substrates the proton stiochimetry for the final product of the reaction of the thiono ester with papain has a pK_a that is consistent with the formation of the dioxygen acid. [For the *N*-benzoylglycine compound the measured pK_a of the product was 4.0, compared to 3.96 for the dioxygen acid and 3.14 for corresponding thiol acid (Table II).] However, for the *N*-benzoylglycine methyl thiono ester, when the k_3 and k_2/K_s values obtained from the pH stat are corrected by using a product pK_a of 3.96, poor agreement is seen with stopped-flow results below pH 5.0. Instead, agreement is obtained by using the pK_a of the thiol acid (Figure 1).

Thus, an apparent mechanistic difference exists between the two thiono esters used in this study. For *N*-benzoylglycine methyl thiono ester the product in the acidic pH range, according to the pH dependencies of k_3 and k_2/K_s, is the thiol acid, whereas from the final proton stoichiometries the carboxylic acid is the product. On the other hand, for *N*-(β-phenylpropionyl)glycine methyl thiono ester the product is the carboxylic acid by both criteria. Clearly, for the *N*-benzoylglycine substrate the oxygen–sulfur exchange occurs after the product initially leaves the enzyme, and for the *N*-(β-phenylpropionyl)glycine substrate the exchange occurs before the product dissociates from the enzyme. In the latter case the exchange must occur after the acyl enzyme stage in the mechanism since, with the thiono ester substrates, the absorption and resonance Raman spectroscopic results clearly show that the acyl enzyme is a dithio ester and not a thiol ester. On the basis of this information and the fact that desulfurization occurs only at acidic pH, there are two stages in the reaction sequence at which the exchange can occur. One possibility is at the tetrahedral intermediate stage involving steps I, IV, and VI in Scheme II, i.e., a proton shift coupled with a pseudoequilibrium between the tetrahedral intermediate and the acyl enzyme. The thiolacyl enzyme formed would then deacylate normally (steps IX–XI). A pH-dependent oxygen–sulfur exchange has been observed during the base-catalyzed hydrolysis of thiono esters (Smith & O'Leary, 1963; Smith & Feldt, 1968; Bruice & Mautner, 1973).

The second possible route for the oxygen–sulfur exchange in the enzyme system could occur via steps I → II → V → VII → VI in Scheme II. In this mechanism the first tetra-

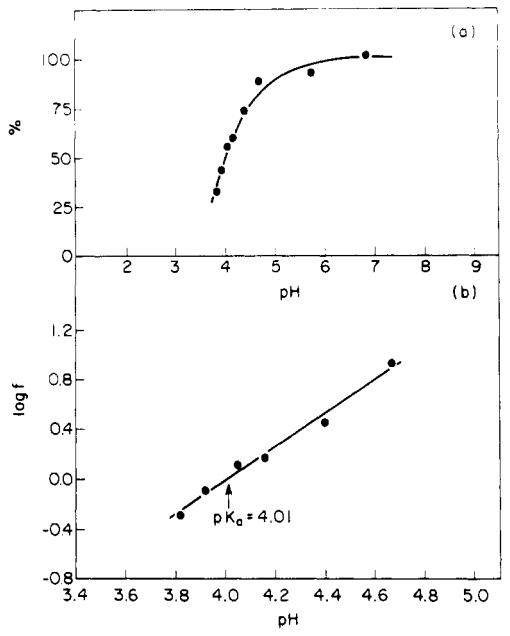
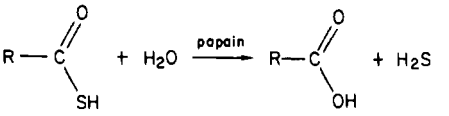


FIGURE 3: (a) Plot of the percent stoichiometry of proton release as a function of pH for the papain-catalyzed hydrolysis of *N*-(β-phenylpropionyl)glycine methyl thiono ester. (b) As in (a) but plotted as log f vs. pH where f = (percent proton release)/(100 − percent proton release); from this plot when log f = 0, then pH = pK_a.

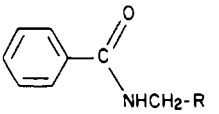
hedral intermediate formed breaks down to give the energetically unfavorable thiono acid (Scheithauer & Mayer, 1979), which rapidly rearranges in the enzyme active site to give the more stable thiol acid. Since protonated thiol acids are powerful acylating agents (Cronyn & Jiu, 1952; Hawkins et al., 1953) and are also good substrates for papain (see below), this bound thiol acid can reacylate the enzyme (steps VII and VI), and the thiolacyl enzyme thus formed can then deacylate normally (steps IX–XI). The two proposed exchange mechanisms differ in the mechanism of the prototropic shift, i.e., in one it occurs in the tetrahedral intermediate and in the other in the first acid product. Since both mechanisms are expected to be pH-sensitive, it is impossible to determine which is responsible for the observed exchange (it is of course possible that both are involved). However, the second mechanism involving reacylation by the thiol acid is favored since it provides an explanation for the product differences between the two substrates, *N*-benzoylglycine methyl thiono ester and *N*-(β-phenylpropionyl)glycine methyl thiono ester. Papain has a relatively higher affinity for the second substrate (k_2/K_s is larger) since it is a close analogue of phenylalanylglycine derivatives, which fulfill papain's requirement for a hydrophobic residue at the S_2 site (Berger & Schechter, 1970). Thus both this substrate and the putative initial product, the corresponding thiol acid, will bind more tightly to the enzyme than *N*-benzoylglycine methyl thiono ester and thiol acid. The tighter binding will favor a reacylation of the enzyme (steps VII and VI, Scheme II) over dissociation of the acid from the enzyme (step VIII), whereas the lower specificity for the enzyme for the *N*-benzoylglycine derivatives will result in a looser binding of the thiol acid product, thus favoring dissociation rather than reacylation. However, in the latter case, the accumulating thiol acid product will itself act as a substrate for papain (see below) with the result that the thiol acid, as its concentration relative to substrate increases, will be converted gradually to the carboxylic acid product.

Thiol Acids as Substrates. It has been reported that the protonated forms of *N*-acyl amino acids are competitive inhibitors of the papain-catalyzed hydrolysis of esters (Sluyterman, 1964), and Smolarsky (1978, 1980), using a chromophoric analogue of phenylalanine, has shown that these inhibitors interact with papain to form acyl enzymes. Therefore, it seemed likely that *N*-acyl amino thiol acids would also interact with papain and that the acylation of the enzyme would result in the hydrolysis of the thiol acids:



This reaction can be monitored by using the pH stat since the thiol acid pK_a's differ from those of the corresponding carboxylic acids by almost 1 unit (Table II). Results obtained by using *N*-benzoylglycine thiol acid as a substrate with papain are given in Table III. Clearly, thiol acids are excellent substrates for the enzyme, and the production of a thiolacyl enzyme (steps VIII → VII → VI, Scheme II) is indicated by the similar values for k_{cat} seen in Table III for the methyl ester, thiol ethyl ester, and thiol acid substrates. The similarity in k_{cat} for these substrates suggests that deacylation occurs from a common intermediate whose hydrolysis is rate-limiting. The much larger value of k_{cat}/K_m for the thiol acid is probably due in part to the greater leaving group ability of the HS^- ion as opposed to the methoxy and ethyl thiolate anions. The demonstration that thiol acids are good substrates for papain adds support to the suggestion made above that the oxygen–sulfur exchange observed during the hydrolysis of thiono esters occurs via the reacylation of the enzyme by the thiol acid product.

Table III: Kinetic Constants Obtained for a Variety of *N*-Benzoylglycine Substrates with Papain[a]



substrate, R =	pH	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
-C(=O)-OCH$_3$	6.0	21.8 ± 2.1	3.48 ± 0.12	160 ± 6
-C(=S)OCH$_3$	5.7	0.30 ± 0.02	0.064 ± 0.002	214 ± 9
-C(=O)-SCH$_2$CH$_3$	6.0	16[c,d]	3.4[c,d]	206[c,d]
-C(=O)SH	3.95	0.77 ± 0.09[b]	2.58 ± 0.17[b]	3340 ± 180[b]
		0.10 ± 0.01[e]	2.58 ± 0.17[e]	24800 ± 1300[e]
	6.0		2.58 ± 0.17[f]	59800 ± 3200[f]

[a] Measurements were made at 20 °C by the pH-stat technique unless otherwise stated. [b] The results were corrected for proton stoichiometries calculated by using the pK_a's of the thiol acid substrate and the carboxylic acid product. The enzyme concentration was 4.6 μM, that of thiol acid was 0.1–2.0 mM, and the reaction was followed by the addition of 20 mM HCl. [c] At 35 °C. [d] Lowe & Williams, 1965b. [e] Assuming only the protonated acid is a substrate. [f] Values calculated assuming a pK_a of 4.1 for k_{cat}/K_m (the pK_a's for the methyl and thiono methyl esters are 4.1 and 4.15, respectively).

Solvent Deuterium Isotope Effect. The effect on the kinetic parameters of replacing the solvent H_2O with D_2O is given in Table IV for the esters and thiono esters of *N*-benzoylglycine and *N*-(β-phenylpropionyl)glycine. The ratios of k_2/K_s values in H_2O and D_2O for both thiono esters and *N*-(β-phenylpropionyl)glycine methyl ester are approximately 0.8. For *N*-benzoylglycine methyl ester the ratio is 1.24 ± 0.18, which is in accord with a value of 1.07 for this substrate reported by Polgár (1979). A range of 0.63–1.45 for the ratio of k_2/K_s in H_2O and D_2O for a variety of esters has been reported by Polgár (1979), and all values determined in the present study fall within this range. The absence of a high k_2/K_s solvent D_2O effect prompted Polgár (1979) to conclude that, in con-

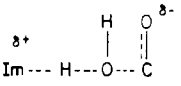
Table IV: Kinetic Solvent Isotope Effects[a]

substrate	solvent	K_m (mM)	k_3 (s^{-1})	k_2/K_s (M^{-1} s^{-1})
N-benzoylglycine methyl ester	H_2O (pH 6)	18.54 ± 4.28	3.18 ± 0.38	171 ± 20
	D_2O (pD 6)[b]	11.36 ± 1.42	1.57 ± 0.10	138 ± 10
	ratio	1.63 ± 0.43	2.03 ± 0.28	1.24 ± 0.18
N-(β-phenylpropionyl)glycine methyl ester	H_2O (pH 6)	6.63 ± 0.47	4.73 ± 0.20	714 ± 25
	D_2O (pD 6)	2.00 ± 0.13	1.78 ± 0.05	892 ± 42
	ratio	3.32 ± 0.32	2.66 ± 0.14	0.80 ± 0.05
N-benzoylglycine methyl thiono ester	H_2O (pH 5.7)	0.301 ± 0.019	0.0644 ± 0.0017	214 ± 9
	D_2O (pD 5.7)	0.153 ± 0.009	0.0386 ± 0.0008	253 ± 11
	ratio	1.97 ± 0.17	1.67 ± 0.06	0.85 ± 0.05
N-(β-phenylpropionyl)glycine methyl thiono ester	H_2O (pH 5.7)	0.110 ± 0.004	0.163 ± 0.002	1480 ± 40
	D_2O (pD 5.7)	0.053 ± 0.003	0.101 ± 0.002	1900 ± 90
	ratio	2.08 ± 0.14	1.61 ± 0.004	0.78 ± 0.004

[a] The kinetic constants were measured at 20 °C by the pH-stat technique. [b] The pD was obtained by adding 0.4 to the pH meter reading.

trast to the serine proteinases (Bender & Kézdy, 1965), general-base catalysis does not occur in the acylation of papain by esters. The present results support this conclusion and indicate that general-base catalysis is not involved in the acylation of papain by thiono esters.

The solvent isotope effect on k_3 is more marked. For esters the ratio $k_3(H_2O)/k_3(D_2O)$ is in the range 2.0–2.75 (Table IV; Whitaker & Bender, 1965; Szawelski & Wharton, 1981) whereas for the two thiono esters used in this study it is 1.6 (Table IV). For the esters the solvent isotope effect on k_3 has been interpreted as evidence for deacylation being promoted by general-base catalysis (Whitaker & Bender, 1965; Szawelski & Wharton, 1981). Szawelski & Wharton (1981) have studied the phenomenon further using the proton inventory technique and conclude that for ester substrates a single proton is being transferred in the transition state for deacylation. From their results and theoretical considerations Szawelski & Wharton (1981) argue that the transition state lies about half-way along a concerted reaction pathway; i.e., the heavy atom reorganization and proton transfer are coupled and are both approximately 50% complete. Therefore, the structure they suggest for the transition state is



where Im is the imidazole group of histidine-159.

The results of a proton inventory study of the methyl ester and thiono ester of *N*-(β-phenylpropionyl)glycine are shown in Figure 4. The linearity of k_n/k_0 against n was tested with three values of n ($n \approx 0$, 0.5, and 1). The linear plots are indicative of the kinetic isotope effect originating from the transfer of one proton in the transition state for deacylation (Szawelski & Wharton, 1981). The fractionation factors determined from the slopes in Figure 3 are 0.45 (ester) and 0.63 (thiono ester). The result for the ester is very similar to those obtained previously for a series of esters (0.4–0.44) by Szawelski & Wharton (1981). Thus, their arguments and conclusions regarding the transition-state structure for the deacylation step in the papain-catalyzed hydrolysis of esters can be applied also in the case of *N*-(β-phenylpropionyl)glycine methyl ester. Although the fractionation factor for the thiono ester (0.63) is somewhat higher than that for the esters (0.4–0.45), it is still in the range of fractionation factors (0.3–0.7) compatible with the transition-state structure proposed for the esters [Szawelski & Wharton (1981), Table 4]. However, other transition-state structures are also compatible with the fractionation factor of 0.63 [Szawelski & Wharton (1981), Table 4]. Although there is no additional experimental evidence to help in the exclusion of the other possible transition

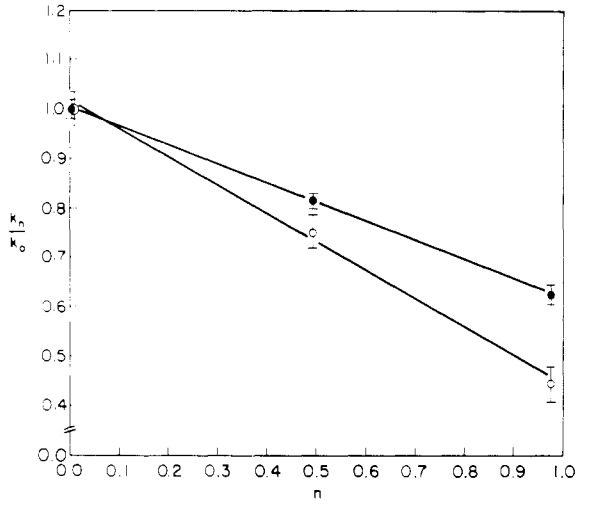
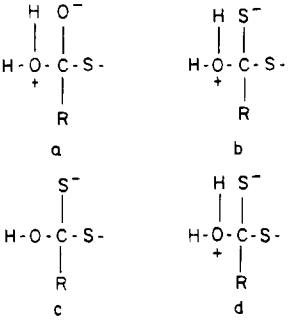


FIGURE 4: Plot of the ratio of the deacylation rates measured in H_2O/D_2O mixtures (k_n) and H_2O alone (k_0) for the substrates *N*-(β-phenylpropionyl)glycine methyl ester (○) and thiono ester (●) against the atom fraction of deuterium n. The points plotted represent the averages of 10 determinations and their standard deviations.

states, theoretical considerations may be of value. Szawelski & Wharton (1981) have pointed out that Marcus's theory (Marcus, 1968) and Jencks's rule for general-acid–general-base catalysis (Jencks, 1972) can be used to argue (Albery, 1975) that the transition state for proton-transfer reactions has a degree of heavy atom reorganization such that the free energy change for proton transfer is zero. This condition is satisfied when the pK_a values of the proton-accepting and proton-donating species are equal, in which case proton transfer is 50% complete in the transition state. Assuming a pK_a of −2 (the pK_a of a protonated alcohol) for structure a, Szawelski &



Wharton (1981) were able to calculate a heavy atom reorganization of 65%. For the thiono esters the pK_a of structure b is expected to be 2–4 pK units lower than that for the ester analogue. This value is suggested by a comparison of the pK_a's

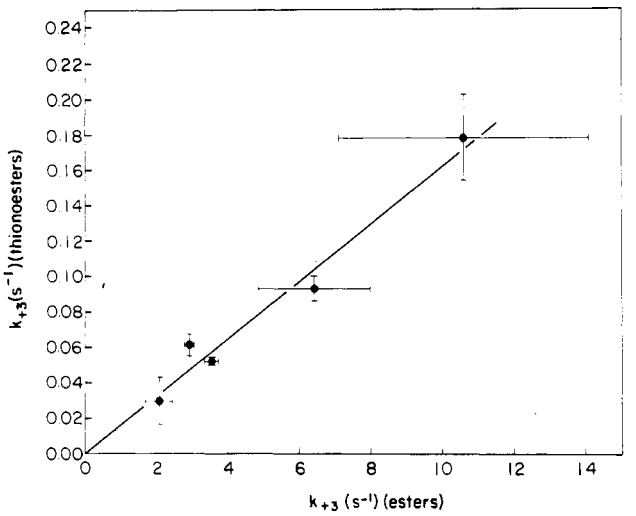


FIGURE 5: Plot showing the correlation in rates of deacylation for para-substituted *N*-benzoylglycine metal esters (abscissa) and thiono esters (ordinate). The values were taken from Table I.

for structure c and the equivalent structure in which O^- replaces S^- (Storer, 1983; Papoff & Zambonin, 1967). A pK_a of −5 for structure d would result in a heavy atom reorganization in the transition state of 55%. Thus the transition-state structures for the deacylation of thiolacyl and dithioacyl papains are probably very similar although the larger fractionation factor for the thiono ester substrate (dithioacyl papain) suggests that the degree of coupling between the heavy atom reorganization and the proton transfer may be less than in the case of the ester substrates.

Para-Substituted N-Benzoylglycine Ester Substrates. We have previously reported the results of a stopped-flow kinetic study on the papain-catalyzed hydrolysis of five para-substituted *N*-benzoylglycine methyl thiono esters (Carey et al., 1984a). The thiono ester results are compared in Table I and Figure 5 with those obtained in this study for the corresponding ester substrates using the pH-stat technique. The values obtained for the ester substrates differ from the values obtained for these same substrates by Hansch et al. (1977). In their study the ordering of the k_3 (k_{cat}) values is H > NO_2 > CH_3 > OCH_3 > Cl compared with ours of NO_2 > Cl > H > CH_3 > OCH_3. Although similar experimental techniques were used in the two studies, we found it necessary to introduce a cosolvent (CH_3CN) in order to maintain the solubility of the substrates (especially the NO_2 and Cl). Even in the presence of this cosolvent the maximum solubility for these two esters is approximately 10 mM. Hansch et al. (1977) did not report the use of a cosolvent, and this may account for the difference between the two data sets.

From Figure 5 there is a clear correlation between the k_3 values for the esters and for the thiono esters. Thus, the conclusion reached regarding the deacylation of the dithioacyl papains produced from thiono ester substrates can also be applied to the thiolacyl papains produced from ester substrates, namely, that the rate-limiting step for deacylation involves breaking an N···S interaction in the acyl enzyme (Carey et al., 1984b) and that the disruption must be partially completed in the transition state for deacylation. The N···S interaction is between the acyl group's glycinic nitrogen atom and the sulfur atom of cysteine-25 and involves a HOMO–LUMO type interaction between the nitrogen lone pair and a vacant orbital on the sulfur. Evidence for the disruption of the N···S interaction in the rate-limiting step came from correlating the strength of the N···S attraction with k_3, the rate constant for deacylation. Disruption of the N···S contact is consonant with

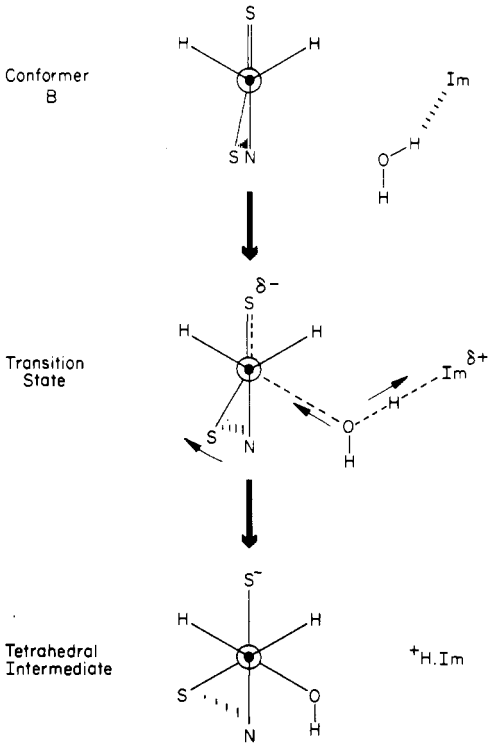


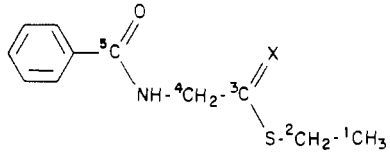
FIGURE 6: Changes occurring near the catalytic center as the acyl enzyme is converted to the tetrahedral intermediate for deacylation. The example shown is that of a dithioacyl enzyme; however, similar changes also occur for thioacyl papains.

a simple conformational analysis. It is likely that the rate-determining step for deacylation involves the conversion of the acyl enzyme to a tetrahedral intermediate (Lowe, 1976). On the basis of preliminary soft-sphere calculations (unpublished work, this laboratory) the sterically favored conformation in the tetrahedral intermediate is one in which the C_α and C-(carbonyl) substituents are gauche, i.e., the thiol sulfur atom moves out of the plane defined by the N–C_α–C(carbonyl) atoms so that the nitrogen–sulfur distance increases and the interaction between these two atoms is broken or weakened. Taking this finding with the results of the solvent isotope study, the deacylation process may be depicted in the manner shown in Figure 6.

Steric Requirements in the Oxyanion Hole. The overall conclusion from this study is that the catalytic mechanisms for the hydrolysis of esters and thiono esters are very similar, and moreover, there is a reasonably close similarity in the kinetic rate constants. At first sight this may appear surprising, since the modification to the ester substrates to produce thiono esters involves an atomic substitution at a position that is closely invovled in the catalytic process. However, the chemical and physical properties of the pairs of compounds, esters and thiono esters (acylation) and thiol esters and dithio esters (deacylation), involved in the catalytic process probably do not differ greatly. An important example of similar properties, of relevance in this context, is that esters, thiol esters, and thiono esters undergo base-catalyzed hydrolysis at approximately the same rate (Asbóth & Polgár, 1983) and that dithio esters undergo the same reaction less than an order of magnitude more slowly (Storer, 1983).

A major difference that much must be taken into account in a discussion of the catalytic mechanisms of ester and thiono ester hydrolysis by papain concerns the atomic sizes of sulfur and oxygen. The oxyanion hole is thought to be an important feature in the mechanism of cysteine proteinases (Drenth et al., 1976), and its ability to accommodate a sulfur atom may

Table V: Comparison of Selected Bond Lengths and Bond Angles of *N*-Benzoylglycine Ethyl Thiol Ester (X = O) and *N*-Benzoylglycine Ethyl Dithio Ester (X = S)



	X = O	X = S[a]
conformation[b]	B	B
bond lengths (Å)		
N···S[c]	2.879	2.867, 2.847
$^4C—^3C$	1.521	1.522, 1.523
$^3C=X$	1.209	1.616, 1.624
$^3C—S$	1.722	1.711, 1.712
$S—^2C$	1.802	1.804, 1.804
bond angles (deg)		
$^4C—^3C=X$	119.9	119.8, 119.7
$^4C—^3C—S$	116.4	113.8, 114.1
X=C—S	123.7	126.4, 126.1
$^3C—S—^2C$	99.8	103.2, 104.2
ϕ'[d]	−88.74	−78.7, −83.5
ψ'[d]	5.38	−15.5, −10.5

[a] The crystals of *N*-benzoylglycine ethyl dithio ester contained two slightly different conformational forms.
[b] For a definition of conformer B see Huber et al. (1982).
[c] The nonbonded N···S distance.
[d] ϕ' and ψ' are the torsional angles ^5C-N-^4C-^3C and N-^4C-^3C-S, respectively.

be a critical requirement for the similarity of the ester and thiono ester mechanisms (Asbóth & Polgár, 1983). The oxyanion hole has been identified in papain by the X-ray crystallographic studies of Drenth et al. (1976). In an acyl enzyme analogue it was shown that the acyl group's C=O oxygen is H-bonded in the oxyanion hole to the side chain of glutamine-19 and the backbone NH of cysteine-25. The ease by which a C=S sulfur atom can replace a C=O oxygen in the oxyanion hole is affected by a number of factors, e.g., the molecular geometries about the acyl group's C=O or C=S group, the differences in C=O and C=S bond lengths, and the differences in the lengths and strength of NH H bonds to C=O and C=S. The comparison, given in Table V, of the structures of *N*-benzoylglycine ethyl thiol ester (Huber et al., 1984) and dithio ester (Varughese et al., 1984) shows that the geometries about the ester C=O and C=S groups are strikingly similar, with the obvious exception that the C=S bond is 0.4 Å longer than C=O. A survey of the literature reveals that the geometries of NH···S and NH···O H bonds are also quite similar (Jeffrey et al., 1984; Mitra & Ramakrishnan, 1977) and that for H bonds of similar strength the N to S distance is 0.5 Å longer than the N to O distance in NH···O (Lautié & Novak, 1980). If it is assumed that the optimum H bonding is retained for C=O and C=S in the oxyanion hole, i.e., the N–H group approaches C=O or C=S at an angle of about 120° to the C=O(S) bond, then the above considerations show that the oxyanion hole NH groups will have to move outward by approximately 0.6 Å upon going from C=O to C=S. It is also important to emphasize that recent studies have shown the NH···S bonds, in certain situations, can be as strong as the corresponding NH···O bonds (Lautié & Novak, 1980; Krueger, 1970).

Therefore, the competence of the oxyanion hole to accommodate the C=S S atom hinges on whether the H-bonding donor N atoms of glutamine-19 and cysteine-25 can adjust their positions by distances of about 0.6 Å. Two pieces of evidence demonstrate that the oxyanion hole is flexible and may be able to encapsulate a larger species. The flexibility is indicated by the atomic temperature factors *B* determined in a refinement of the X-ray crystallographic structure of papain to 1.65 Å (J. Drenth, personal communication). The mean amplitudes of vibration of the side chain nitrogen of glutamine-19 and the backbone nitrogen of cysteine-25 are 0.4 (B = 12.41) and 0.27 Å (B = 5.83), respectively. Moreover, the ability of the oxyanion hole to expand is indicated by the necessity to accommodate the anionic oxygen atom of the tetrahedral intermediates that lie on the acylation and deacylation pathway. The C–O^- bond length is about 0.23 Å longer than the C=O length.

It is unlikely that the assumption, implicit in the above discussion, that the substrate remains a fixed immovable entity and that the enzyme adjusts itself around the larger S atom is totally correct. Rather, it is probable that the substrate makes subtle adjustments in its geometry, concurrent with those in the enzyme, when the C=O to C=S change is made. One way in which the acylated substrate can change geometry with minimal expenditure or energy is to change the torsional angles in the glycinic RC(=O)NH–CH_2–C(=S) bonds, the so-called ψ, and ϕ' angles (Huber et al., 1982). It was shown recently for glycine-based dithio esters that fairly large correlated changes in ψ' and ϕ' can occur while the basic acyl enzyme conformer B-type geometry (Varughese et al., 1984) is maintained. In turn, these findings suggest that the enzyme-bound dithioacyl group can adjust its geometry about the ϕ' and ψ', angles and remain on the reaction pathway.

In a recent paper Asbóth & Polgár (1983) argued against the importance of the oxyanion hole in the hydrolytic mechanism of cysteine proteinases. Part of their case was that the ease with which papain and other cysteine proteinases accept thiono esters as substrates is incompatible with the strict steric requirements of the oxyanion hole, although we have argued, that with some adjustments, thiono esters probably can be accomodated in the oxyanion hole. Support for the assertion of Asbóth and Polgár came from the work of Tsunoda & Yasunobu (1966), who reported that the residue corresponding to glutamine-19 in papain was replaced in chymopapain B by valine. Thus, one of the amino acids needed to form the oxyanion hole appears to be absent in chymopapain B, suggesting either a lack of importance for the oxyanion hole or a lack of a conserved mechanism for the cysteine proteinases. The latter is in conflict with previous reports from this laboratory which suggest that the deacylation process is highly conserved among the cysteine proteinases (Carey et al., 1983, 1984a; Brocklehurst et al., 1984). The nonconservation of glutamine-19 also appears to be anomalous in light of the fact that this residue is found in all other 12 cysteine proteinases so far sequenced or partially sequenced: papain (Light et al., 1964; Mitchel et al., 1970; Husain & Lowe, 1970), actinidin (Carne & Moore, 1978), ficin (Wong & Liener, 1964), stem bromelain (Goto et al., 1976), fruit bromelain (Murachi, 1976), rat liver cathepsins B and H (Takio et al., 1983), porcine spleen cathepsin B (Takahashi et al., 1984), asclepains A and B (Lynn et al., 1980), chymopapain A (D. C. Watson, M. Yaguchi, and K. R. Lynn, private communication), and streptococcal proteinase (Tai et al., 1976). Thus, either cymopapain B is a unique cysteine proteinase or the sequence data for that enzyme may have been misinterpreted. The data were obtained for a fragment that contains a free cysteine –SH group (Tsunoda & Yasunobu, 1966), but since it is known that cymopapain B has two free –SH groups (Brockelhurst & Salih, 1983), it is possible that the sequence data obtained do not correspond to the section including residue 19.

Taken in toto, the sequence results and the identification of putative oxyanion holes in papain (Drenth et al., 1976) and actinidin (Baker, 1980) by X-ray crystallography provide

strong evidence for the existence of an oxyanion site. In the present work, a comparison of the papain-catalyzed hydrolysis of esters and thiono esters has revealed a modest difference in rate constants for the two classes of substrates, strong similarities in their solvent isotope and pH dependences, and a correlation in the k_3 rate constants for para-substituted *N*-benzoylglycine esters and thiono esters. These experimental findings provide a sound basis for asserting that the kinetic properties and the mechanisms of the papain-catalyzed hydrolysis of esters and thiono esters are markedly similar and, therefore, that the mechanisms for dithioacyl intermediates also involves the use of an oxyanion hole in the active site.

The conclusion of this study, concerning the close similarity in the kinetic and mechanistic properties of papain-catalyzed hydrolysis of esters and thiono esters, provides some confidence that the structural detail that has been elicited for dithioacyl enzymes by resonance Raman spectroscopy can be used to define the properties of their thiolacyl analogues.

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Studies of the Structure of Fructose-6-phosphate 2-Kinase:Fructose-2,6-bisphosphatase†

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ABSTRACT: Some physicochemical properties of a homogeneous preparation of a bifunctional enzyme, fructose-6-phosphate 2-kinase:fructose-2,6-bisphosphatase, were determined. The molecular weight of the enzyme is 101 000 as determined by high-speed sedimentation equilibrium. The molecular weight of dissociated enzyme is 55 000 in 6 M guanidinium chloride by sedimentation equilibrium and in sodium dodecyl sulfate by polyacrylamide gel electrophoresis. A value of 4.7 was observed for the isoelectric point. Tryptic peptide maps and high-performance liquid chromatography of the trypsin-digested enzyme revealed approximately 60 peptides. Amino acid analysis of the enzyme shows that it contains 27 lysine and 36 arginine residues per 55 000 daltons. No free N-terminal amino acid residue was detectable, suggesting that it is blocked. Hydrolysis of the enzyme by carboxypeptidases A and B releases tyrosine followed by histidine and arginine, indicating that the amino acid sequence at the carboxyl terminus is probably -Arg-His-Tyr. Tryptic digestion of [^{32}P]phosphofructose-6-phosphate 2-kinase:fructose-2,6-bisphosphatase yields a ^{32}P-labeled peptide detected by tryptic peptide mapping and high-performance liquid chromatography. Thermolysin digestion of CNBr-cleaved ^{32}P-enzyme also yields a single ^{32}P-peptide. These results indicate that fructose-6-phosphate 2-kinase:fructose-2,6-bisphosphatase is a dimer of 55 000 daltons and the subunits are very similar, if not identical.

The synthesis of fructose 2,6-bisphosphate (fructose-2,6-P_2) from fructose 6-phosphate (fructose-6-P) and ATP is catalyzed by fructose-6-P-2-kinase (Fru-6-P-2-kinase) (eq 1) (Furuya

$$\text{fructose-6-P} + \text{ATP} \rightleftharpoons \text{fructose-2,6-P}_2 + \text{ADP} + \text{H}^+ \quad (1)$$

& Uyeda, 1981; El-Maghrabi et al., 1981; Van Schaftingen & Hers, 1981a). The degradation of fructose-2,6-P_2 is catalyzed by fructose-2,6-bisphosphatase (Fru-2,6-bisphosphatase) (eq 2) (Richards et al., 1982; Van Schaftingen et al., 1982;

$$\text{fructose-2,6-P}_2 + \text{H}_2\text{O} \rightarrow \text{fructose-6-P} + \text{P} \quad (2)$$

Furuya et al., 1982; El-Maghrabi et al., 1982a), and both enzyme activities reside in the same protein (Van Schaftingen et al., 1982; El-Maghrabi et al., 1982a; Sakakibara et al., 1984a).

The enzyme has been purified to apparent homogeneity (El-Maghrabi et al., 1981; Sakakibara et al., 1984a). By molecular sieve filtration on Sephadex G200, we have earlier determined the molecular weight of the partially purified enzyme as 100 000 using nine marker proteins (Furuya et al., 1982b). El-Maghrabi et al. (1981) reported a molecular weight value of 85 000-90 000 by gel filtration on Sephadex G100. The molecular weight of the enzyme subunit has been estimated as 55 000 by polyacrylamide gel electrophoresis under denaturing conditions (Furuya et al., 1982b). However, we calculate a value of 51 500 from the data presented by El-Maghrabi et al. (1984a).

Fructose-6-P-2-kinase:Fru-2,6-bisphosphatase is regulated in part by phosphorylation and dephosphorylation. The en-

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