$CO_2HCH(OH)CHFCO_2H$, 74806-81-8; malate dehydrogenase, 9001-64-3.

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Reactivity of Small Thiolate Anions and Cysteine-25 in Papain toward Methyl Methanethiosulfonate†

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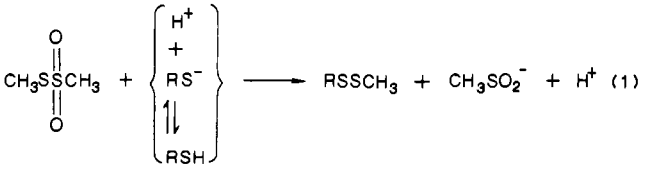
ABSTRACT: The dependence on thiol pK of the second-order rate constant (k_S) for reaction of thiolate anions with MMTS was shown to follow the Brønsted equation $\log k_S = \log G + \beta pK$ with $\log G = 1.44$ and 3.54 and $\beta = 0.635$ and 0.309 for aryl and alkyl thiols, respectively. The reactivity toward MMTS of the protonated thiol group was found to be negligible in comparison to that of the thiolate anion. For 2-mercaptoethanol the reactivity toward MMTS of the protonated form of the thiol group was shown to be at least 5×10^9 smaller than that of the thiolate anion. The pH dependence of the second-order rate constant for reaction of the thiolate group of Cys-25 at the active site of papain was determined and shown to be consistent with the previously determined low pK for Cys-25 and its electrostatic interaction with His-159. The small dependence of the reactivity of Cys-25 on thiol pK ($\beta \sim 0.09$) suggested that the charge–charge interactions that act through space to perturb the pK of the nucleophile at the active site of papain and perhaps other enzymes may serve to increase the fraction of nucleophile present in the reactive basic form without introducing the decrease in nucleophilic reactivity seen in model systems where pK's are lowered primarily by charge–dipole interactions.

In 1975 Kenyon and his co-workers (Smith et al., 1975; Nishimura et al., 1975) demonstrated the utility of methyl methanethiosulfonate (MMTS)[1] for selective modification of thiol groups in proteins, via the reaction depicted in eq 1.[2]



Since then MMTS has gained wide use as a probe of the function of thiol groups in proteins [e.g., see Bruice & Kenyon (1977), Lewis et al. (1978), Bloxam et al. (1979), Marshall & Cohen (1980), Gavalanes et al. (1982), Claiborne et al. (1982), and Kopczynski & Babior (1984)]. A major advantage of this reagent is that it blocks a thiol sulfur atom with

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[1] Abbreviations: bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; BzArgPNA, N^{α}-benzoyl-L-arginine *p*-nitroanilide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid disodium salt; MCA, 7-amino-4-methylcoumarin; MMTS, methyl methanethiosulfonate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); PDT, potentiometric difference titration; ZArg–MCA, N^{α}-benzyloxycarbonyl-L-arginine–7-amido-4-methylcoumarin.

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a small neutral non-hydrogen bonding methylthio group and thereby produces a minimal (and hopefully negligible) perturbation in the structure of the target protein. Additionally, the ionization behavior of thiol groups in proteins and other compounds can be deduced from the pH dependence of the protons given off in the reaction depicted in eq 1 (Lewis et al., 1976, 1980).

This work, which establishes the dependence of the rate constant for reaction of MMTS with simple thiols and the thiol group at the active site of papain on their degree of protonation and pK, provides a basis for interpreting the reactivity of MMTS with thiol groups in proteins.

Experimental Procedures

Materials. *N*,*N*-Bis(2-hydroxyethyl)glycine (bicine), 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2), and dithiothreitol (DTT) were from Calbiochem. Mixed bed resin AG-50 1X-8 was from Bio-Rad. Methyl methanethiosulfonate (MMTS), ethylenediaminetetraacetic acid disodium salt (EDTA), cysteine ethyl ester hydrochloride, 4-mercaptopyridine, 4-methoxybenzenethiol, and 4-aminobenzenethiol were from Aldrich. 4-Aminobenzenethiol was converted to its HCl salt and recrystallized from ethanol–ether. 5-Mercapto-2-nitrobenzoic acid was prepared by reacting Nbs_2 with 0.9 equiv of DTT. Other aryl thiols and methyl 2-mercaptoacetate were generously supplied by Dr. Donald Hupe. All other alkyl thiols were obtained as described previously (Lewis et al., 1980). N^{α}-Benzoyl-L-arginine *p*-nitroanilide (BzArgPNA) was from the Protein Research Foundation, Minoh-shi, Osaka, Japan. N^{α}-Benzyloxycarbonyl-L-arginine–7-amido-4-methylcoumarin hydrochloride (ZArg–MCA) was from Sigma.

Papain was isolated from crude dried papaya latex and purified by affinity chromaography by the method of Burke et al. (1974) and was reacted with MMTS and stored as the methylthio derivative of Cys-25 (Lewis et al., 1976; Smith et al., 1975).

Methods. Pseudo-first-order rate constants for reaction of low molecular weight thiols with MMTS were measured at 25 °C, $\Gamma/2$ 0.15, in buffered solutions which had been purged with argon, with the MMTS in 20–100-fold molar excess of the thiol. Reactions were monitored by continuously recording the time dependence of absorbance at a single wavelength in the UV or visible (with nitroaryl thiols) region of the spectrum. Pseudo-first-order rate constants were obtained from the slopes of linear plots of ln $|A_f - A|$ vs. time, where $|A_f - A|$ is the absolute value of the difference between the final absorbance and the absorbance at a given time. All rate constants were independent of the wavelength chosen to monitor the reaction. Second-order rate constants were calculated by dividing the observed pseudo-first-order rate constants by the concentration of MMTS. Evaluation of second-order rate constants at several MMTS concentrations verified that they were independent of the MMTS concentrations. The acid dissociation constants for the thiol group in 4-aminobenzenethiol and 5-mercapto-2-nitrobenzoic acid were determined by spectrophotometric titration.

The catalytic activity of papain was assayed with BzArgPNA by the method of Johnson et al. (1981). Thiol concentrations were determined by the generation of the thiol anion from Nbs_2 as measured at 412 nm in 0.10 M phosphate buffer, pH 8.0, by using a molar absorptivity of 1.42×10^4 M^{-1} cm^{-1} (Riddles et al., 1979). Concentrations of papain were determined from the thiol titer. These values were 95% of that estimated from the absorbance at 280 nm by using a molar absorptivity of 5.77×10^4 M^{-1} cm^{-1} (Skalski et al., 1973).

Kinetic parameters for reaction of MMTS with papain were determined at 25 °C, $\Gamma/2$ 0.15, in buffered solutions containing 5% acetonitrile at the indicated final pH values. The final concentration of buffer species was 0.025 M except for bicine buffers which contained 0.075 M bicine. The buffers used were potassium phosphate monobasic/HCl (pH ≤3.6), acetic acid–sodium acetate (pH 3.6–5.5), potassium phosphate monobasic/sodium phosphate dibasic (pH 6–7.5), and bicine/sodium hydroxide (pH 8–9.5). Concentrated solutions of active papain were prepared fresh each day by activating methylthiopapain (2.3 mg/mL) in 10 mM phosphate buffer (pH 6.5), 1 mM EDTA, with 1 mM DTT. After 10 min at room temperature when full activity toward BzArg-PNA was observed, 4 mL of the solution of papain was stirred with 1.5 mL of AG-50 1-X8 mixed bed resin for 20 min to remove the DTT and low molecular weight ions. The resulting solution of desalted papain was separated from the resin, adjusted to contain 5 mM EDTA–1 mM acetate (pH 4.5), and filtered through a 0.45-μm Millipore HV filter.

Two protocols (a and b) were used in studies of the reaction of MMTS with papain. (a) For reactions at papain concentrations of 40 nM (pH 3.1–8.5), solutions composed of ZArg–MCA (usually 345 μM), MMTS (0.56 μM), and the desired reaction buffer with 2.5 mM EDTA were thermally equilibrated at 25 °C in a fluorescence cuvet. An aliquot of a concentrated solution of papain was added and the ensuing reaction monitored fluorometrically (excitation 380 nm, emission 440 nm) by using a ratio fluorometer built by Dr. David Ballou and Gordon Ford of this department. (b) Reactions at 1–6 μM papain were monitored in a stopped-flow fluorometer built by Kinetic Instruments of Ann Arbor, MI. Reactions were initiated by shooting a solution (at 25 °C) containing papain (2–12 μM) against an equal volume of a solution (at 25 °C) containing ZArg–MCA (0.5–0.9 mM) and MMTS (10–125 μM). For runs between pH 4 and pH 9.5, the papain solution contained buffer, 5 mM EDTA, 5% acetonitrile, and salt so as to produce the indicated final pH and an ionic strength of 0.15 upon dilution with a solution of ZArg–MCA and MMTS containing 0.075 M NaCl in 5% acetonitrile. For runs between pH 2.5 and pH 4, 5% acetonitrile and buffer at an ionic strength of 0.05 were present in the solution containing the ZArg–MCA and MMTS, whereas the papain solution contained 0.25 M NaCl, 5% acetonitrile, and 5 mM EDTA. The solution containing the buffer was alternated in this way to avoid prolonged exposure of MMTS above pH 8 and papain below pH 4, conditions that cause time-dependent decomposition of these materials. Control experiments demonstrated that decomposition was negligible when reagents were incubated separately at the pH extremes for the times used to study the reaction of papain with MMTS. Measurements of the dependence of initial velocity on the concentration of ZArg–MCA were performed in buffered solutions spectrophotometrically (360 nm) and fluorometrically.

Prior to use, solutions containing ZArg–MCA were filtered through 0.45-μm Millipore HV filters. The concentration of ZArg–MCA solutions was determined after filtration from the absorbance ($\epsilon_{325} = 2 \times 10^4$ M^{-1} cm^{-1}) of an aliquot of the solution which had been diluted into acetate buffer (pH 4.5).

The concentration of MMTS in stock solutions was determined from the loss of absorbance at 412 nm after addition of a sample of the solution to a reaction mixture (pH 8)

[2] Although reaction of MMTS with other groups in proteins has been documented (Kluger & Tsui, 1980), the reactivity of these groups toward MMTS is several orders of magnitude lower than that of thiolate groups.

Table I: pH Dependence of $[H]k_{obsd}/K$ for Reaction of Mercaptoethanol with MMTS (25 °C, Γ/2 0.15)

pH	$10^6[H]k_{obsd}/K$ (M^{-1} s^{-1})	pH	$10^6[H]k_{obsd}/K$ (M^{-1} s^{-1})
5.65	3.2	2.14	3.0
5.00	3.0	1.30	3.0
4.00	2.8	0.95	3.3

composed of DTT and excess Nbs_2 which had an absorbance of about 0.6 prior to addition of the MMTS.

Results and Discussion

The pH dependence of the observed second-order rate constant (k_{obsd}) for reaction of MMTS with thiols containing no other ionizable groups should be given by eq 2, where K

$$k_{obsd} = \frac{Kk_S}{[H] + K} + \frac{[H]k_{SH}}{[H] + K} \quad (2)$$

represents the acid dissociation constant for the thiol group and k_S and k_{SH} represent the rate constants for reaction of MMTS with the thiolate anion and the protonated thiol group, respectively. Since k_{SH} was expected to be much smaller than k_S, we attempted to evaluate this parameter by measuring rate constants for the reaction thiols with MMTS at low pH values ($[H] \gg K$) to maximize the relative contribution of the k_{SH} term in eq 2.

When $[H] \gg K$, eq 2 reduces to eq 3. Values of $[H]k_{obsd}/K$

$$\frac{[H]k_{obsd}}{K} = k_S + \frac{[H]k_{SH}}{K} \quad (3)$$

at several pH values are listed in Table I for the reaction of MMTS with mercaptoethanol. The relative constancy of $[H]k_{obsd}/K$ with pH allowed us to set an upper limit of 0.3 $\times$ 10^6 M^{-1} s^{-1} for $[H]k_{SH}/K$ at pH 0.95. Since $K = 10^{-9.65}$, the second-order rate constant (k_{SH}) for attack of the protonated thiol on MMTS must be less than 6 $\times$ 10^{-4} M^{-1} s^{-1} or at least 5 $\times$ 10^9 times smaller than the rate constant k_S for attack by the thiolate anion. Thus, for all practical purposes reaction of a thiol group with MMTS can be assumed as occurring via the thiolate anion, especially when the observed second-order rate constant is much greater than 6 $\times$ 10^{-4} M^{-1} s^{-1}. Consistent with this conclusion, the pH dependence of k_{obsd} for all thiols with no other ionizable groups was observed to be within experimental error (±10%) of that predicted by the relationship (eq 4) obtained by setting k_{SH} to zero in eq 2.

$$k_{obsd} = \frac{Kk_S}{[H] + K} \quad (4)$$

Values of k_S were determined by using eq 4 to relate measured values of k_{obsd} to k_S and K or by measuring k_{obsd} at pH values where $[H] \ll K$ and $k_{obsd} = k_S$.

Figure 1 and Table II depict the dependence on thiol pK of the second-order rate constant (k) for the attack of thiolate anions on MMTS. The experimentally determined dependencies of rate constant on thiolate basicity (thiol pK) could be represented by the Brønsted equation

$$\log k_S = \log G + \beta pK \quad (5)$$

with $\log G = 1.44$ and $\beta = 0.635$ for aryl thiols and $\log G = 3.54$ and $\beta = 0.309$ for the alkyl thiols. Interestingly, the pK dependencies of the rate constants for the attack of aryl and alkyl thiolate group on Nbs_2 also yields two Brønsted correlations (Wilson et al., 1977). In the latter case, however, similar β values (0.48 and 0.49), but different G values, were obtained for aryl and alkyl thiolate groups. Although the reason for the different Brønsted correlations for the reactions

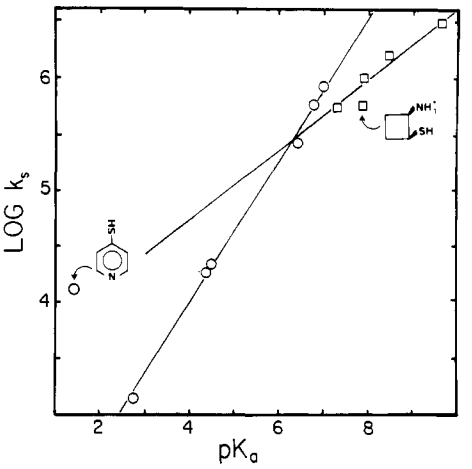


FIGURE 1: Dependence on thiol pK of the second-order rate constant for reaction of aryl (○) and alkyl thiolate (□) anions with MMTS at 25 °C, Γ/2 0.15.

Table II: Dependence of the Second-Order Rate Constant for Reaction of Thiolate Anions with MMTS on Thiol pK

	pK	k^a (M^{-1} s^{-1})
4-mercaptopyridine	1.43[b]	1.3 $\times$ 10^4
2,3,4,5-tetrafluorobenzenethiol	2.75[c]	1.4 $\times$ 10^3
5-mercapto-2-nitrobenzoic acid[d]	4.37	1.8 $\times$ 10^4
4-nitrobenzenethiol	4.50[c]	2.2 $\times$ 10^4
benzenethiol	6.43[c]	2.7 $\times$ 10^5
4-methoxybenzenethiol	6.77[e]	5.9 $\times$ 10^5
4-aminobenzenethiol	7.00	8.6 $\times$ 10^5
cysteine ethyl ester[f]	7.30[g]	5.6 $\times$ 10^5
cis-2-mercaptocyclobutylamine[f]	7.89[h]	5.7 $\times$ 10^5
methyl 2-mercaptoacetate	7.91[c]	1.0 $\times$ 10^6
2-mercaptoethylamine[f]	8.46[h]	1.6 $\times$ 10^6
2-mercaptoethanol	9.65[h]	3.1 $\times$ 10^6

[a] All rate constants are the average of at least four separate determinations. [b] Albert and Barlin (1959). [c] Hupe and Jencks (1977). [d] pK and rate constants given for the carboxylate-containing form. [e] De Maria et al. (1973). [f] pK and rate constant for the species with a protonated amino group. [g] Reuben and Bruice (1976). [h] Lewis et al. (1980).

of aryl and alkyl thiolates with MMTS is not known, interactions between the π system of thiolate anions with MMTS may somehow give rise to the observed alterations in the Brønsted parameters β and G. Thus, one might expect the Brønsted parameters for thiolate reactivity to be a function of the π system to which it is conjugated. It is therefore not surprising that the reactivity of the aromatic heterocyclic compound 4-thiopyridine (pK = 1.43) deviates from the Brønsted correlation for substituted benzenethiols. The plot of Figure 1 also reveals that *cis*-2-mercaptoaminocyclobutane is about 1.8-fold less reactive than a primary alkyl thiol with the same pK. The low reactivity of this compound might well be a consequence of the increased steric hindrance of a thiolate group attached to a secondary carbon atom.

Equations 4 and 5 together with the values of G and β observed for alkyl thiols lead to eq 6 for relating the reactivity

$$\log k_{obsd} = 3.54 - 0.691\ pK - \log ([H] + K) \quad (6)$$

of an alkyl thiol to its pK and the pH. To a first approximation, one might expect the second-order rate constant for reaction of MMTS with a thiol group in a protein to be given by eq 6. The use of eq 6 to predict the pH dependence of the reactivity of a protein thiol group is complicated, however, by the possibility that the pK of a protein thiol group may itself be a function of pH. For example, ionization of a neighboring group could perturb the electrostatic environment and pK of

the reactive thiol group. In addition to this complication, several factors could cause the reactivity of a protein thiol group to deviate from that predicted by eq 6. For example, limited accessibility of the thiol group might lower its reactivity. Furthermore, the reactivity of a protein thiol group whose pK has been perturbed by charge–charge interactions may deviate from that predicted by eq 6, wherein the dependence of thiolate reactivity on thiol pK reflects primarily charge–dipole interactions. To determine how the nucleophilic reactivity of thiol groups in proteins might differ from that of thiol groups in simple low molecular weight organic compounds, we examined the pH dependence of the reactivity toward MMTS of a protein thiol (Cys-25 at the active site of papain) whose ionization behavior has been studied by other methods.

Preliminary experiments indicated that papain reacted with MMTS[3] too rapidly to allow us to monitor the reaction by periodic removal of samples from the reaction mixture for assays of enzymic activity. To circumvent this problem, papain (initial concentration $[E_0]$) was reacted with MMTS in the presence of the fluorogenic substrate ZArg–MCA (initial concentration $[S_0]$), while the time-dependent release of MCA, the fluorescent product of papain-catalyzed substrate hydrolysis, was continuously monitored. For the inactivation of papain by MMTS, where the inactivation reaction is irreversible and competitively blocked by substrate, it can be demonstrated that the time dependence of product release should be given by eq 7–9.[4] F_f and $[S_f]$ represent the

$$\ln ([S] - [S_f]) = \ln \left(\frac{k_{cat}[S_0][E_0]}{K_M k_{obsd}[MMTS]} \right) - k'_{obsd} t \quad (7)$$

$$\ln (F_f - F) = \ln \left(\frac{k_{cat} \Delta F_T [E_0]}{K_M k_{obsd}[MMTS]} \right) - k'_{obsd} t \quad (8)$$

$$k'_{obsd} = \frac{K_M}{K_M + [S_0]} k_{obsd}[MMTS] \quad (9)$$

fluorescence and concentration of substrate after the enzyme has been inactivated, and ΔF_T represents the change in fluorescence expected for complete hydrolysis of the substrate. The derivation of eq 7–9 is based on the assumption that the concentrations of substrate and MMTS do not change significantly, relative to their initial values, during the inactivation reaction, and that if an E·MMTS complex forms, its concentration is small relative to the concentration of unmodified enzyme. Examination of eq 8 reveals that the pseudo-first-order rate constant k'_{obsd} can be evaluated from the first-order approach of the fluorescence to its final value, provided, of course, the enzyme catalyzes formation of a measurable amount of fluorescent product before it is inactivated. Equation 10, obtained by setting $t = 0$ in eq 7, relates the final

$$[S_0] - [S_f] = [P_f] = \frac{k_{cat}[S_0][E_0]}{K_M k_{obsd}[MMTS]} \quad (10)$$

concentration of hydrolysis product ($[P_f]$) to the specificity constant (k_{cat}/K_M) for enzymic catalysis, the initial substrate concentration, the ratio of papain ($[E_0]$) to [MMTS], and the second-order rate constant for reaction of the thiol group with MMTS.

[3] The specificity of the reaction of the thiol group at the active site of papain with MMTS has been documented by Smith et al. (1975) and Lewis et al. (1976).

[4] The derivation of eq 7–9 has been discussed previously [e.g., see Tian & Tsou (1982) and Leytus et al. (1984)].

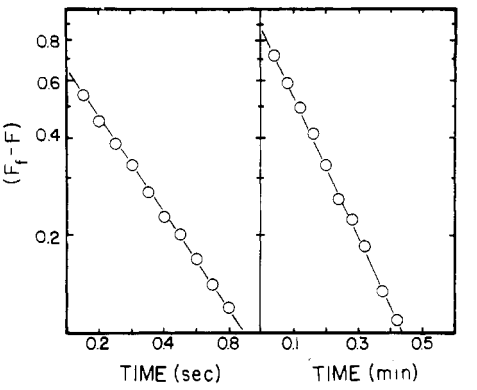


FIGURE 2: Determination of the second-order rate constant for the reaction of papain with MMTS at 25 °C, pH 6.5, $\Gamma/2$ 0.15. Depicted are first-order approaches of fluorescence intensity to a final value. The left panel depicts the reaction of 1.2 μM papain, 12 μM MMTS, and 287 μM ZArg–MCA ($k'_{obsd} = 1.69\ s^{-1}$). The right panel depicts reaction of 0.040 μM papain with 0.56 μM MMTS and 345 μM ZArg–MCA ($k'_{obsd} = 8.46 \times 10^{-2}\ s^{-1}$). Substitution of the values of [MMTS], k'_{obsd}, and the $K_m/(K_m + [S_0])$ (0.709 for the left panel and 0.673 for the right panel) in eq 9 yields values for k_{obsd} of $1.99 \times 10^5\ M^{-1}\ s^{-1}$ (left panel) and $2.24 \times 10^5\ M^{-1}\ s^{-1}$ (right panel).

The upper limit of $[S_0]$ was fixed by its solubility, whereas the upper limit of $[E_0]/[MMTS]$ was set at 0.1 to ensure that the relative concentration of MMTS did not change significantly during the reaction when eq 8 and 9 were used to evaluate k'_{obsd}. The values of less than 0.01 observed for the ratio of the specificity constant for the fluorescent substrate and k_{obsd} fixed the fractional conversion of substrate to <0.1% when papain was inactivated with $[MMTS] > 10[E_0]$. The high sensitivity of the fluorometric assays made it feasible to continuously monitor the release of this level of product below pH 8.5. At pH values above 8.5 the fluorescence yield of MCA decreased, and higher degrees of conversion were necessary for accurate evaluation of rate constants. To increase the yield of MCA, the initial value of $[E_0]/[MMTS]$ was increased to 0.4–0.67, and the method described under the Appendix was used to evaluate k_{obsd}. Since less than 1.0% of the substrate was hydrolyzed in all determinations, the requirement for a small change in relative substrate concentration during inactivation was satisfied. When $[E_0]/[MMTS] \leq 0.1$, k'_{obsd} was evaluated from slopes of linear plots of ln $(F_f - F)$ vs. time; values of k_{obsd} were calculated from k'_{obsd} by using eq 9. The term $K_M/(K_M + [S_0])$ in eq 9 was between 0.66 and 1.0. This term was evaluated from measurements of initial velocity in the absence of MMTS by using eq 11 (which follows from the Michaelis–Menten equation)

$$\frac{K_M}{K_M + [S_0]} = \frac{(V_0 - V_2)[S_2]}{([S_0] - [S_2])V_2} \quad (11)$$

where $[S_0]$ represents the substrate concentration used to monitor the reaction with MMTS and V_0 the corresponding initial velocity measured in the absence of MMTS. $[S_2]$ and V_2 are substrate and initial velocities measured in the absence of MMTS with $[S_2]$ usually about $0.2[S_0]$.

The two plots depicted in Figure 2 illustrate evaluations of k'_{obsd} at pH 6.5 at enzyme and MMTS concentrations which differ by a factor of more than 20. The values of k'_{obsd}, however, yield upon substitution in eq 9, essentially the same value [2.1 ($\pm$0.1) $\times 10^5\ M^{-1}\ s^{-1}$] for k_{obsd}. A similar independence of the value of k_{obsd} on the concentration of MMTS was observed at all pH values tested. These observations establish a second-order rate law for the reaction of papain and MMTS and verify the assumption that, under the experimental conditions used, there is little or no buildup of an

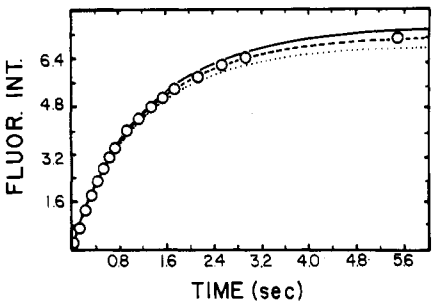


FIGURE 3: Determination of k_{obsd} from the time dependence of fluorescence intensity at 25 °C, pH 8.53, Γ/2 0.15. The concentrations of papain, MMTS, and ZArg–MCA were 3.13, 6.1, and 424 μM, respectively. The solid, dashed, and dotted lines are fits to the data (circles) with k''_{obsd} fixed at 2.1×10^5 M^{-1} s^{-1}, 2.2×10^5 M^{-1} s^{-1}, and 2.3×10^5 M^{-1} s^{-1}, respectively. The best-fit value of k''_{obsd} (2.2×10^5 M^{-1} s^{-1}) and the value of 0.671 observed for $[S]/(K_m + [S_0])$ yield upon substitution in eq A3 of the appendix a value of 3.28×10^5 M^{-1} s^{-1} for k_{obsd}.

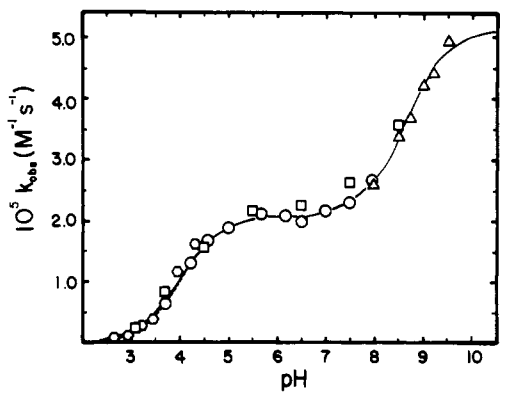
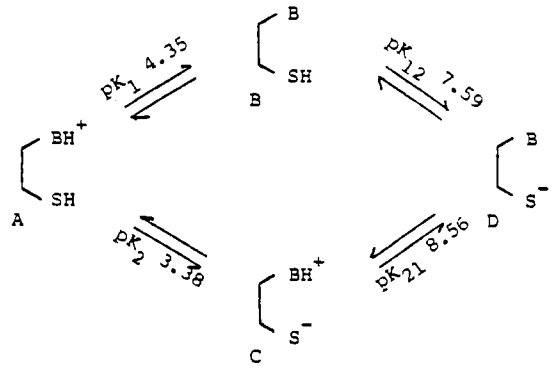


FIGURE 4: pH dependence of k_{obsd} at 25 °C, Γ/2 0.15. The solid line was calculated from eq 12, and the dashed line was calculated from eq 16. The initial concentrations of papain, MMTS, and ZArg–MCA were respectively the following: (□) 0.04, 0.56, and 345 μM; (○) 1.2, 12, and 287 μM; (△) 3.1, 6.1, and 424 μM; (○) 5.8, 61, and 400 μM.

E·MMTS complex as a reaction intermediate. Figure 3 illustrates the evaluation of k_{obsd} from the fit of the digitally integrated differential equations (given under the Appendix) to data obtained with $[F_0]/[MMTS] > 0.5$. The value (3.4×10^5 M^{-1} s^{-1}) of k_{obsd} obtained by this method is within experimental error (10%) of values of k_{obsd} evaluated from the fit of eq 8 to data obtained under pseudo-first-order conditions ($[E_0]/[MMTS] < 0.1$). Additionally, the value of k_{obsd} was independent of the substrate concentration (data not shown). Taken together these observations verify the analytical methods used to determine the pH dependence of k_{obsd} depicted in Figure 4.

The pH dependence of k_{obsd} resembles that of a double titration curve and is similar to previously reported pH dependencies for the reactivity of Cys-25 toward other electrophiles such as α-haloamides [e.g., see Polgar (1983)]. This complex behavior is not unexpected in light of the ionization behavior of Cys-25 in papain. Potentiometric difference titrations (Lewis et al., 1976), NMR titrations (Johnson et al., 1981a; Lewis et al., 1981), and fluorescence titrations (Johnson et al., 1981b) support the view that the pK of the active center thiol group in papain changes from 3.38 to 7.59 (Γ/2 0.15) when the neighboring active center His-159 is deprotonated. The interacting ionizations of Cys-25 and His-159 are illustrated in Scheme I along with values for the microscopic equilibrium constants. In light of the two acid dissociation constants for the thiol group, species C and D might be expected to exhibit different nucleophilic reactivities toward

Scheme I: Microscopic Equilibrium Constants for Ionization of Cys-25 (SH) and His-159 (BH^+) at the Active Site of Papain [29 °C, Γ/2 0.15, from Lewis et al. (1976)]



MMTS. Thus, the observed rate constant for the reaction of papain with MMTS should be given by

$$k_{obsd} = f_C k_{SC} + f_D k_{SD} = \frac{K_2 k_{SC}/K_I + K_{II} k_{SD}/[H]}{1 + [H]/K_I + K_{II}/[H]} \quad (12)$$

where f_C and f_D represent the fraction of papain that exists as species C and D, respectively, and k_{SC} and k_{SD} their corresponding nucleophilic reactivities. The macroscopic equilibrium constants K_I and K_{II} are related to the microscopic equilibrium constants of Scheme I by eq 13–15.

$$K_I = K_1 + K_2 \quad (13)$$

$$K_{II}^{-1} = K_{12}^{-1} + K_{21}^{-1} \quad (14)$$

$$K_1 K_{12} = K_2 K_{21} \quad (15)$$

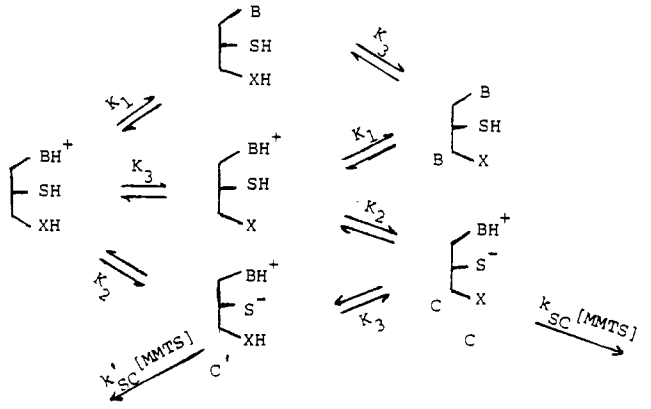
The best fit of the pH dependence of k_{obsd} to eq 12 yielded values of 4.0, 8.6, 2.1×10^5 M^{-1} s^{-1}, and 5.1×10^5 M^{-1} s^{-1} for pK_I, pK_{II}, $K_2 k_{SC}/K_I$, and k_{SD}, respectively. The value of 8.6 obtained for pK_{II} is the same as that found by potentiometric difference titration (Lewis et al., 1976). Interestingly, the second-order rate constant k_{SC} for reaction of the thiolate ion in D is within 67% of the value of 7.6×10^5 M^{-1} s^{-1} predicted for a thiol group with a pK of 7.59 (the pK of the thiol group in B) by the Brønsted plot in Figure 1.

The value of pK_I of 4.0 obtained from the fit of the data to eq 12 is 0.66 pK unit higher than that expected from the value determined from pH difference titrations. Although this discrepancy may reflect in part an error in the determination of pK_I by the PDT method, it is important to note that the pK = 4 ionization observed in the pH rate profile for reaction of papain with MMTS may not be equivalent to pK_I of Scheme I. Rather, this pK = 4 ionization may reflect the presence of a second group that upon ionization causes a subtle structural change near the active site so as to increase the access of MMTS to Cys-25. Such a pK = 4.0 ionization has been shown (Lewis et al., 1978; Schack & Kaarsholm, 1984) to increase the rate of reaction of Cys-25 with amide and ester substrates. Assuming that the thiolate anion in C is less accessible and less reactive when the pH 4 group is protonated, the acid limb of the pH rate proile for the reaction of Cys-25 with MMTS should be given by eq 16, assuming the acid–base equilibria

$$k_{obsd} = \frac{\frac{K_2}{K_I} k_{SC} + \frac{K_2}{K_3 K_1} k_{SC}[H]}{1 + \frac{K_I + K_3}{K_1 K_3}[H] + \frac{[H]^2}{K_I K_3}} \quad (16)$$

depicted by Scheme II. The dashed line in Figure 4 shows the fit of the data to eq 16 with K_I set to the value $10^{-3.34}$, i.e., $K_1 + K_2$, as determined by the PDT method (Lewis et al.,

Scheme II: Ionization Equilibria That Modulate the Nucleophilic Reactivity of Cys-25 in Papain



1976), and K_3, K_2k_{SC}/K_I, and $K_2k'_{SC}/K_I$ set at 10^{-4}, 2.1×10^5 M^{-1} s^{-1}, and 4×10^4 M^{-1} s^{-1}, respectively. It is clear from the fit of the dashed line to the data in Figure 4, that the pH rate profile for reaction of papain with MMTS is also consistent with the existence of a pK = 4 group which upon ionization increases the accessibility of Cys-25 so as to increase its reactivity toward MMTS by about 5-fold.

The intrinsic reactivity of the thiolate anion in C can be calculated from the value of K_2k_{SC}/K_I which was obtained from the fits of the data in Figure 4 to eq 16 and the fraction of B and C present as ion pair C (K_2/K_I). The value (0.91) of K_2/K_I evaluated by the PDT method (Lewis et al., 1975) yields a value of 2.3×10^5 M^{-1} s^{-1} for k_{SC}. This value is about 6 times greater than the value (3.8×10^4 M^{-1} s^{-1}) predicted by the Brønsted plot (Figure 1) for an alkyl thiolate anion with a basicity similar to that of the thiolate anion in C (parent thiol pK = 3.38).

It is unlikely that the six-fold higher than expected reactivity of the thiolate group in C is due to acid-catalyzed elimination of methylsulfinic acid [pK = 2.12 (Lewis et al., 1976)] by the neighboring imidazolium ion of His-159, since reaction of thiolate anions with MMTS does not appear to be subject to acid catalysis. If it were, hydronium ion catalysis of attack of thiolate anions on MMTS would give rise to a significant pH-independent term (at [H] $\gg$ K) in the expression for the dependence of k_{obsd} on [H]. To within experimental error, however, k_{obsd} was strictly proportional to the fraction of thiol existing as thiolate anion (i.e., proportional to $[H]^{-1}$ at $H >> K$). Furthermore, the simple structure of MMTS together with the first-order dependence on MMTS concentration observed for the rate of reaction of Cys-25 with MMTS makes it unlikely that MMTS forms a noncovalent complex with papain wherein reaction of Cys-25 and MMTS might be enhanced by proximity and orientational effects. With respect to these considerations MMTS differs from the amide and ester substrates of papain which form a noncovalent complex with papain and undergo acid-catalyzed nucleophilic attack.

Assuming that the thiolate anions in C and D and those in the model system all react with MMTS via a similar transition state, the deviation in reactivity of Cys-25 from that predicted by the Brønsted relationship for simple alkyl thiols indicates that the factors responsible for the difference between the pK of the thiol groups in A and B affect thiolate reactivity differently than the electronic effects that give rise to the difference in the pK of the alkyl thiols in the model system. For example, the enhanced reactivity of the thiolate in C may be a consequence of the pK of the thiol group in A being altered from that in B primarily via charge–charge interactions involving the adjacent His residue,[5] whereas in the model alkyl thiol system the pK of the thiol group is altered primarily via charge–dipole interactions. Studies of disulfide interchange by Snyder (1984) suggest that through-space charge–charge interactions have little or no effect on the nucleophilic reactivity of low molecular weight thiolate anions toward neutral disulfides. Consistent with this conclusion we observe only a 2.4-fold difference in the reactivities of the thiol groups in C and D, where there exists a 4.2 pK unit difference in the pK values of the parent thiols (A and B).

The pK dependence of the reactivity of Cys-25 toward MMTS is characterized by a β value of 0.09, which is substantially lower than the β value of 0.31 observed for the pK dependence of reactivity observed for simple alkyl thiols. The low β value observed for Cys-25 suggests the possibility that perturbations in pK resulting from through-space charge–charge interactions might have very small effects on the nucleophilic reactivity of thiolate anions (and perhaps other nucleophiles) toward neutral substrates. Such charge–charge interactions might allow an enzyme to lower the pK of an active site thiol (or other group) and thereby increase the fraction present in the reactive basic form without suffering the concomitant loss in nucleophilic reactivity seen when pK's are lowered via charge–dipole interactions.

It is important to note that the estimate of the fraction of B and C existing as an ion pair as determined by the PDT method was based on the assumption that groups other than His-159 have a negligible effect on the ionization behavior of Cys-25 and that neutralization of the negative charge of the thiolate anion of Cys-25 with a proton and methylthio group have equivalent effects on the ionization behavior of His-159. Errors in either of these assumptions could introduce errors in the estimation of K_2/K_I. It is unlikely, however, that the estimate of the fraction of B and C present as ion pair is in gross error, since NMR studies in D_2O (Johnson et al., 1981a,b; Lewis et al., 1981) indicate extensive protonation of His-159 in papain at pH 4.0, consistent with the notion that (in D_2O at least) the equilibrium concentration of C is greater than that of B. These NMR studies have also verified that methylthiolation of Cys-25 decreases the pK of His-159 in a manner consistent with that expected for loss of a thiolate imidazolium ion pair.

Additionally, the failure to observe significant rates for reaction of protonated thiols with MMTS together with the substantial rate constant (2.1×10^5 M^{-1} s^{-1}) observed at the low pH rate plateau (Figure 4) strongly suggests that a substantial fraction of Cys-25 exists as a thiolate anion at pH 5. A lower limit of 0.41 can be estimated from the ratio of the rate constants ($2.1 \times 10^5/5.1 \times 10^5$) for the high and low pH plateaus for the fraction (K_2/K_I) of species B and C which exists as the ion pair C. This estimate of the minimum value for the fraction ion pair assumes that the through-space charge–charge interactions responsible for the change in pK of Cys-25 do not alter its nucleophilic reactivity toward MMTS (i.e., β = 0 so that the reactivity toward MMTS of the thiolate anion in C is equal to that in D). Allowance for a decreased

[5] Van Duijnen et al. (1979) have concluded from molecular orbital calculations that the electrostatic field arising from the dipolar α-helical polypeptide chain perturbs the pK's of Cys-25 and His-159 so as to stabilize the ion pair C more than its uncharged protomer, B. This electrostatic field (which can be envisaged as decreasing pK_2 more than pK_1 in Scheme I) cannot account for the difference between the pK's of the thiol group in A and B, since the same helix dipole is present in both species. An estimate of the effect of the helix dipole on the pK of Cys-25 in both A and B might be obtained from appropriate comparisons of the pK of the thiol group in B (pK = 7.6) with that of the thiol group in N^{α}-acylcysteinylamides.

reactivity of C relative to D ($\beta \geq 0$) would increase the estimate for the fraction ion pair, [C]/([B] + [C]).

Creighton and co-workers (Creighton et al., 1980; Wandinger & Creighton, 1980) have estimated K_2/K_1 from the magnitude of solvent deuterium isotope effects on the rate of alkylation of Cys-25. Using a set of assumptions different from those used in the PDT method, these investigators have estimated that two-thirds of an equilibrium mixture of B and C exists as the thiolate–imidazolium ion pair. In light of the different approaches, the two estimates (0.67 and 0.91) for the extent of ion pair formation appear in tolerable agreement. Recent observations of solvent deuterium isotope effects on the equilibrium constant for formation of thiohemiacetals between Cys-25 in papain and aldehydes, however, are not easy to reconcile with a substantial extent of ion pair formation (Bone & Wolfenden, 1985). Further studies of the active site of papain and models for reactions of thiolate–imidazolium ion pairs with aldehydes may be required to explain this discrepancy.

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APPENDIX

For studies where $[E_0]/[MMTS_0]$ was greater than 0.1, values of k_{obsd} were evaluated from fits to the experimental data of predicted time dependencies of fluorescence (F) as calculated by Runge–Kutta digital integration of differential eq A1 and A2. [E′] represents the total concentration of

$$\frac{dF}{dt} = \frac{V_f^0}{[E_0]}[E'] \tag{A1}$$

$$-\frac{d[E']}{dt} = -\frac{d[MMTS]}{dt} = \frac{d[EM]}{dt} = k''_{obsd}[MMTS][E'] \tag{A2}$$

$$k''_{obsd} = k_{obsd}\frac{K_m}{K_m + [S_0]} \tag{A3}$$

unmodified enzyme at time t, [EM] represents the concentration of the inactive methylthio derivative of papain, and V_f^0 represents the value of dF/dt at zero time (t). Values of V_f^0 and k''_{obsd} were systematically varied until the best fit of the digitally integrated rate equations to the data was obtained. The value of k_{obsd} was evaluated from eq A3 by using the best-fit value of k''_{obsd} along with the value of $K_m/(K_m + [S_0])$, which was determined in separate studies as described in the discussion of eq 11 in the text. In all experiments less than 1% of the substrate was consumed so that corrections for substrate depletion were negligible.

Registry No. MMTS, 2949-92-0; L-Cys, 52-90-4; $HO(CH_2)_2SH$, 60-24-2; p-$O_2NC_6H_4SH$, 1849-36-1; PhSH, 108-98-5; p-$MeOC_6H_4SH$, 696-63-9; p-$H_2NC_6H_4SH$, 1193-02-8; L-CysOEt, 3411-58-3; $HSCH_2CO_2Me$, 2365-48-2; $HS(CH_2)_2NH_2$, 60-23-1; 4-mercaptopyridine, 4556-23-4; 2,3,4,5-tetrafluorobenzenethiol, 3467-86-5; 5-mercapto-2-nitrobenzoic acid, 15139-21-6; *cis*-2-mercaptocyclobutylamine, 36455-65-9; papain, 9001-73-4.

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