Inhibition of Papain by *N*-Ethylmaleimide†

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ABSTRACT: The rate of inactivation of papain by *N*-ethylmaleimide was studied, both in the presence and absence of substrates. Contrary to previous reports the reaction between papain and *N*-ethylmaleimide was found to be completely irreversible and first order with respect to *N*-ethylmaleimide concentration, with no evidence for the binding of *N*-ethylmaleimide to papain prior to reaction with the active sulfhydryl group. It is suggested that the conflicting results reported in the literature arise from using papain that has been activated and subsequently used without removing the activator from the enzyme solution. The substrate *N*-benzoylglycine ethyl ester fully protects the enzyme against *N*-ethylmaleimide inactivation whereas the substrate α-*N*-benzoyl-L-arginine ethyl ester provides only partial protection.

Inhibition studies of the enzyme papain have largely been concerned with the inhibition of the enzyme's catalytic activity by chemical modification of the essential cysteine residue. The sulfhydryl group at the active site of papain has been specifically alkylated by iodoacetamide (Finkle and Smith, 1958), 1-chloro-3-tosylamido-2-propanone (Husain and Lowe, 1965), Tos-PheCH_2Cl[1] (Bender and Brubacher, 1966; Whitaker and Perez-Villasenor, 1968; Wolthers, 1969), dibromoacetone (Husain and Lowe, 1968), Tos-LysCH_2Cl (Whitaker and Perez-Villasenor, 1968; Wolthers, 1969), chloroacetic acid (Sluyterman, 1968; Chaiken and Smith, 1969a), chloroacetamide (Chaiken and Smith, 1969b), both antipodes of α-iodopropionic acid and α-iodopropionamide (Wallenfels and Eisele, 1968), and MalNEt (Feng *et al.*, 1965; Morihara, 1967; Anderson and Vasini, 1970). There are conflicting reports in the literature regarding the nature of the reaction between MalNEt and papain. The present study provides a basis for a clearer understanding of the reaction between papain and MalNEt. A model is presented which satisfactorily accounts for the conflicting reports in the literature.

Materials and Methods

Papain. Mercuripapain in 70% ethanol (Worthington Biochemical Corp.) was centrifuged at 12,000*g* for 15 min at 2°. The supernatant was removed and mercuripapain was dissolved in 0.05 M acetate buffer, pH 5.2, μ = 0.038, [EDTA] = 10^{-5} M, to give a mercuripapain solution approximately 2 × 10^{-3} M in papain. The mercuripapain solutions were stored at 2–4°.

Mercuripapain was activated to papain by dissolving 0.2 mmol (24 mg) of L-cysteine in each milliliter of mercuripapain solution (Brubacher and Bender, 1966; Bender *et al.*, 1966). After standing for 5–7 min the solution was passed through a 1.2 × 17 cm column of Sephadex G-25 which had been preequilibrated with 0.05 M acetate buffer, pH 5.2, μ = 0.038, [EDTA] = 10^{-5} M. The papain eluted between the 9- and 14-ml eluate volume. The fractions containing 90–95% of the total protein (determined spectrophotometrically at 280 nm) were combined and stored at 2–4°, in a flask flushed with nitrogen, until the enzyme was used. This is the stock enzyme solution.

The normality of active papain solutions was determined spectrophotometrically using BzArgOEt as a substrate (Bender *et al.*, 1966).

For assays of relative concentrations of papain the activity toward ZGlyONph was determined spectrophotometrically. To a 1.00-cm absorbance cell was added 3.00 ml of a solution which was 1 × 10^{-4} M in ZGlyONph and 1 × 10^{-3} M in EDTA in 0.05 M acetate buffer, pH 5.2, μ = 0.038, at a temperature of 25.0°. In some cases the solution was made 6.6 × 10^{-4} M in cysteine at this point by adding 20 μl of freshly prepared 0.1 M cysteine solution. (Under these conditions, spontaneous hydrolysis of ZGlyONph is negligible.) Then 100 μl of papain was added and the absorbance was recorded until about 30% of the ZGlyONph was hydrolyzed. The absorbance curve is nearly linear since the initial substrate concentration is some 10-fold greater than K_m under these conditions. (Kirsch and Igelstrom (1966) give K_m = 0.93 × 10^{-5} M at pH 6.8, which should apply at pH 5.2 as well since neutral substrates exhibit pH-independent K_m values (Lucas and Williams, 1969; Sun and Chou, 1963.) The initial slope is a measure of the relative papain concentration. The spectrophotometer was a Hitachi Perkin-Elmer Model 139; absorbance readings were recorded at 10-sec intervals over a period of 3–5 min and then plotted to obtain the slope.

Kinetics of Inactivation by MaLNEt in the pH-Stat. METHOD A. This method was used when no substrate was present. To 500 μl of a solution of MalNEt (3.0–18.0 × 10^{-3} M) in aqueous 0.1 M phosphate buffer, pH 6.0, μ = 0.3, [EDTA] = 1 × 10^{-3} M, was added 100 μl of approximately 1 × 10^{-4} M papain. At intervals, 100 μl of this solution was withdrawn and added to 5.00 ml of 0.01 M BzArgOEt in the pH-Stat (previously adjusted to pH 6.0, and 25.0°). The 50-fold dilution into the assay solution effectively quenches further reaction of MalNEt with papain. The rate of base uptake (0.100 M KOH) is a measure of the remaining enzyme activity. A plot of these rates on a logarithmic scale *vs.* time is linear. The first-order inactivation rate constant is obtained from the slope.

METHOD B (Sluyterman, 1968). The general procedure for these runs is shown schematically in Figure 1. Substrate (5 ml) in an aqueous solution that is 0.3 M in KCl and 10^{-3} M in EDTA was added to a water-jacketed reaction vessel whose

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[1] Abbreviations used are: BzArgOEt, α-*N*-benzoyl-L-arginine ethyl ester; MalNEt, *N*-ethylmaleimide; ZGlyONph, *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester; BzGlyOEt, *N*-benzoylglycine ethyl ester; Tos-PheCH_2Cl, 1-chloro-3-tosylamido-4-phenyl-2-butanone; Tos-LysCH_2Cl, 1-chloro-3-tosylamido-7-amino-2-heptanone; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

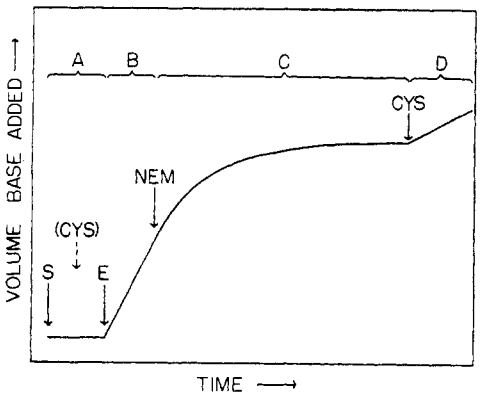

FIGURE 1: Schematic representation of a typical inactivation reaction of papain by MalNEt (NEM) as monitored in the pH-Stat. The arrows indicate the sequence of addition of substrate (S), papain (E), MalNEt, and cysteine (CYS). Cysteine was not always added in region A. The four regions are labeled A–D for ease of reference in the text.

temperature was maintained at 25.0 ± 0.1°. An oxygen-free atmosphere was maintained by the introduction of water-saturated nitrogen gas into the reaction vessel, just above the surface of the reaction mixture. In some runs the solution was made 5×10^{-4} M in cysteine at this point by adding 50 μl of 0.05 M cysteine. The pH of the reaction mixture was adjusted to 6.0 with 0.100 M KOH before approximately 2×10^{-6} mmol of active papain was added. After several minutes, during which time about 5% of the substrate was hydrolyzed, 250 μl of 0.20 M MalNEt was added to the reaction mixtures. Inactivation was allowed to proceed until less than 1% of the original activity remained. In some runs, 100 μl of 1.0 M cysteine was added at this point, resulting in increased enzymic activity (region D in Figure 1). The method of extracting k_i from the data is described in the Results section.

Kinetics of Inactivation Monitored Spectrophotometrically. A discontinuous method of assay (Anderson and Vasini, 1970) was also used to follow the rate of papain inactivation by MalNEt. The inactivation reaction mixture, equilibrated at 25.0°, contained $2.50\text{–}15.00 \times 10^{-3}$ M MalNEt, approximately 2×10^{-6} M papain, and 1×10^{-5} M EDTA in aqueous 0.10 M phosphate buffer, pH 6.0, $\mu = 0.30$ M. At various intervals, a 100-μl aliquot was withdrawn and assayed spectrophotometrically by its activity toward ZGlyONph (no cysteine in the assay solution). Since the inactivation mixture is diluted 30-fold when introduced into the assay solution, the inactivation of papain by MalNEt during the assay is negligible. The enzyme activity was plotted on a logarithmic scale *vs.* time (in this case the length of the inactivation before the assay was begun). The rate constant for inactivation, k_i, was determined from the slope of the first-order plot.

Chemicals. BzArgOEt, mp 130–132° (Mann Research Laboratories) and mp 128–130° (Fluka), ZGlyONph, mp 122–124° (Cyclo Chemical), and MalNEt, mp 41–43° (Eastman Organic Chemicals), were used without further purification. The MalNEt was titrated spectrophotometrically against cysteine and found to be >99% pure. BzGlyOEt, mp 57–59° (Mann Research Laboratories), was recrystallized from boiling water before use, mp 58.5–59.5°. All other chemicals were reagent grade and used without further purification.

Results

It is standard procedure in this laboratory to prepare

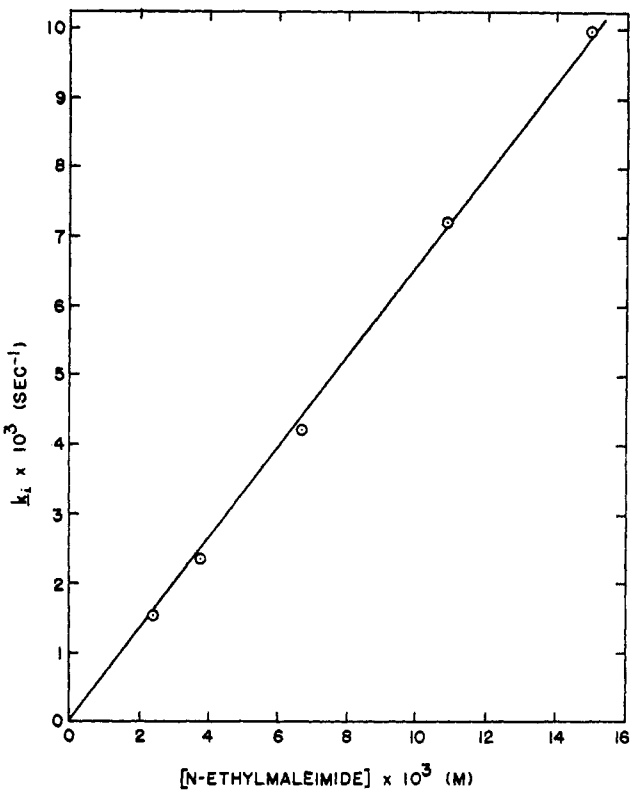

FIGURE 2: Plot of pseudo-first-order rate constant *vs.* MalNEt concentration for the inactivation of papain by MalNEt. The straight line was obtained by an unweighted least-squares regression analysis. 25.0°, pH 6.00, ionic strength 0.30 M, enzyme concentration 1.5×10^{-5} M.

papain for kinetic studies by treating a mercuripapain solution with a thiol compound such as cysteine, and then passing the solution through a Sephadex G-25 column to obtain active papain free of activator. Such enzyme preparations slowly lose activity (less than 2%/day). Part of this loss is irreversible (probably autolysis), part is reversible, upon incubation with cysteine. The latter probably arises from an oxidation of the essential SH group.

Thus activator-free papain solutions contain three types of protein molecules: E_a, active papain; E_{ia}, inactive papain which may be activated by reducing agents such as cysteine; and E_u, papain which has no activity and no potential activity and can be ignored since it generally has no effect in kinetic studies.

$$[E]_{tot} = [E_a] + [E_{ia}] + [E_u] \quad (1)$$

The kinetics of the inactivation of papain by MalNEt are straightforward when activator-free (freshly-prepared) active papain is used. These results will be presented first, followed by the more complex results obtained using (older) papain preparations that were preincubated with cysteine to restore higher activity levels.

Kinetics of Inactivation of Activator-Free Papain by MalNEt. When MalNEt is in large excess compared to the papain concentration, the kinetics of the inactivation reaction are first-order in papain (pH-Stat Method A) for reactions in which the initial enzyme concentration was varied tenfold. Further, the pseudo-first-order rate constant, k_i, is directly proportional to the MalNEt concentration. The data are plotted in Figure 2. The second-order rate constant for the reaction, 0.661 M^{-1} sec^{-1}, is obtained from the slope in Figure 2 as determined by a least-squares regression analysis. One determination using

the spectrophotometric method at a MalNEt concentration of 9.5×10^{-3} M yielded $k_i = 6.31 \times 10^{-3}$ sec^{-1}, which agrees well with the value 6.28×10^{-3} sec^{-1} read from Figure 2.

The second-order rate constant for the reaction of MalNEt with cysteine is 153 M^{-1} sec^{-1} at pH 6 (Gorin *et al.*, 1966), some 230-fold faster than with papain.

The effect of the presence of substrate on the rate of inactivation of papain by MalNEt was studied using pH-Stat method B.

Sluyterman (1968) has proposed the mechanism of eq 2 for the reaction of covalent inhibitors with papain in the presence of substrate. When the substrate concentration is constant, and the inhibitor is in large excess, the inactivation is

$$\begin{array}{ccccccc} E + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES & \xrightarrow[-P_1]{k_2} & ES' & \xrightarrow{k_3} & E + P_2 \\ \downarrow{\scriptstyle k_{if}[I]} & & \downarrow{\scriptstyle k_{ic}[I]} & & & & \\ E_i & & E_i & & & & \end{array} \qquad (2)$$

pseudo first order, with a first-order rate constant, k_i, given by eq 3 (Sluyterman, 1968) where k_f ($= k_{if}[I]$) and k_c ($= k_{ic}[I]$) are the pseudo-first-order rate constants for the reaction of the inhibitor with the thiol group in the free enzyme, and in the enzyme–substrate complex, respectively. From progress curves such as that in Figure 1, k_i may be obtained

$$k_i = \frac{k_c k_3}{k_2 + k_3} + \left(k_f - \frac{k_c k_3}{k_2 + k_3}\right)\left(\frac{K_m}{[S] + K_m}\right) \qquad (3)$$

from first-order plots of the changing slope in region C with appropriate correction for decreasing substrate concentration (Sluyterman, 1968). Such plots are indeed linear for the MalNEt reaction in the presence of BzArgOEt and BzGlyOEt. Since this procedure is tedious, most k_i values were obtained by computer fitting the data to the integrated equation for the rate of production of P_2 (hydrogen ion) derived using the usual steady-state approximation (eq 4). An additional empirical

$$[H^+] = \left(\frac{[S]}{[S] + K_m}\right)B(1 - e^{-k_i t}) \qquad (4)$$

$$B = \frac{k_2 k_3 [E]_0}{k_i(k_2 + k_3)} \qquad (5)$$

term, linear in time, was added to take account of the small residual activity (less than 1%) observed at the end of region C. The correction for the changing substrate concentration was incorporated by programming the computer to calculate the term $[S]/([S] + K_m)$ (see eq 4) for each data point, where $[S] = [S]_0 - [H^+]$. This correction was generally small since [S] changed by less than 10% during the reactions. An independently determined value of K_m was supplied. The curve fitting was performed on the University of Waterloo IBM 360-75 computer with program NLMAX which uses a modified Gauss–Newton iteration method to produce a (least-squares) best fit.

The observed pseudo-first-order rate constants of inactivation at varying BzArgOEt and BzGlyOEt concentrations are plotted as k_i *vs.* $K_m/([S] + K_m)$ in Figure 3 according to eq 3. Values of K_m used were 0.017 M for BzArgOEt (determined in this work), and 0.013 M for BzGlyOEt (Sluyterman, 1968).

At zero substrate concentration eq 3 yields $k_i = k_f$. The BzArgOEt curve and the BzGlyOEt curve converge to this value within experimental error (Figure 3). At infinite substrate concentration eq 3 yields $k_i = k_c k_3/(k_2 + k_3)$. From Figure 3 it can be seen that at infinite BzGlyOEt concentration, k_i is equal to zero, the same effect found for this

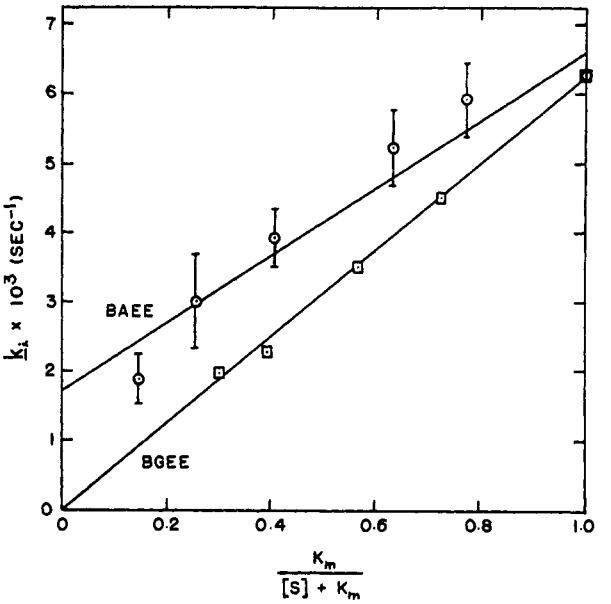

FIGURE 3: The dependence of k_i on substrate concentration (eq 3). The straight lines were obtained from an unweighted least-squares regression analysis. The MalNEt concentration was 9.50×10^{-3} M in all cases; enzyme concentration, 6×10^{-7} M; 25.0°; pH 6.00; ionic strength, 0.30 M; EDTA concentration, 1×10^{-3} M. For BzArgOEt (BAEE) each data point is the average of three runs, with standard deviations. BGEE is *N*-benzoylglycine ethyl ester.

substrate toward other inactivating reagents (Sluyterman, 1968; Whitaker, 1969). At infinite BzArgOEt concentration, k_i is approximately one-fourth as large as k_f. The value of the second-order rate constant at infinite BzArgOEt concentration is 0.180 M^{-1} sec^{-1}.

Some experiments were performed to investigate whether papain that has been inactivated by MalNEt can be reactivated by treatment with thiol reagents. Relative enzyme activities were determined using the ZGlyONph assay procedure in three different assays. (1) When unmodified papain is assayed with no cysteine present in the assay solution, the slope of the absorbance *vs.* time curve is proportional to $[E_a]$ (see eq 1). (2) When cysteine is present in the assay solution the slope is proportional to the concentrations of E_a plus E_{ia} (see eq 1) and is defined as $[E_c]$.

$$[E_c] = [E_a] + [E_{ia}] \qquad (6)$$

(3) When the papain used was previously fully inactivated by MalNEt, the assay performed in the presence of cysteine gives a slope proportional to an enzyme concentration defined as $[E_{nc}]$. The active enzyme in this assay can have come from two sources: E_{ia}, which was present from the beginning but was untouched by MalNEt since its thiol group is in an oxidized form; and the hypothetical E_r, which is regenerated by the action of cysteine on enzyme which was inactivated by reaction with MalNEt.

$$[E_{nc}] = [E_{ia}] + [E_r] \qquad (7)$$

Combining eq 6 and 7 gives a relationship for calculating E_r (eq 8). The results of the three assays for five stock enzyme

$$[E_r] = [E_{nc}] + [E_a] - [E_c] \qquad (8)$$

solutions of different ages are shown in Table I. Clearly, $[E_r]$ is zero within experimental error, *i.e.*, cysteine does not reactivate enzyme that has been inactivated by reaction with MalNEt. Thus, $[E_{nc}] = [E_{ia}]$.

Liener (1961) has found similar results in the reaction of

TABLE I: Relative Activities of Papain before and after Inactivation by MalNEt.[a]

Stock Papain Soln No.	$[E_{nc}]^b$	$[E_a]^c$	$[E_c]^d$	$[E_r]^e$	$100[E_r]/[E_c]$, %
1	0.25	2.17	2.40	+0.02	0.8
2	0.54	2.06	2.54	+0.06	2.4
3	1.02	1.40	2.46	−0.04	−1.6
4	1.24	1.50	2.84	−0.10	−3.5
5	1.48	2.51	3.78	+0.19	5.0

[a] Assayed by activity towards ZGlyONph. Entries are the initial slopes of absorbance *vs.* time curves corrected for spontaneous hydrolysis. [b] Enzyme was fully inactivated by reaction with 9.3×10^{-3} M MalNEt at pH 6.00 prior to the assay which was performed in the presence of cysteine. [c] Assay performed in the absence of cysteine. [d] Assay performed in the presence of cysteine. [e] Calculated from eq 8.

MalNEt with ficin. Using ficin preparations several weeks old he found that the amount of enzyme activity (assayed in the presence of 2,3-dimercaptopropanol) which remained after MalNEt inhibition was the same as that which could be activated by prior treatment with 2,3-dimercaptopropanol before inhibition by MalNEt.

Reaction of MalNEt with Papain in the Presence of Cysteine. Active papain solutions more than a week or two old contain a significant amount of E_{ia}. Preincubation with 5×10^{-4} M cysteine reactivates this fraction in less than 1 min, but the behavior of the enzyme toward MalNEt as presented in this section differs in several ways from what has been described previously.

In a typical experiment using papain preincubated with cysteine, 50 μl of 0.05 M cysteine was added to the reaction vessel of the pH-Stat in time region A (Figure 1), giving a final concentration of 0.5×10^{-3} M. When MalNEt is added (final concentration of 2.5–15 $\times 10^{-3}$ M) it consumes the cysteine essentially instantaneously since the thiol of cysteine is 230-fold more reactive than that of papain. After the inactivation of papain by MalNEt is complete, the addition of excess cysteine (final concentration 20×10^{-3} M) causes the instantaneous reappearance of enzymic activity (region D in Figure 1), which, since MalNEt-inactivated papain cannot be reactivated (Table I), must arise from some other source. The active enzyme so produced will be referred to as E_{enc}, the subscripts indicating that the enzyme was treated successively with cysteine, MalNEt, and cysteine.

Three important facts concerning this recovered enzymic activity are as follows. (1) The fraction of enzymic activity recovered (E_{enc}) relative to the initial activity (slope in region D divided by slope in region B of Figure 1 after minor corrections for changed substrate concentration) is independent of the initial enzyme concentration for runs using a stock enzyme solution of fixed age. (2) The relative amount of enzymic activity recovered in region D increases with increasing age of the stock enzyme solution, and approximately equals the amount of E_{ia} in the stock solution. (3) The recovered enzymic activity, E_{enc} has a K_m of 0.015 M which is, within experimental error, identical with the value of 0.017 M for native papain for our conditions.

These results suggest that during the MalNEt reaction wih papain that was preincubated with cysteine, some other inactivator is present that inactivates papain reversibly. This is confirmed by additional kinetic observations which are of a qualitative nature. (1) Experiments in which the inactivation reaction (region C, Figure 1) was interrupted by the addition of cysteine (in excess of MalNEt) at various times showed that the inactive enzyme which is ultimately reactivated in region D (E_{enc}) is formed much more rapidly than the rate of inactivation of papain by MalNEt. (2) First-order plots of the data from the inactivation by MalNEt of papain that had been preincubated with cysteine were biphasic also indicating a fast disappearance of part of the enzymic activity followed by a slower inactivation rate, the latter in agreement with the data in Figure 2. Before the complications associated with preincubated papain were fully realized, some values of k_i were determined by drawing best straight lines through the data in the early part of such plots. Plots of these rate constants *vs.* MalNEt concentration were curved, suggesting, incorrectly, a saturation effect.

Discussion

MalNEt has been used for some time for the quantitative determination of sulfhydryl groups in dilute aqueous solution (Gregory, 1955; Alexander, 1958; Roberts and Rouser, 1958). Although MalNEt is not completely specific toward thiol compounds under certain conditions (Smyth *et al.*, 1960, 1964; Brewer and Riehm, 1967; Freedberg and Hardman, 1971; Arndt *et al.*, 1971), it seems most likely that at pH 6 MalNEt causes loss of papain activity by reaction with the thiol groups of cysteine-25. The following observations support this conclusion. At pH 6 in aqueous solution the amino acids, lysine, L-isoleucine, and α-*N*-benzoyl-L-histidine methyl ester did not react detectably with MalNEt under conditions for which the inactivation of papain by MalNEt is essentially complete (B. Glick, unpublished observations). The inactivation of papain by excess MalNEt is clearly first order, ruling out formation of a modified enzyme which is partially active, which might be possible if a site other than cysteine was modified by MalNEt.

The reaction between MalNEt and papain is first order with respect to both reagents and characterized by a second-order rate constant of 0.661 M^{-1} sec^{-1} at pH 6.00 and 25.0°. The second-order rate constant for the reaction of MalNEt with cysteine at pH 6 is 153 M^{-1} sec^{-1} (Gorin *et al.*, 1966), some 230-fold larger than the papain rate constant. Thus, MalNEt is different from most other alkylating reagents. The latter usually attack the thiol of papain more rapidly than that of cysteine; for example, chloroacetic acid reacts 30,000 times faster with papain than with cysteine at pH 6 (Sluyterman, 1968).

Papain is not unique in reacting more slowly than simple thiols with MalNEt. Table II lists rate constants for the reaction of MalNEt with thiol groups in some other enzymes. They are generally similar to the rate of reaction of MalNEt with papain. Clearly, addition of SH at the surface of an enzyme to a double bond is hindered for whatever electronic and/or steric reasons, whereas substitution for halide ion is generally enhanced compared to the reaction of model thiol compounds.

The results of Table I demonstrate that papain which has been inhibited by MalNEt cannot be reactivated by addition of excess cysteine, a behavior to be expected of a thioether product. Reports of partial reactivation are most likely due to complications arising from the presence of *in situ* activator.

When papain is preactivated with cysteine, it appears that

an inactivating substance is released which is freed to reinactivate papain when the cysteine is removed by reaction with the added MalNEt. (This complication would not arise with other alkylating reagents that react with cysteine more slowly than with papain.) We have not attempted to identify the postulated reversible inactivator, but one reasonable possibility is suggested in eq 9. The symbol E–S_{ox} represents

$$E\text{–}S_{ox} + CySH \rightleftharpoons E\text{–}SH + CyS_{ox} \quad (9)$$

papain with a thiol group in an (undefined) state of oxidation higher than SH. Under activating conditions, the excess cysteine present forces the equilibrium essentially completely to the right producing an equivalent of oxidized cysteine, CyS_{ox}. When MalNEt is added it upsets the equilibrium by reacting rapidly with reduced cysteine, thus permitting CyS_{ox} to reoxidize E–SH to E–S_{ox} (reverse of eq 9). Later, when cysteine is again added in excess, E–S_{ox} is reactivated giving enzyme E_{cno} as it was earlier defined.

There is a report (Feng *et al.*, 1965) that up to 26% of papain that has been inactivated by MalNEt can be reactivated by adding cysteine. Since these workers used cysteine to preactivate the papain, before inactivating with MalNEt, we feel that the apparent reversal is not due to the reversibility of the MalNEt reaction, but to an *in situ* reversible inhibitor as discussed above in connection with eq 9.

Morihara (1967) found that native papain (nonactivated) which had been incubated with MalNEt for 17 hr could be activated by cysteine but not by cyanide. Although this seems to support his rather complex model for papain activation, there is a simpler explanation. When the activator added is cysteine, it immediately consumes all the MalNEt, and activates the inactive but activatable papain fraction (E_{ia}) which is present from the beginning but is untouched by MalNEt since its thiol group is involved in a disulfide bond with cysteine (Sluyterman, 1967a; Klein and Kirsch, 1969a,b). When cyanide ion is the activator, it does not consume MalNEt (B. Glick, unpublished observations) but it does activate E_{ia} which then can react with the MalNEt present to become irreversibly inactivated. Consequently, as observed, no activity remains when an assay is performed 70 min after the addition of cyanide ion.

In a recent study of papain inhibition by maleimide derivatives (Anderson and Vasini, 1970), *N*-butyl-, *N*-pentyl-, and *N*-hexylmaleimide appeared to bind to the enzyme giving enzyme–inhibitor complexes with essentially identical intracomplex inhibition rate constants. On the assumption that MalNEt also binds, these workers calculated a dissociation constant of 3×10^{-3} M for the MalNEt–papain complex. The excellent linearity of Figure 2 demonstrates quite clearly that if MalNEt does bind to papain, the dissociation constant must be larger than 0.1 M.

It has been found that saturating levels of BzArgOEt do not affect the rate of papain inactivation by chloroacetic acid (Sluyterman, 1968), chloroacetamide (Whitaker, 1969), or Tos-PheCH_2Cl (Wolthers, 1969). That is, according to eq 3, $k_f = k_c k_3/(k_2 + k_3)$. If $k_f = k_c$, as Sluyterman argues, then $k_3 \gg k_2$. This disagrees with an earlier kinetic study in which it was concluded that $k_2/k_3 = 3.2$ for this substrate (Whitaker and Bender, 1965). Sluyterman rejected the alternative explanation that k_c is larger than k_f by exactly the factor $(k_3 + k_2)/k_3$ as too fortuitous a compensation. In addition, the noncovalently bound substrate, if it has any effect at all, might be expected to hinder the approach of the alkylating agent to the active sulfhydryl group causing k_c to be smaller than k_f (Brocklehurst *et al.*, 1968).

TABLE II: Rate of Reaction of MalNEt with Enzyme Sulfhydryl Groups.

Enzyme	Conditions	k (M^{-1} sec^{-1})
Papain[a]	25.0°, pH 6.0	0.661
D-Amino acid oxidase[b]	25°, pH 7	0.453
Propionyl-CoA carboxylase[c]	22°, pH 7	0.97
Sheep kidney mitochondrial phosphoenolpyruvate carboxykinase[d]	20°, pH 7	16.3
Yeast alcohol dehydrogenase[e]	25°, pH 7	0.223

[a] This work. [b] Fonda and Anderson (1969). [c] Edwards and Keech (1967). [d] Barns and Keech (1968). [e] Heitz *et al.* (1968).

The latter objection may be discarded if BzArgOEt binds to papain primarily in a nonproductive mode leaving the sulfhydryl group "uncovered," as Brocklehurst *et al.* (1968) have suggested. Indeed, Whitaker (1969) found that when papain is saturated with α-*N*-benzoyl-D-arginine ethyl ester, a competitive inhibitor of papain, the reactivity of the sulfhydryl group is 6-fold greater than in the free enzyme. As for Sluyterman's objection, the work reported in this paper shows that there is an alkylating agent, namely MalNEt, which does not exhibit this fortuitous compensation. It has also been shown that BzArgOEt partially protects papain from inactivation by cyanate ion (Sluyterman, 1967b).

Support for the nonproductive binding of BzArgOEt is available from activation studies of Nbs_2-inhibited papain (B. R. Glick and L. J. Brubacher, unpublished results) and nonactivated papain (Sluyterman, 1966). BzArgOEt binds to both Nbs_2-inhibited and nonactivated papain suggesting that the BzArgOEt binding site is adjacent to, but does not include, the active sulfhydryl residue.

The apparent curvature of the BzArgOEt data in Figure 3 lends further support to the nonproductive binding hypothesis which allows for nonlinear behavior in such plots (Brocklehurst *et al.*, 1968).

Hinkle and Kirsch (1971) explain Whitaker's observation that the reactivity of the active sulfhydryl group towards alkylating agents increases in the presence of α-*N*-benzoyl-D-arginine ethyl ester by the elimination of the nonproductive binding mode for the alkylating agent by the substrate. They have, in addition, concluded that, "the principal mode for the binding of *p*-nitrophenyl esters of *N*-acylamino acids to papain and ficin is nonproductive."

BzArgOEt appears to protect papain against different alkylating agents with varying degrees of efficiency. BzArgOEt offers no protection against alkylation by chloroacetic acid (Sluyterman, 1968), chloroacetamide (Whitaker, 1969) and Tos-PheCH_2Cl (Wolthers, 1969), partial protection against alkylation by MalNEt, and full protection against alkylation by Tos-LysCH_2Cl (Wolthers, 1969).

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References

Alexander, N. M. (1958), *Anal. Chem. 30*, 1292.
Anderson, B. M., and Vasini, E. C. (1970), *Biochemistry 9*,

3348.
Arndt, D. J., Simon, S. R., Maita, T., and Konigsberg, W. (1971), *J. Biol. Chem. 246*, 2602.
Barns, R. J., and Keech, D. B. (1968), *Biochim. Biophys. Acta 159*, 514.
Bender, M. L., Begué-Cantoń, M. L., Blakely, R. L., Brubacher, L. J., Feder, J., Gunter, C. R., Kézdy, F. J., Killheffer, J. V., Marshall, T. H., Miller, C. G., Roeske, R. W., and Stoops, J. K. (1966), *J. Amer. Chem. Soc. 88*, 5890.
Bender, M. L., and Brubacher, L. J. (1966), *J. Amer. Chem. Soc. 88*, 5880.
Brewer, C. F., and Riehm, J. P. (1967), *Anal. Biochem. 18*, 248.
Brocklehurst, K., Crook, E. M., and Wharton, C. W. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 2*, 69.
Brubacher, L. J., and Bender, M. L. (1966), *J. Amer. Chem. Soc. 88*, 5871.
Chaiken, I. M., and Smith, E. L. (1969a), *J. Biol. Chem. 244*, 5095.
Chaiken, I. M., and Smith, E. L. (1969b), *J. Biol. Chem. 244*, 5087.
Edwards, J. B., and Keech, D. B. (1967), *Biochim. Biophys. Acta 146*, 576.
Feng, Z. M., Zhu, J. Z., and Sun, Y. K. (1965), *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao 5*, 285.
Finkle, B. J., and Smith, E. L. (1958), *J. Biol. Chem. 230*, 669.
Fonda, M. L., and Anderson, B. M. (1969), *J. Biol. Chem. 244*, 666.
Freedberg, W. B., and Hardman, J. K. (1971), *J. Biol. Chem. 246*, 1439.
Gorin, G., Martic, P. A., and Doughty, G. (1966), *Arch. Biochem. Biophys. 115*, 593.
Gregory, J. D. (1955), *J. Amer. Chem. Soc. 77*, 3922.
Heitz, J. R., Anderson, C. D., and Anderson, B. M. (1968), *Arch. Biochem. Biophys. 127*, 627.
Hinkle, P. M., and Kirsch, J. F. (1971), *Biochemistry 10*, 2717.
Husain, S. S., and Lowe, G. (1965), *Chem. Commun.*, 345.
Husain, S. S., and Lowe, G. (1968), *Chem. Commun.*, 310.
Kirsch, J. F., and Igelstrom, M. (1966), *Biochemistry 5*, 783.
Klein, I. B., and Kirsch, J. F. (1969a), *Biochem. Biophys. Res. Commun. 34*, 575.
Klein, I. B., and Kirsch, J. F. (1969b), *J. Biol. Chem. 244*, 5928.
Liener, I. E. (1961), *Biochim. Biophys. Acta 53*, 332.
Lucas, E., and Williams, A. (1969), *Biochemistry 8*, 5125.
Morihara, K. (1967), *J. Biochem. (Tokyo) 62*, 250.
Roberts, E., and Rouser, G. (1958), *Anal. Chem. 30*, 1291.
Sluyterman, L. A. AE. (1966), *Biochim. Biophys. Acta 113*, 577.
Sluyterman, L. A. AE. (1967a), *Biochim. Biophys. Acta 139*, 430.
Sluyterman, L. A. AE. (1967b), *Biochim. Biophys. Acta 135*, 439.
Sluyterman, L. A. AE. (1968), *Biochim. Biophys. Acta 151*, 178.
Smyth, D. G., Blumenfeld, O. O., and Konigsberg, W. (1964), *Biochem. J. 91*, 589.
Smyth, D. G., Nagamatsu, A., and Fruton, J. S. (1960), *J. Amer. Chem. Soc. 82*, 4600.
Sun, Y. K., and Chou, C. L. (1963), *Sci. Sinica 12*, 201.
Wallenfels, K., and Eisele, B. (1968), *Eur. J. Biochem. 3*, 267.
Whitaker, J. R. (1969), *Biochemistry 8*, 4591.
Whitaker, J. R., and Bender, M. L. (1965), *J. Amer. Chem. Soc. 87*, 2728.
Whitaker, J. R., and Perez-Villasenor, J. (1968), *Arch. Biochem. Biophys. 124*, 70.
Wolthers, B. G. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 2*, 143.