Reaction of the Sulfhydryl Group of Papain with Chloroacetic Acid*

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SUMMARY

The kinetics of the alkylation of the essential sulfhydryl group of papain with chloroacetic acid was studied. The variation of the second order rate constant with pH at 4.5° and 30.5°, \( \Gamma / 2 = 0.07 \), is bell-shaped, with rate constants at a maximum near neutrality, approaching zero at low pH, and approaching a positive minimum at high pH. These profiles are described by a formulation which relates the apparent rate constants both with the states of dissociation of the sulfhydryl and other groups in papain and with the absolute rate constants for sulfhydryl reaction under specific conditions of pH. This formulation, as well as the absolute rate constants and the dissociation constants, have been used to suggest the presence of groups near the active center sulfhydryl group which affect the pH dependence of both the alkylation and the catalytic reactions of papain.

The alkylation of the catalytically essential sulfhydryl group of papain by chloroacetamide is described in the preceding paper (1). In the present investigation, the kinetics of the reaction with chloroacetate was studied in attempts to understand the basis for the highly reactive nature of the thiol group and its role in the catalytic mechanism of the enzyme.

Gerwin (2) first demonstrated that the pH dependence for the alkylation of the thiol group of streptococcal proteinase differs markedly for chloroacetamide, which yields an S-shaped curve approaching a maximum at high pH, and for chloroacetate, which shows a bell-shaped curve with a maximum near neutrality. After our studies were completed, similar findings were reported for papain by Wallenfels and Eisele (3) in studies with neutral and anionic alkylating agents. Our interpretations of these phenomena differ somewhat from those of these investigators.

MATERIALS AND METHODS

Reagents used included chloroacetic acid (Matheson Coleman and Bell) and bromoacetic acid (Eastman Organic Chemicals), both of which were recrystallized from ligroine before use. Other materials and methods are described in the preceding paper (1). A single preparation of papain, Preparation A (1), was used for all of the studies reported.

RESULTS

Kinetics of Alkylation of Papain by Chloroacetic Acid—The reaction of papain with chloroacetic acid follows pseudo-first order reaction kinetics with respect to enzyme and true second order kinetics for enzyme and inhibitor under all conditions of temperature, pH, ionic strength, and inhibitor concentration that were studied. Some examples are shown in Fig. 1.

The apparent second order rate constants, \( k_a \), were calculated from plots such as those of Fig. 1B; these constants were invariant over the entire range of inhibitor concentration studied, from 0.07 to 0.27 mm at pH 5.79 and from 0.13 to 1.33 mm at pH 9.25.

pH Dependence of Alkylation with Chloroacetic Acid at Ionic Strength 0.07—The variation of \( k_a \) with pH for the chloroacetic acid-papain reaction at both 4.5 and 30.5°, \( \Gamma / 2 = 0.07 \), is shown in Fig. 2. At low pH, \( k_a \) values were calculated based on the amount of \( \text{ClCH}_2\text{COO}^- \) present. The assumption was made that inhibition by the uncharged species, \( \text{ClCH}_2\text{COOH} \), is negligible at low pH. This assumption seems valid based on the inability of chloroacetamide to inhibit at low pH values (1). The concentration of chloroacetate ion was calculated from the reported values for the \( pK_a \) (4) of 2.82 at 4.5° and 2.88 at 30.5°. Determinations of \( k_a \) at 30.5° could not be performed below pH 3.15, at which level papain becomes susceptible to irreversible acid denaturation.

The data of Fig. 2, A and B, respectively, can be described by the relationship

\[
k_a = \frac{k_bK[H^+] + k_bK'[H^+] + K_bK[H^+] + K_bK'}{[H^+] + K[H^+] + K[H^+] + K[H^+]} \tag{1}
\]

This is derived from the scheme

\[
\begin{array}{c}
\text{EH}_2 \\
\xrightarrow{K_1} \text{EH} \\
\xrightarrow{K_1'} \text{E} + \text{H}^+ \\
+I \quad k_4 \\
\text{EI} \\
\end{array}
\]

† Computing assistance was obtained from the Health Sciences Computing Facility, UCLA, sponsored by National Institutes of Health Grant FR-3.
which assumes that chloroacetate can react with an enzyme species which has either, or both, of two ionizable groups dissociated (E or EH) but not with the enzyme form in which both groups are protonated. Specification of E as a reactive species implies that \( K_d \) should approach \( K_d \) at high pH, a phenomenon which seems apparent in Fig. 2A in the approach of \( K_d \) to a positive minimum in this pH range. Although this seems evident for the data obtained at 4.5° (Fig. 2A), the data at 30.5° (Fig. 2B) are complicated by an irreversible, slow loss of activity at the higher temperature. Nevertheless, the decrease of the values of \( K_d \) at high pH in Fig. 2B is more gradual than would be expected for the scheme where only one form of enzyme (EH, in Equation 2) can react with \( I \) to form an alkylated derivative. Nonlinear least squares computer fit (1) of the data of Fig. 2 to Equation 1 gives rise to the best fit theoretical curves shown in Fig. 2 and the calculated values for \( k_3 \), \( k_4 \), \( pK'_{3} \), and \( pK'_{4} \) given in Table I. \( \Delta H_{f,\varphi} \) values described by the variation of each of \( pK'_{3} \) and \( pK'_{4} \) with temperature (1) are listed in Table II. Also in Table II are the values for the activation parameters for the variation of \( k_3 \) and \( k_4 \) with temperature (1).

**Reaction with Bromoacetate**—A bell-shaped pH-\( K_d \) profile with a flat optimum between pH 5.5 and 7.5, similar to that measured for chloroacetate, was found for bromoacetate inhibition of papain at 4.5° and \( T/2 = 0.07 \). As expected, the rate of alklylation with the bromo compound was exceedingly rapid, being at its maximum almost 200 times greater than with the chloro derivative. The values for the parameters derived from the use of Equation 1 were: \( K_d = 139 \text{ m}^{-1} \text{sec}^{-1} \); \( k_4 = 9.3 \text{ m}^{-1} \text{sec}^{-1} \); \( pK'_{3} = 3.56 \) and \( pK'_{4} = 8.63 \). These values are, however, less certain than those obtained with the chloro compound because of the experimental problem of measuring this rapid reaction with the present methods. Measurements at temperatures higher than 4.5° were not feasible because of the high rates.

**Variation of \( k_d \) with Ionic Strength for Reaction of Chloroacetic Acid with Papain at 30.5°**—For the chloroacetic acid reaction, \( k_3 \) increases dramatically with ionic strength at pH 9.86, increases less so at pH 7.28, and decreases slightly at pH 4.28, as shown in Fig. 3. The significance of these changes is discussed below.

**DISCUSSION**

The curves for the \( k_d \)-pH profiles shown in Fig. 2 for both 4.5° and 30.5° are essentially bell-shaped, although \( k_d \) does not fall to zero normally at high pH but instead tends to reach a plateau at a low, finite value at 4.5°. These data are described best by Equations 1 and 2, which imply that the reaction of papain with chloroacetate proceeds at a high rate dependent upon the presence of the deprotonated form of a group described by \( pK'_{3} \) and the protonated form of a group described by \( pK'_{4} \), and at a lower rate when the latter group is deprotonated. It is, of course, the nature of these groups that is of interest.

The ascending limbs of the curves in Fig. 2 on the acid side appear to be simple dissociation curves throughout. \( pK'_{3} \) is 3.97 at 4.5° and 3.15 at 30.5°; these values suggest a carboxyl group. However, the data yield a value for \( \Delta H_{f,\varphi} < 0 \) of 10.7 kcal per mole, whereas the heats of ionization for the carboxyl groups of most compounds are normally close to zero (5). It cannot be ascertained, therefore, whether \( pK'_{3} \), in fact represents the simple dissociation of some group other than a carboxyl or whether it reflects carboxyl ionization superimposed upon a separate temperature-dependent event. These \( pK'_{3} \) values appear to be too high to correspond to the \( pK'_{3} \) values of the carboxyl group of chloroacetic acid (4). It also seems unlikely that \( pK'_{3} \) describes the pH-dependence of the intrinsic reactivity of chloroacetate; increasing pH would increase hydrolysis of an alkyl halide to an alcohol (6), a reaction which is slow and would decrease the concentration of the halogen-containing alkylating agent.

A carboxylic ester has been implicated in the alkylation of the sulphydryl group of papain previously by Wallenfels and Eisele (8). The \( pK'_{3} \) values derived from the acid limbs of the bell-shaped pH-\( k_d \) profiles for the reaction of \( v(+) \)- and \( v(-) \)-iodopropionic acid with papain at 25° (8) were calculated to be 3.2 and 4.0, respectively. Much earlier, Stockell and Smith (7) indicated that the papain-catalyzed hydrolysis of synthetic substrates appears to require the dissociated form of a group with \( pK'_{3} \) near 4 for both alylation and decylylation steps; the heat of dissociation associated with this group is essentially zero. In the molecular structure of papain deduced from x-ray analysis
FIG. 2. Variation of apparent second order rate constants with pH for the inhibition of activated papain by chloroacetic acid, T/2 = 0.07. A, 4.5°; chloroacetic acid concentration is 0.667 mM. B, 30.5°; chloroacetic acid concentration is 0.167 mM. Rate constants were calculated according to plots such as those shown in Fig. 1B. The solid lines are the k2-pH computer-derived best fit curves described by Equation 1. The broken lines in A and B are the theoretical pH-ks curves which describe the reaction of chloroacetamide with activated papain at T/2 = 0.07 at 4.5° and 30.5°, respectively (1).

TABLE I

<table>
<thead>
<tr>
<th>Temperature</th>
<th>k1</th>
<th>k2</th>
<th>pK1</th>
<th>pK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5°</td>
<td>0.72 ± 0.04</td>
<td>0.28 ± 0.06</td>
<td>3.97 ± 0.1</td>
<td>9.03 ± 0.1</td>
</tr>
<tr>
<td>30.5°</td>
<td>4.22 ± 0.17</td>
<td>0.219 ± 0.05</td>
<td>3.15 ± 0.1</td>
<td>8.10 ± 0.1</td>
</tr>
</tbody>
</table>

TABLE II

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔH1.0,pK2</td>
<td>10.8 ± 3 kcal/mole</td>
</tr>
<tr>
<td>ΔH1.0,pK1</td>
<td>12.2 ± 3 kcal/mole</td>
</tr>
<tr>
<td>ΔH1.4</td>
<td>11.9 ± 1 kcal/mole</td>
</tr>
<tr>
<td>ΔH1.4</td>
<td>11.9 ± 2 kcal/mole</td>
</tr>
<tr>
<td>ΔS1.4</td>
<td>-10.3 ± 2 e.u.</td>
</tr>
<tr>
<td>ΔS1.4</td>
<td>-11.0 ± 2 e.u.</td>
</tr>
<tr>
<td>ΔF1.0,A</td>
<td>16.3 ± 0.4 kcal/mole</td>
</tr>
<tr>
<td>ΔF1.0,A</td>
<td>14.0 ± 2 kcal/mole</td>
</tr>
</tbody>
</table>

of the crystals (8), there are apparently at least two carboxyl groups of aspartyl residues near the sulphydryl.

The basic limbs of the bell-shaped curves in Fig. 2 are somewhat more complex than the acidic limbs. However, Equations 1 and 2 indicate that this limb can be described simply by pK'4, which has the values of 9.09 and 8.10 at 4.5° and 30.5°, respectiv}

FIG. 3. Variation of apparent second order rate constants with ionic strength for the reaction of chloroacetic acid with activated papain, T/2 = 0.07. Inhibitor concentration is 0.167 mM at pH values 4.26 and 7.23 and 0.667 mM at pH 9.86. The solid straight lines were drawn to fit the experimental data. μ, ionic strength.
containing a sulphydryl anion; thus, the reaction of chloroacetate with papain at basic pH should depend in some manner on the pK of the sulphydryl group; (b) the pK′ and ΔH\textsubscript{i,0,pK} values correspond approximately to those values assigned to the sulphydryl group from its reaction with chloroacetamide at \( \Gamma/2 = 0.07 \) (pK values are 9.25 at 4.5° and 8.58 at 30.5° and \( \Delta H_{i,0,pK} = 8.3 \text{ kcal per mole} \) (1)). However, although schemes which involve more than two dissociating groups fail to fit the k\textsubscript{2}-pH profiles for chloroacetate inhibition as analyzed by computer best fit (9), the computer method cannot be used reliably for such complex schemes. Thus, the possibility cannot be excluded that the basic limits of the curves in Fig. 2 represent the summation of the dissociation of both a sulphydryl and one or more other groups on the protein which influence sulphydryl reactivity.

The value of pK′\textsubscript{4} at 30.5° for the chloroacetate reaction (pK′ = 8.10) is close to the pK for the basic limb of the k\textsubscript{2}-pH profile for the reaction of papain with L(-)α-iodopropionic acid, reported to be 7.82 at 25°, \( \Gamma/2 = 0.08 \) (3). In the latter case, it was suggested that the basic limb described the dissociation of an imidazole group. The pK′\textsubscript{4} values found in the present study would appear to be somewhat high for an imidazole group alone. Also, \( \Delta H_{i,0,pK} \) is higher than the values for the heat of ionization (about 7 kcal per mole) normally found for an imidazole group (5).

The value which \( k_2 \) appears to approach at high pH in both parts of Figs. 2, A and B, is \( k_4 \). This constant is assumed in Equations 1 and 2 to be the absolute second order rate constant for the chloroacetate-papain reaction when the group (or groups) described by pK′\textsubscript{4} are deprotonated. At high pH, the sulphydryl group should be dissociated, based on the chloroacetamide reaction studies (1); thus, \( k_4 \) probably represents the absolute second order rate constant for chloroacetate reaction with the simple sulphydryl anion of papain. This seems likely, since, based on the relative reaction rates of halogen acetates and acetamides with simple sulphydryl compounds at high pH, chloroacetate should react at a finite, although much slower, rate than chloroacetamide. This order of relative reaction rate is implicit in the values of the absolute second order rate constants \( k_2 \) (chloroacetamide at high pH (11)) and \( k_3 \) (chloroacetate at high pH) at \( \Gamma/2 = 0.07 \). At 4.5°, \( k_3/k_2 = 0.023 \); at 30.5°, this ratio is 0.038. The ratio of rate constant for chloroacetamide and chloroacetate and the sulphydryl group of glutathione at high pH is about 0.07 (2, 10). For the reaction of L(-)α-iodopropionic acid with papain (3), it was assumed that \( k_2 \) falls to zero at high pH.

The activation parameters calculated for the temperature variation of \( k_2 \) and \( k_4 \) are similar to each other: for \( k_2 \), \( \Delta H^* = 11.0 \text{ kcal per mole}, \Delta S^* = -19.3 \text{ e.u.}, \) and \( \Delta F^* = 16.3 \text{ kcal per mole} \); for \( k_4 \), \( \Delta H^* = 11.9 \text{ kcal per mole}, \Delta S^* = -11.0 \text{ e.u.}, \) and \( \Delta F^* = 14.9 \text{ kcal per mole} \). This correspondence lends support to the formulation of Equations 1 and 2, especially to the implication that \( k_4 \) indeed does approach the limiting value \( k_4 \). The activation parameters described by \( k_2 \) and \( k_4 \) are quite similar to those described both for the reaction of chloroacetamide with papain at both \( \Gamma/2 = 0.07 \) and \( \Gamma/2 = 0.50 \) (1) and for the papain-catalyzed hydrolysis of N-acetyl-L-arginimamide (7). This correspondence indicates that the reaction of the thiol group of papain with chloroacetate is analogous to that of papain with its substrates and with the inhibitor chloroacetamide.

There are two striking anomalies associated with the k\textsubscript{2}-pH profiles for chloroacetate inhibition in Fig. 2. First, in the pH range of sulphydryl dissociation, pH 7 to 10, \( k_2 \) increases with decreasing pH, whereas, for all model system alkylations of a sulphydryl group by a halogen acetate (11), the rates decrease with decreasing pH in this region. Second, the rate of reaction of papain at low pH values is greater with chloroacetate than with chloroacetamide, whereas, in all of the model systems studied, halogen acetamides are better alkylating agents for sulphydryl groups than halogen acetates at any pH (11).

The above facts lead to the proposal that, near neutral pH, at which level there is little ionization of the sulphydryl group some factor allows chloroacetate not only to react but to do so at a high rate. This factor may be the concomitant protonation of the imidazole of histidine-158, known to be near the sulphydryl group in the three-dimensional structure (8). This could facilitate the approach of chloroacetate to the sulphydryl. In fact, although there is little sulphydryl anion present at the low pH values at which chloroacetate still reacts, facilitated approach could effect the efficient reaction of the inhibitor with the sulphydryl anion as soon as it is formed. However, that the sole driving force for the reaction of chloroacetate with papain at low pH values is facilitated binding by imidazole is inconsistent with the effect of ionic strength on chloroacetate inhibition at low pH. Such facilitated binding would be expected to manifest a strong decrease in rate with an increase in ionic strength, whereas the data of Fig. 3 (pH 4.26) show only a slight change in rate.

In addition to the electrostatic interaction, it is possible that the imidazole of histidine-158 also influences the sulphydryl group in such a manner as to enhance its nucleophilicity. Such an interaction might be analogous to the imidazole-serine hydroxyl hydrogen bond found in the active sites of both subtilisin (12) and chymotrypsin (13). In addition, the imidazole or sulphydryl may interact at appropriate pH with the carboxylate anion presumably described by pK′\textsubscript{4}. This latter interaction also could be necessary for the “activation” of the sulphydryl group is consistent with the acid limbs of the pH-dependent profiles for both papain alkylation and papain-catalyzed hydrolysis of synthetic substrates lacking an ionizable group in the neutral pH range.

The following conclusions thus can be drawn for the chloroacetate inhibition of papain. Chloroacetate, like chloroacetamide, reacts with the sulphydryl anion at high pH values, at rates expected for a relatively unperturbed sulphydryl group. However, under conditions of pH where the imidazole of histidine-158 presumably is protonated, the chloroacetate reaction is abnormally rapid and a maximal rate is found near pH 5 to 6. Such enhanced alkylation may be due to electrostatic interaction of the imidazole of histidine-158 with chloroacetate. The high rate of reaction of the sulphydryl with both chloroacetate and chloroacetamide may be due to interactions of the sulphydryl, imidazole, and carboxylate groups of the enzyme. Such intraprotein interactions may cause increased nucleophilicity of the sulfur anion; this possibility may also explain the low pH, low ionic strength enhancement found for the rate of chloroacetamide inhibition (1).
pH not only with alkylating agents such as chloroacetate but, most important, with their substrates. It should be recalled that in the case of neutral substrates, such as hippurylamide and others, the pH optimum is in the region of pH 6 to 7, as it is also for substrates such as N-acylated arginine esters and amides (14). In contrast, anionic substrates, such as benzyloxy carbonyl-L-isoglutamine (14) and benzyloxy carbonyl glycylglycine (15) show an even more acidic pH optimum, indicating the presence of an anionic group near the active site that results in some electrostatic repulsion.

Thus, the problem remains as to why chloroacetate is a better inhibitor of papain than chloroacetamide at neutral pH, whereas neutral and cationic compounds are better substrates than anionic compounds at neutral pH. It would appear, therefore, that rates of alkylation and enzymic catalysis are not completely parallel reactions for papain. For example, alkylation with the neutral chloroacetamide occurs at maximal rates at pH values above 10, at which level enzymic catalysis is essentially negligible. This suggests that the sulfhydryl anion is readily alkylated under conditions where the changes in state of other groups, e.g. imidazole, alter the catalytic reactivity of the sulfhydryl group. Indeed, it is not yet clear whether the active catalytic form of the sulfhydryl group is the undissociated state or the anion.

Although streptococcal proteinase and papain show many similarities, the two enzymes do manifest some differences in behavior and many differences in the sequences, particularly near the active thiol groups (16-18).

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