Evidence for Histidine in the Active Site of Papain

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Papain was irreversibly inhibited by 1,3-dibromoacetone, a reagent designed to react first with the active-site cysteine residue and subsequently with a second nucleophile. The molecular weight of the inhibited enzyme was indistinguishable from that of papain itself, and no evidence of dimeric or oligomeric species was found. The optical-rotatory-dispersion curves of chloroacetone-inhibited papain and 1,3-dibromoacetone-inhibited papain were essentially similar. Amino acid analysis of the 1,3-dibrom[2-14C]acetone-inhibited enzyme and the performic acid-oxidized material clearly showed that a cysteine and histidine residue had been alkylated through the thiol and N-1 of the imidazole group respectively. These groups must therefore be within 5 Å of each other in the tertiary structure of papain. Possible mechanistic implications are briefly discussed.

In recent years the use of irreversible inhibitors, in particular active-site-directed inhibitors, has provided perhaps the most direct evidence for the presence of an amino acid residue in the active site of an enzyme (see Baker, 1967). With the exception of X-ray-crystallographic methods, this has been the only method available for locating such residues in the amino acid sequence of proteins. The recently reported tertiary structures of ribonuclease A and S (Kartha, Bello & Harker, 1967; Wyckoff et al. 1967) and α-chymotrypsin (Matthews, Sigler, Henderson & Blow, 1967) provide valuable confirmatory evidence for the validity of this approach.

Papain is the most extensively studied enzyme in a group of proteases that depend on a cysteine residue for their proteolytic activity. Papain contains only one free thiol group, which is alkylated by iodoacetate (Light, Frater, Kimmel & Smith, 1964) and by the active-site-directed irreversible inhibitor chloromethyl toluene-p-sulphonamido- methyl ketone (Husain & Lowe, 1965). By the use of radioactive inhibitors cysteine-25 has been identified as the residue alkylated by both these reagents.

During the hydrolysis of a substrate by papain, an acyl-enzyme intermediate is formed through the thiol group of this cysteine residue (Lowe & Williams, 1965a; Brubacher & Bender, 1966). Kinetic studies have led to the suggestion that an imidazole group of a histidine residue also plays an essential role in the mechanism of action of this enzyme (Lowe & Williams, 1965b, c).

Since alkylating reagents appear to react exclusively with cysteine-25 in papain, a bifunctional alkylating reagent would be expected to first react with cysteine-25 and then with a second nucleophile within a locus defined by the structure of the reagent. 1,3-Dibromoacetone was chosen as the bifunctional reagent for the following reasons. (i) This reagent is structurally similar to known reagents that inhibit papain through alkylation of the active-site cysteine residue. The reagent should further be capable of alkylation of a second nucleophile within a range of about 5 Å from the cysteine residue, and forming a stable covalent bond. (ii) Baeyer–Villiger (performic acid) oxidation of the inhibited protein should give both $S$-carboxymethylcysteine sulphone and the carboxymethyl derivative of the neighbouring nucleophilic residue. (iii) The synthesis of the reagent from bromoacetyl chloride as outlined below:

\[
\text{CH}_3\text{N}_2 + \text{CH}_2\text{Br} \cdot \text{COCl} \rightarrow \text{CH}_2\text{Br} \cdot \text{CO} \cdot \text{CH}_2\text{Br} \rightarrow \text{CH}_2\text{Br} \cdot \text{CO} \cdot \text{CH}_2\text{Br} + \text{HBr}
\]

was readily adaptable for the preparation of 14C-labelled material. Further, with either of the radioactive bromoacetates, the reagent would be symmetrically labelled, and hence both Baeyer–Villiger oxidation products would be radioactive. (iv) The intermediate diazo-ketone is potentially a valuable reagent for identifying a carboxylic acid group close to the active-site cysteine residue.

A preliminary account of this work has been published (Husain & Lowe, 1968).

MATERIALS AND METHODS

Papain. Twice-crystallized papain was prepared from granular papaya latex (we are grateful to the Wallerstein Co., New York, N.Y., U.S.A., for a generous gift of this
material) by the method of Kimmel & Smith (1954). The activator was separated from the activated enzyme on a column of Sephadex G-25 by elution with 0-05M-sodium acetate buffer, pH 5-6, containing EDTA (0-1mM).

Enzyme concentrations were determined from the extinction at 278 m\(\mu\) (Glazer & Smith, 1961). Enzyme activity was assayed by the rate of hydrolysis of methyl hippurate on a Radiometer pH-stat (Lowe & Williams, 1965c).

**Preparation of 1,3-dibromoacetone.** Bromoacetyl bromide (4-64g.) in dry ether (25ml.) at 0\(^\circ\) was added slowly to an ethereal solution of diazomethane (approx. 2-6g. in 100ml.) at 0\(^\circ\). After 5 min. acetic acid was added dropwise until the excess of diazomethane had decomposed; the solvent was then removed under reduced pressure. The residue was chromatographed on Whatman silica gel SG31 (100g.) in benzene and the diazo-ketone eluted with benzene containing ether (5\%, v/v). The product (2-00g.) had \(\nu_{\text{max.}}^\text{KBr} \approx 2000\text{cm}^{-1}
\((\text{CH} = \text{N=N})\) and 1642 cm\(^{-1}\) (C=O) (Found: C, 22-2; H, 1-8; Br, 48-9; N, 17-4. \(\text{C}_9\text{H}_3\text{BrN}_2\text{O}\) requires C, 22-1; H, 1-8; Br, 49-0; N, 17-2\%).

Constant-boiling HBr was added dropwise to a solution of the diazo-ketone (0-46g.) in acetic acid (1ml.) until all the diazo-ketone had reacted. After 5 min. the solvent was removed under reduced pressure and the residue crystallized from light petroleum (b.p. 60-80\(^\circ\)) to give colourless needles of 1,3-dibromoacetone (0-37g.), m.p. 27-28\(^\circ\) [Wegynd & Schmied-Kowarzik (1949) give m.p. 26-5\(^\circ\)]. \(\nu_{\text{max.}}^\text{KBr} \approx 1720\text{cm}^{-1}\) (C=O) (Found: C, 46-6; H, 1-8; Br, 73-6. Calc. for \(\text{C}_9\text{H}_3\text{BrN}_2\text{O}\): C, 46-7; H, 1-9; Br, 74-0\%).

**Preparation of 1,3-dibromo[\(2,14C\)]acetone.** Bromo[\(1,14C\)]-acetic acid (2-76mg., 0-2mc, specific activity 10-1mc/mole) was diluted with bromoacetic acid (66-4mg.) and dissolved with dry benzene (2ml.). Oxaly chloride (0-12ml.) was added and solution warmed for 5 min. and then refluxed for 2 hr. The excess of oxaly chloride and most of the benzene were removed under reduced pressure by fractional distillation. The fractionating column was washed with dry ether (5ml.) and the washings were added to the acid chloride.

The ethereal solution of the acid chloride was cooled to 0\(^\circ\) and slowly added to an ethereal solution of diazomethane [4ml., derived from 0-4g. of \(N\)-nitrosomethyl]urea] at 0\(^\circ\). When addition was complete, excess of diazomethane was decomposed by adding a drop of acetic acid. The solvent was removed in a rotary evaporator and the residue in dry benzene was applied to a 1mm.-thick plate (20cm. x 20cm.) of silica gel HF\(_{254}\) and developed with dry benzene. The yellow band of 1-bromo-3-diazo[\(2,14C\)]acetone (\(R_F\) 0-14) was scraped off and extracted with ether.

The ethereal solution of 1-bromo-3-diazo[\(2,14C\)]acetone at 0\(^\circ\) was saturated with dry Br\(_2\)-free HBr. The solution was brought to 20\(^\circ\) and after 15 min. the ether was removed in a rotary evaporator. Chromatography of the residue on a column (25cm. x 1cm.) of Whatman silica gel SG31 with dry benzene gave 1,3-dibromo[\(2,14C\)]acetone (31mg.), which had \(R_F\) 0-27 (identical with that of a characterized sample of 1,3-dibromoacetone) on a silica-gel thin-layer chromatoplate eluted with dry benzene.

**Inhibition of papain.** 1,3-Dibromo[\(2,14C\)]acetone (0-43mg., 2\(\mu\)mole) in acetone (0-5ml.) was added to papain (41mg., approx. 2\(\mu\)mole) in 0-05M-sodium acetate buffer, pH 5-6 (30ml.). Inhibition of the enzyme was rapid (less than 10min.) and complete. The inhibited enzyme was dialysed against several changes of water for 2 days.

**Molecular weight of the inhibited enzyme.** The molecular weight of the inhibited enzyme was determined on a column of Sephadex G-75 that had been calibrated with haemoglobin (mol. wt. 68000) and papain (mol. wt. approx. 21000) (see Fig. 1).

**Optical rotatory dispersion of the inhibited enzyme.** The optical-rotatory-dispersion curves of chloroacetone-inhibited papain and 1,3-dibromoacetone-inhibited papain were measured in 0-05M-sodium acetate buffer, pH 5-6, containing EDTA (0-1mM), in a Bendix Polaromatic 62 instrument, between 320 and 240 m\(\mu\). By taking \(\alpha_0 = 220 m\mu\), the Moffitt parameters were \(b_0 = 166 \pm 22\) and \(b_0 = 162 \pm 12\) respectively.

**Amino acid analyses.** Protein hydrolysates were prepared for amino acid analysis on the Technicon AutoAnalyzer or Eva Electrosequenium Ltd. analyser by treating 0-1\(\mu\)mole of protein with constant-boiling HCl (2ml.) in a sealed evacuated tube at 110\(^\circ\) for 2 hr. The acid was removed under vacuum and the residue taken up in 0-1N-HCl (1ml.).

**Performic acid oxidation.** The inhibited enzyme (1\(\mu\)mole) was oxidized with performic acid at \(-10^\circ\) for 6 hr. by the method of Hirs (1956).

**Radioactivity.** All radioactivity measurements were made in solution in an Isotopes Development Ltd. scintillation counter type 6012 with scaler 1700.

**RESULTS AND DISCUSSION**

The irreversible inhibition of papain by a molar equivalent of 1,3-dibromoacetone at pH 5-6 was rapid and complete. The molecular weight of the inhibited enzyme, as determined on a column of Sephadex G-75, was indistinguishable from that of active papain and there was no evidence of dimeric or oligomeric species (Fig. 1). The optical-rotatory-dispersion curves of chloroacetone-inhibited papain and 1,3-dibromoacetone-inhibited papain were also essentially similar.

The amino acid analyses of papain and 1,3-dibromoacetone-inhibited papain are compared in Table 1. The loss of one histidine residue in the inhibited enzyme is evident. The loss of a half-cystine residue is not convincingly demonstrated owing to its instability during acid hydrolysis. The basic amino acid analyses of papain treated with 1,3-dibromoacetone for 10 and 60 min. are compared with that of the native enzyme in Table 2. These analyses show that the alkylation of the histidine residue is complete in less than 10 min.

To identify histidine positively as one of the alkylated amino acid residues and to determine the site of alkylation (i.e. N-1 or N-3), papain inhibited with 1,3-dibromo[\(2,14C\)]acetone was oxidized with performic acid and hydrolysed. The hydrolysate was applied to the Technicon AutoAnalyzer and the column eluted with the buffer system of Thomson & Miles (1964). The eluate from the column not required by the analytical system was collected and
Table 1. Amino acid analyses of papain and 1,3-dibromoacetone-inhibited papain

The amino acid analyses were performed in triplicate and averaged. The protein was assumed to contain 10 leucine residues (Light et al. 1964).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Papain</th>
<th>Inhibited papain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>17.5</td>
<td>18.0</td>
</tr>
<tr>
<td>Thr</td>
<td>7.2</td>
<td>7.2</td>
</tr>
<tr>
<td>Ser</td>
<td>10.8</td>
<td>11.1</td>
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<tr>
<td>Glu</td>
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<tr>
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<td>14.2</td>
<td>14.5</td>
</tr>
<tr>
<td>Cys</td>
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<td>4.1</td>
</tr>
<tr>
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<td>1.1</td>
</tr>
<tr>
<td>Arg</td>
<td>10.9</td>
<td>10.5</td>
</tr>
</tbody>
</table>

The radiochromatogram shown in Fig. 2 determined. The radioactivity found in fractions 10–40 was due to S-carboxymethylcysteine sulphone or its acid-catalysed degradation products (or both). The only other radioactive peak in the whole amino acid chromatogram emerged on the front edge of the alanine peak. To identify this peak, N\textsuperscript{\textdegree}-acetylhistidine was alkylated with iodoacetic acid as described by Crestfield, Stein & Moore (1963). The mixture of amino acids was run on the Technicon AutoAnalyzer. Four peaks emerged: one was unchanged histidine and the remaining three were identified by their relative positions in the chromatogram and the ratio of areas of the peaks, when compared with the data of Crestfield et al. (1963). 1,3-Bis(carboxymethyl)histidine was unresolved from S-carboxymethylcysteine, 1-carboxymethylhistidine from alanine and 3-carboxymethylhistidine from cystine. The radioactive peak eluted with alanine (Fig. 2) can therefore be identified as 1-carboxymethylhistidine; the chromatogram shows no evidence for 3-carboxymethylhistidine.

These results provide direct evidence for the presence of an imidazole group of a histidine residue within a range of \(5\lambda\) from the active-site thiol group, thus supporting the proposal made on the basis of kinetic studies that such a residue was involved in the mechanism of action of papain. Attention has already been drawn to the similarity of the amino acid sequence around cysteine-25 in papain and the active-site serine residue of trypsin (Lowe, 1966). It may well be therefore that the mechanism of action of the serine and cysteine proteinases is very similar. If this is so then the thiol and imidazole groups are probably hydrogen-bonded and act in concert.

The pH-dependence for the acylation of papain by all substrates studied is bell-shaped with apparent \(pK_1\) between 3.9 and 4.3 and \(pK_2\) between 8.0 and 8.5 (Whitaker & Bender, 1965, and references cited therein). The alkylation of papain by the chloromethyl ketone derived from \(N\)-toluene-p-sulphonyl-L-phenylalanine (TPCK), however, shows sigmoid pH-dependence with apparent \(pK\) 8.25, the rate of inhibition rising with pH (Bender & Brubacher, 1966). The alkylation of papain by \(L(-)\)-\(\alpha\)-iodopropionamide also shows sigmoid pH-dependence with apparent \(pK\) 7.99 (Wallenfels & Eisele, 1968). The inhibition of papain by \(L(-)\)-\(\alpha\)-iodopropionic acid, however, shows bell-shaped pH-dependence with apparent \(pK\) 4.0 and 7.8.

Table 2. Basic amino acids in the hydrolysates of papain and 1,3-dibromoacetone-inhibited papain

The protein was assumed to contain 9 lysine residues (Light et al. 1964). The amino acid lost is indicated by the italicized values.
If the thiol and imidazole groups are hydrogen-bonded in the active enzyme, the thiol group must be the hydrogen donor since sulphur is unable to accept hydrogen bonds. The pK for the protonation of such a hydrogen-bonded system would be lower than that of the free imidazole group. The apparent pK 4.0 is quite compatible with such a system. It is not possible to identify the proton lost from the thiol–imidazole system on deprotonation, but the observed apparent pK is between that of a free thiol or imidazolium ion, as might be expected (Fig. 3). All the available pH-dependence data for the inhibition and mechanism of action of papain can be interpreted in terms of this system, and it is unnecessary to invoke the participation of a carboxylic acid group.

The bell-shaped pH-dependence observed for the inactivation of papain by L(−)-α-iodopropionic acid, together with the fact that the limiting second-order rate constant is 25 times that for the reaction of the anionic thiolate–imidazole system of papain with L(−)-α-iodopropionamide, suggests that L(−)-α-iodopropionate must be quite strongly attracted to the active enzyme. The high-pH limb of the bell-shaped profile is readily interpreted as a repulsion of the negatively charged inhibitor by the thiolate–imidazole system, whereas the low-pH limb is expected owing to the lack of sufficient nucleophilicity in the free thiol group. By hydrogen-bonding, the thiol–imidazole system is polarized, which adds to its nucleophilicity and its attraction for L(−)-α-iodopropionate.

The second-order rate constant, as well as the energy and entropy of activation, are very similar for the alkylation of papain and cysteine by L(−)- and D.L.-α-iodopropionamide respectively (Wallenfels & Eisele, 1968). It therefore appears that there is little or no attraction between papain and L(−)-α-iodopropionamide and consequently the thiolate–imidazole system is required for alkylation to occur.

The selective alkylation of N-1 of the active-site imidazole group of papain by 1,3-dibromoacetone suggests that its orientation with respect to the thiol group is constrained, presumably with N-3 directed towards the thiol group, as indicated in Fig. 3.

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REFERENCES