Reaction of Papain with α-Bromo-β-(5-imidazolyl)propionic Acid*

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ABSTRACT: 5-Vinylimidazole, 5-(2-bromoethyl)imidazole, D,L-α-chloro-β-(5-imidazolyl)propionic acid, and D,L-α-bromo-β-(5-imidazolyl)propionic acid were tested as reagents for synthetic introduction of an imidazole at the active site of papain. Only D,L-α-bromo-β-(5-imidazolyl)propionic acid (BIP) was found to react. At pH 8.0 with a 50-fold molar excess of BIP the half-time of inactivation of the enzyme was 6.5 min. The apparent second-order rate constant for the process follows a bell-shaped pH dependence with maximal values in the vicinity of pH 6.0–8.0. The catalytic sulfhydryl group (cysteine-25) was demonstrated to be the site of alkylation by loss of sulfhydryl titer from the modified enzyme and a corresponding incorporation of 0.6 mole of ^{14}C reagent/mole of protein. The α-carboxyl group of the reagent appears to be essential since 5-(2-bromoethyl)imidazole was ineffective. A proposal is made to explain the reactivity patterns of alkylating agents toward papain in terms of the disposition of groups in the catalytically active form of the enzyme. The modified enzyme contains, in addition to the imidazole at histidine-159, a second synthetically introduced imidazole at residue 25. The modified site should in part resemble the catalytic site of ribonuclease A since it contains two proximal imidazoles, however it lacks activity toward either RNA or cyclic 2′,3′-cytidylic acid. The study shows that BIP has potential as a reagent for exploring reactivity patterns of sulfhydryl groups as well as for introducing imidazole groups into proteins.

The sulfhydryl group of papain located at cysteine-25 of the primary sequence has been extensively studied and implicated in the catalytic activity of this proteinase (Glazer and Smith, 1971). X-Ray diffraction studies (Drenth *et al.*, 1970) as well as chemical studies (Husain and Lowe, 1968) have also implicated an imidazole (histidine-159) in the catalytic activity. These groups lie in a cleft on the enzyme's surface which forms the active site (Drenth *et al.*, 1970).

The reactivity of the single SH group of this enzyme has been explored by a variety of haloacetic acids and their derivatives (Arnon, 1970). Chloroacetic acid, for example, has been found to be 30,000 times more reactive to this group than to the SH group of cysteine at pH 6.0 (Sluyterman, 1968). The carboxyl group of α-halo acids appears to have a specific function in enhancing and altering the pH profile of reactivity of these agents (Chaiken and Smith, 1969b; Wallenfels and Eisele, 1968), although the phenomenon is not fully understood.

Ribonuclease, in contrast to papain, is an enzyme which has two imidazole groups at its active site (histidines-12 and -119; Heinrikson *et al.*, 1965). The imidazole function of histidine has also been implicated in the catalytic activity of a host of other enzymes. It is, therefore, of importance to explore the microenvironmental requirements for these cooperative effects in enzyme catalysis. One approach to evaluating these requirements is to arrange functional groups on a preformed template and study these arrangements for catalytic behavior. Accordingly, this study utilizes the active site of papain as a preformed template and α-bromo-β-(5-imidazolyl)propionic acid and related compounds as alkylating agents in a first attempt to convert an active site containing an imidazole and an SH group to a site containing two imidazole groups. Study of these reagents also supplies additional information regarding the reactivity profile of the SH group of papain.

Materials and Methods

The following list gives the sources of commercially obtained materials: acetonitrile, Eastman Chemical Co.; *N*-benzoyl-L-arginine ethyl ester, *N*-carbobenzoxylglycine-*p*-nitrophenyl ester, 2′:3′-cyclic cytidinemonophosphoric acid (sodium salt), EDTA, histamine-free base, *p*-hydroxymercuribenzoate, mercaptoethanol, urocanic acid, RNA from yeast (type XI), and papain (twice crystallized), Sigma Chemical Co.; D,L-histidine monohydrate, Mann Research Laboratories: D,L-histidine-2-^{14}C, New England Nuclear Corp.; 48% hydrobromic acid (redistilled before use), Matheson Coleman & Bell; 1,4-bis[2-(4-methyl-5-phenyloxazole)]benzene, 2,5-diphenyloxazole, Packard Instrument Co.; Triton X-100, Rohm-Haas Corp.; Sephadex G-25 (medium), Pharmacia Fine Chemicals, Inc.; Linde Molecular Sieves (Type 3A pellets), Linde Division, Union Carbide Corp.; dithiothreitol, Calbiochem. Other reagents were generally reagent grade. HBr (48%) was redistilled before use and distilled deionized water was used throughout the study. Microanalyses were done by Galbraith Laboratories, Knoxville, Tenn. Amino acid determinations were done on a Model NC-1 Technicon Analyzer.

Two procedures were used to activate papain. In the early phases of the study the enzyme was activated as described by Morihara (1967) but using ten times the amount of mercaptoethanol. In other experiments dithiothreitol (DTT)[1] was used

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[1] Abbreviations used are: BIP, D,L-α-bromo-β-(5-imidazolyl)propionic acid; CIP, D,L-α-chloro-β-(5-imidazolyl)propionic acid; BEI,

under the following typical conditions: 0.1 ml of papain (1.43×10^{-3} M), 0.1 ml of 0.1 M EDTA, 0.2 ml of water, and 0.1 ml of DTT (1.71×10^{-2} M) in pH 6.0, 0.2 M acetate buffer were incubated at 37° for 5 min. Under either set of conditions papain remains fully active for 40 min. In all cases kinetic measurements were limited to the first 30 min after activation. Concentrations of papain were determined spectrophotometrically using $\epsilon_{280\ nm}$ of 5.1×10^4 M^{-1} cm^{-1} and mol wt of 21,000 (Light *et al.*, 1964).

Assay of Papain Activity. A modification of the assay procedure of Kirsch and Ingleström (1966) was employed. One milliliter of Z-Gly-*p*-nitrophenyl ester (7.58×10^{-4} M in 30% acetonitrile) and 0.25 ml of 0.2 M acetate buffer (pH 6.0) were placed in one side of a split-compartment cuvet (Yankeelov, 1963). The other compartment contained activated enzyme and acetate buffer to a total volume of 1.25 ml. After equilibration in the spectrophotometer at 37° the $A_{340\ nm}$ of the unmixed cuvet was adjusted to zero. The cuvet was mixed and the $A_{340\ nm}$ recorded at 10-sec intervals for 2 min. Initial velocities were calculated from the slope of the resulting plot of $A_{340\ nm}$ against time and an $\epsilon_{340\ nm}$ of 5.5×10^3 M^{-1} cm^{-1} for the *p*-nitrophenolate ion (Perez-Villaseñor, 1967). The observed rates were corrected for nonspecific hydrolysis by buffer. The overall character of the kinetic results were verified using a titrimetric assay with benzoyl-L-arginine ethyl ester (Smith and Parker, 1958).

Isotopically labeled (^{14}C) reagents and protein were determined using a Packard Tri-Carb liquid scintillation counter Model 3380. The glass counting vials contained 1.0 ml of aqueous sample and 10 ml of scintillator solution containing 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazole)]benzene. Labeled reagent concentration was determined by a modified Pauly method (Ray, 1967). The maximum $A_{510\ nm}$ and an $\epsilon_{510\ nm}$ of 2.78×10^4 M^{-1} cm^{-1} was used to calculate concentrations of BIP-α-^{14}C. The nuclear magnetic resonance (nmr) spectrum of 5-(2-bromoethyl)-imidazole was obtained on a Varian T-60 spectrometer. Thin-layer chromatography was performed on cellulose foils or glass plates using butanol–acetic acid–water (12:3:5, v/v) as developer unless otherwise noted.

Papain was separated from excess activators and inhibitors on a Sephadex G-25 column (1.5 × 44 cm). Ammonium acetate (0.1 M), pH 6.8 (Klein and Kirsch, 1969), or sodium acetate–acetic acid, pH 5.0 buffer (0.2 M in acetate), was eluent. The sulfhydryl group of papain was determined with *p*-mercuribenzoate (Boyer, 1954; Benesch and Benesch, 1962) after removal of activator. Half-cystine was determined by performic acid oxidation according to the method of Hirs (1956). Protein was hydrolyzed as described by Moore and Stein (1963). Ribonuclease activity was determined with RNA as substrate by the method of Kalnitsky *et al.* (1959) and with cytidine 2′:3′-cyclic monophosphate as substrate according to Hummel *et al.* (1958).

The pH dependence of the apparent second-order rate constants ($\bar{k}_2$) of alkylation was studied using a 50-fold molar excess of BIP and papain concentrations of 7.5×10^{-5} to 7.0×10^{-4} M. Inactivations were performed at 37° in two buffer systems. For pH 3.5–5.5, a sodium acetate–acetic acid system (0.2 M in acetate) was used. For pH 6.0–10.0, a wide-range buffer (Gerwin, 1967) was employed. The wide range buffer was adjusted to the desired pH by adding 6 N NaOH to 50 ml of stock solution which was 2 mM in EDTA and 0.1 M each in boric acid, sodium acetate, and monosodium phosphate. The ionic strength was adjusted by addition of 1 M NaCl so that upon dilution to 100 ml, $\mu = 0.19$. Second-order rate constants ($\bar{k}_2$) were calculated according to eq 1 which treats the reaction as pseudo first-order and also according to eq 2 which takes into consideration reagent depletion due to alkylation of enzyme. In eq 1 A_0 and A represent initial enzymatic velocities at time zero and time t, respectively. In eq 2 EI, E_0, and I_0 represent the concentration

$$\bar{k}_2 = \frac{2.303 \log A_0/A}{[I_0]t} \quad (1)$$

$$\bar{k}_2 = \frac{2.303}{((I_0) - (E_0))t} \log \frac{(E_0)((I_0) - (EI))}{(I_0)((E_0) - (EI))} \quad (2)$$

of inactivated enzyme, and initial concentration of native enzyme, and inhibitor, respectively.

D,L-α-*Chloro-β-(5-imidazolyl)propionic acid* (*CIP*) *and* D,L-α-*bromo-β-(5-imidazolyl)propionic acid monohydrate* (*BIP*) were prepared as previously described (Yankeelov and Jolley, 1972).

D,L-α-*Bromo-β-(5-imidazolyl)propionic Acid-α-*^{14}C. Radiolabeled BIP was prepared by the following modification of the original procedure (Yankeelov and Jolley, 1972). D,L-Histidine-*2-*^{14}C (5 mg, specific activity 50 mCi/mmole) was dissolved in 0.5 ml of redistilled 48% HBr and the solution was chilled in an ice-salt bath to below 0°. A tenfold excess of sodium nitrite (24.4 mg in 0.1 ml of water) was added in 10-μl portions with stirring over a 45-min period so as to avoid significant heating of the solution. Stirring was continued for 1 hr at 0° after the addition was complete. The dark solution was evaporated to dryness under reduced pressure and 100 mg of analytical quality carrier BIP was added. After two crystallizations from 0.5 ml of water (60°), the sample (specific activity 4.89×10^{-2} mCi/mmole) migrated in thin-layer chromatography as a single radioactive, Pauly-positive component (R_F 0.53). A second sample of BIP-α-^{14}C (specific activity 1.54×10^{-2} mCi/mmole) was also prepared and used in labeling studies with comparable results.

5-(2-Bromoethyl)imidazole Hydrochloride (*BEI-HCl*). Histamine, free base (1 g, 8.9 mmoles), was dissolved in 25 ml of 48% HBr (redistilled). The solution was cooled to 0° in an ice-salt bath and a 20-fold molar excess of $NaNO_2$ in 20 ml of water was added dropwise with stirring while keeping the temperature below 5°. The mixture was stirred for 60 min at 0° after the addition was complete and was brought to dryness at reduced pressure while gradually raising the temperature to 60°. The resulting mass was extracted with acetone and residual salt removed by filtration. The acetone solution was concentrated under reduced pressure to a yellow oil which was dissolved in 50 ml of water. An equal volume of 0.1 M, pH 9.0 borate buffer was added and the solution was extracted with ether. The extract was dried over Type 3A Molecular Sieves and the ether removed. The residue was dissolved in 1 ml of ethanol–ether (1:10, v/v). The solution was saturated with dry HCl and BEI·HCl precipitated. After drying at 60 μ over P_2O_5 at 50° the white solid (0.49 g, 26%, mp 105–107°) migrated as a single Pauly-positive spot in thin-layer chromatography (R_F 0.70). Nmr parts per million downfield from acetone as reference peak showed 6.46 (1 H), 5.20 (1 H), 1.50 (2 H), 1.12 (2 H). *Anal.* Calcd for $C_5H_8BrClN_2$: C, 28.39; H, 3.81; Br, 37.78; Cl, 12.03; N, 13.24. Found: C, 27.13; H, 3.51; Br, 38.81; Cl, 13.87; N, 12.25; ash, 1.16.

5-(2-bromoethyl)imidazole; DTT, dithiothreitol; Z, benzyloxycarbonyl; BAEE, benzoyl-L-arginine ethyl ester; IP, imidazolylpropionate; IP-papain, papain modified with BIP.

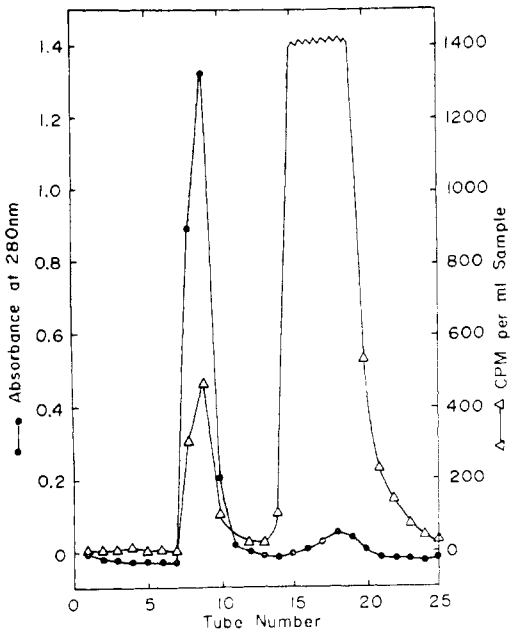


FIGURE 1: Sephadex G-25 (medium) chromatography of reaction mixture of BIP-α-^{14}C-treated papain. Papain (7.7 mg/ml) was treated for 30 min with a 50-fold excess of BIP (1.54×10^{-2} mCi/mmole) at 37°. One milliliter of the reaction mixture was applied to a 1.5×44 cm column of Sephadex G-25 which had been equilibrated with 0.1 M, pH 6.8 ammonium acetate buffer. The column was eluted with the same buffer at 75 ml/hr. Fractions of 4 ml were collected and 1.0 ml aliquots were taken for scintillation counting (●, A_{280} nm; △, cpm/ml).

5-Vinylimidazole. This compound was prepared from urocanic acid (Overberger and Vorchheimer, 1963) and was obtained in 35.9% yield (lit. 36%); mp 83.5–85.0° (lit. 83.2–84.5°); $\epsilon_{243\ nm}$ 12,590 (lit. 12,600).

Results

Behavior of 5-(2-Bromoethyl)imidazole and Related Compounds with L-*Cysteine.* Before evaluating the behavior of 5-(2-bromoethyl)imidazole with papain it was of interest to examine its reactivity toward cysteine, which was done in the following way. An aqueous solution of BEI·HCl (0.01 M) and L-cysteine·HCl·$0.5H_2O$ (0.01 M) was brought to pH 8.0 with 0.5 M NaOH. The pH was maintained at 8.0 for 2 hr (25°). Thin-layer chromatography after 60, 90, and 120 min revealed progressive formation of a ninhydrin- and Pauly-positive product of R_F 0.19. A parallel reaction was performed for a period of 90 min (25°) substituting cysteine in 100-fold molar excess (1.0 M). After reaction under the latter conditions (which avoids polymerization of alkylating agents) amino acid analysis revealed only one product, a basic amino acid, which emerged 90 min after arginine from the 130-cm column in 93% yield (assuming the ninhydrin constant to be that of histidine). This product could also be separated from residual reactants by "desalting" on AG 50W-X2 resin using a 1–8 M HCl gradient. The Pauly-positive amino acid was concentrated to a syrup and finally lyophilized from water. Reduction of the purified amino acid with Raney nickel produced a ninhydrin-positive component with the same mobility as alanine as well as a new Pauly-positive component (presumably 5-ethylimidazole) with the same mobility as BEI. These experiments demonstrate that BEI is cysteine reactive and the properties of the product are consistent with formation of 5-(2-(*S*-L-cysteinyl)ethyl)imidazole. Under comparable conditions α-chloro-β-(5-imidazolyl)propionic acid and 5-(vinyl)imidazole did not react with cysteine. The reaction of BIP with L-cysteine was studied previously (Yankeelov and Jolley, 1972).

Site of Reaction of BIP. Although papain is known to react specifically with a number of reagents at its essential sulfhydryl group (Finkle and Smith, 1958; Sluyterman, 1968; Wallenfels and Eisele, 1968), the observation that a specific tyrosine residue (123) of activated papain is reactive to diisopropyl fluorophosphate (Chaiken and Smith, 1969c) made it desirable to determine the site of alkylation with BIP independently. Accordingly, several properties of the modified protein were examined. Activated papain was allowed to react with a 100-fold excess of reagent for 30 min at pH 6.0, 37°. At least 95% of its esterase activity was abolished as determined by Z-Gly-*p*-nitrophenyl ester assay. In the titrimetric assay with BAEE as substrate no base uptake was observed for the modified protein over a period of 24 min. A control sample of active papain produced a 40% hydrolysis of BAEE under identical conditions. Previous study of the behavior of BIP with free amino acids at pH 8.0 or 6.0 revealed its reactivity was restricted to cysteine (Yankeelov and Jolley, 1972). Furthermore, when mercaptoethanol–EDTA-activated papain was separated from excess reagents by Sephadex G-25 chromatography, the active enzyme had 0.50–0.78 mole of SH group/mole of protein as measured with *p*-mercuribenzoate. This content of SH group for active enzyme is consistent with earlier reports[2] (Finkle and Smith, 1958; Sluyterman, 1967). IP-papain under the same conditions gave values of 0.22–0.29 mole of SH group/mole of protein. The observation is reminiscent of residual titers of 0.18 and 0.15 SH group per mole of papain inactivated with L-1-chloro-3-tosylamido-7-amino-2-heptanone and L-1-tosylamido-2-phenylethyl chloromethyl ketone, respectively (Perez-Villaseñor, 1967) and may suggest a reaction of nonactivatable papain with the mercurial.

Amino acid analyses of acid-hydrolyzed IP-papain using a modified elution gradient (Yankeelov and Jolley, 1972) showed the presence of the expected pair of diastereoisomers but accounted for only 0.20 residue of derivatives or about 35% of the amount expected from the radiolabeling study (*vide infra*). This low yield of derivatives was surprising since the free amino acid derivatives are about as stable as serine to hydrolytic conditions (Yankeelov and Jolley, 1972). Perhaps this yield may be improved by adding an appropriate scavenger to the hydrolysis mixture or changing the conditions of acid hydrolysis (Liu and Chang, 1971). Amino acid analyses of performic acid oxidized IP-papain revealed 5.6 residues of cysteic acid while unmodified protein yielded 6.5 residues. Both determinations were based on the alanine content of the hydrolysates of oxidized protein.

Radiolabeling with BIP-α-^{14}C. When mercaptoethanol–EDTA-activated papain treated with BIP-α-^{14}C at pH 8.0, 37° for 30 min was subjected to Sephadex G-25 chromatography, labeled protein separated from excess reagent as shown in Figure 1. Fractions 8 and 9 were found to contain 0.59 and 0.61 mole of IP-α-^{14}C incorporated per mole of papain. The remainder of fractions 8 and 9 were pooled and tested for trichloroacetic acid soluble counts after treatment with Raney nickel. Only 3% of the radioactivity was released. Apparently the active-site cleft which contains the modified cysteine is

[2] When this work was essentially complete methods for separating papain with 1.0 SH group/mole of enzyme appeared (Blumberg *et al.*, 1970; Sluyterman and Wijdenes, 1970).

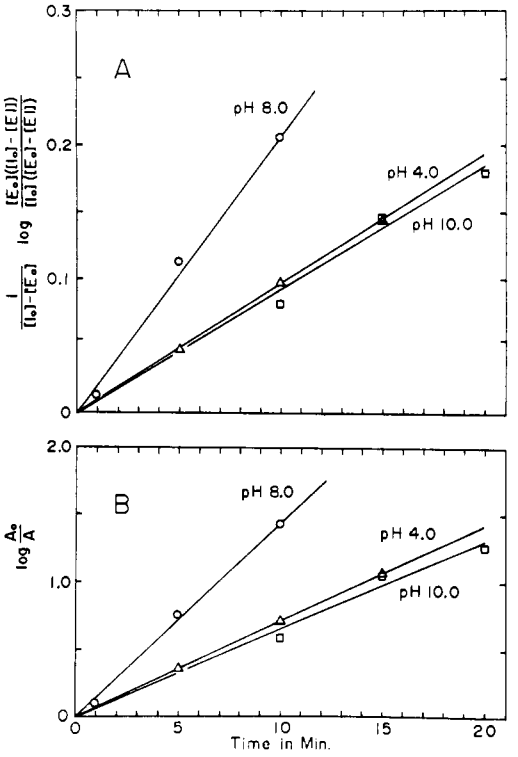


FIGURE 2: Second- and first-order plots of BIP inactivation of papain at 37°. Panel A: second-order plot at three pH values. Panel B: corresponding first-order plots. BIP concentration was 7.14×10^{-3} M. Enzymatic activity was determined spectrophotometrically with Z-Gly-*p*-nitrophenyl ester as substrate (Δ, pH 4.0, ○, pH 8.0; □, pH 10.0). The solid lines were drawn to fit the experimental data.

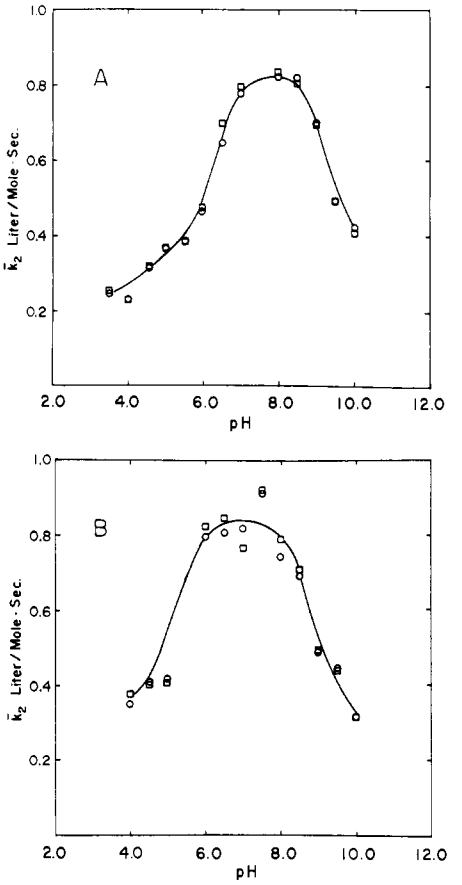


FIGURE 3: Variation of apparent second-order rate constants with pH for the inhibition of activated papain by 50-fold molar excess of BIP at 37°. Panel A: papain activated with mercaptoethanol–EDTA; panel B: papain activated with DTT–EDTA. Details found in Materials and Methods. (○, $\bar{k}_2$ calculated from eq 1; □, $\bar{k}_2$ calculated from eq 2). The solid curves were drawn to fit the experimental data.

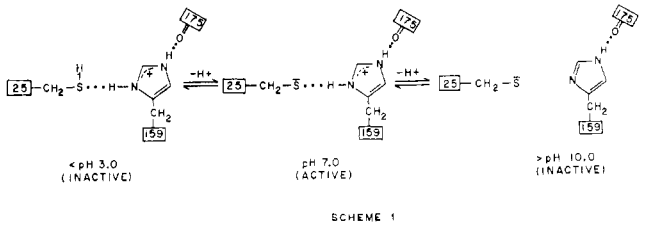
not as accessible to the catalyst as the aminoacyl group of Cys-tRNA^{Cys} (Chapeville *et al.*, 1962).

Kinetics of Alkylation of Papain by α-Bromo-β-(5-imidazolyl)propionic Acid. The reaction of either mercaptoethanol- or DTT-activated papain with BIP followed pseudo-first-order kinetics and true second-order kinetics to at least 70% completion with a 50-fold excess of alkylating agent. Examples of these plots at several pH values are given for DTT-activated enzyme in Figure 2. Similar data were obtained for mercaptoethanol-activated enzyme.

pH Dependence of Alkylation with BIP. The pH dependence of the alkylation rate was studied at ionic strength 0.19 at 37°. Figure 3A shows the pH profile of $\bar{k}_2$ dependence of mercaptoethanol-activated enzyme while Figure 3B shows the corresponding profile for DTT-activated enzyme. Although the pH range for maximal values of $\bar{k}_2$ is broadened and shifted somewhat to lower pH in the case of DTT-activated enzyme, the bell-shaped curve is clearly a characteristic of BIP rather than conditions of activation. The maximal $\bar{k}_2$ for enzyme activated with a high concentration of mercaptoethanol (>100-fold excess) was near pH 8.0 while a corresponding region of pH 6.0–8.0 was observed with enzyme activated with minimal amounts of DTT (12-fold excess). Quantitative evaluation of these profiles in terms of pK's of ionizable groups on the enzyme are of questionable value because of the complicating presence of an imidazole (pK_a = 7.0) in the reagent itself which ionizes over the range of interest. The results, however, are indicative of a dominant role for the carboxylate group of BIP. Wallenfels and Eisele (1968) and Chaiken and Smith (1969b) have observed similar pH dependences for the reactions of papain with α-iodopropionic acid and chloro- and bromoacetic acids, respectively. Gerwin first observed this type of pH dependence for the action of chloroacetate on the SH enzyme streptococcal proteinase (Gerwin, 1967). The lack of reactivity of 5-(2-bromoethyl)imidazole (decarboxylated BIP) and 5-vinylimidazole with papain further points up the importance of the carboxyl group of the reagent for enhancing reactivity with the enzyme.

Discussion

Disposition of Groups at the Active Site. Papain reacts rapidly with α-bromo-β-(5-imidazolyl)propionic acid to produce a modified enzyme containing an imidazolylpropionate group bound through its α-carbon atom to the cysteine-25 sulfur atom of the active site. The pH dependence of the $\bar{k}_2$ of this reaction is bell shaped. This behavior is also characteristic of reactions of papain with chloroacetic acid (Chaiken and Smith, 1969b) and L-α-iodopropionic acid (Wallenfels and Eisele, 1968). While the two simpler reagents are maximally reactive at *ca.* pH 6.0, BIP is maximally reactive at *ca.* pH 7.0, being also somewhat dependent on the procedure used for activating the enzyme. It is likely that this displacement is due to the introduction of an imidazole group into the reagent, but interpretation is complicated because the reagent is racemic. The detailed character of the pH profile may in fact reflect a dominant reaction of one of the two enantiomers. The L isomer of iodopropionic acid, for example, was found



SCHEME I

to be 12 times more reactive to papain than the D isomer; the latter was largely featureless in its pH dependence (Wallenfels and Eisele, 1968). It is likely, therefore, that a stereoselective reaction is also occurring in the case of BIP. The results obtained here, however, clearly reveal that the reactivity of BIP to papain near neutral pH is dependent on the carboxyl group and not the imidazole since decarboxylated BIP (5-(2-bromoethyl)imidazole) was unreactive over the same pH range. This general requirement for a carboxyl group in alkylating agents effective for papain near neutral pH has an important bearing on the disposition of groups at the active site and may be interpreted with more confidence in the light of recent studies of both the primary and three-dimensional structure of the enzyme (Glazer and Smith, 1971).

Husain and Lowe (1968) have proposed a catalytic mechanism for papain which invokes an unusually low pK (*ca.* 4.0) for the imidazole of histidine-159 to account for the acid limb of the pH–activity profile of the enzyme. Chaiken and Smith (1969b) have already emphasized that the pH range for maximal rates of alkylation of papain with chloroacetic acid parallels the range of maximal enzymatic activity for most substrates including hippurylamide and N-acylated arginine esters and amides. While the mechanism suggested by Husain and Lowe (1968) does in part correlate pH profiles of alkylation and enzymatic activity, emphasis is placed on an *imidazole* with an unusually low pK and hydrogen bonding of the *unionized thiol* group is discounted (Lowe, 1970). An alternate mechanism (Sluyterman and Wolthers, 1969), which requires a protonated imidazole for activity, does not explain the acid limbs of pH profiles in terms of *catalytic* groups. The proposal which we advance here differs from the previous two suggestions in that it accounts for both the profile of proteolytic activity and special reactivity patterns of papain with α-haloacids and amides in terms of *catalytic* groups while emphasizing a low pK for the *thiol* group as a result of hydrogen bonding. The suggestion is made, of course, on the assumption that the structural information determined by X-ray diffraction studies is applicable to considerations of the enzyme in solution. The crystallographic studies (Drenth *et al.*, 1970) as well as chemical studies (Husain and Lowe, 1968) indicate that the sulfur atom of cysteine-25 is within hydrogen-bonding distance of N-1 of the imidazole of histidine-159.

Scheme I shows progressive changes in interactions between the imidazole and mercaptan of the catalytic site as the pH is increased. At pH 3.0 both cysteine-25 and histidine-159 are protonated. Under these conditions sulfur alone can act as acceptor in hydrogen-bond formation. Consequently, it is the SH group, rather than the imidazole, which would be expected to have an abnormally low pK.[3] Loss of this proton produces the active form of the enzyme in which the sulfur anion is tightly bound in a hydrogen bond with the protonated imidazole. In this constellation the mercaptide ion is anomalously low in reactivity to uncharged agents such as chloroacetamide (Chaiken and Smith, 1969a), but unusually reactive to anions of α-halo acids. Binding of the carboxylate anion to the imidazolium ion properly orients the α carbon with respect to the sulfur while also releasing nucleophilicity of the latter. In catalytic action the imidazolium group of the active form of Scheme I would be ready to participate in general acid catalysis while concommitantly releasing the sulfur anion for attack on a peptide carbon. Obviously, the behavior of any given alkylating agent is altered as additional binding groups are introduced until considerable deviation from this simple picture may result with complex reagents. At high pH, the catalytic site finally loses a proton from its imidazole so that special reactivity is lost to α-halo acids. By the same process the mercaptide ion resumes reactivity more nearly in accord with that of model compounds. Although sulfur is often considered to be too low in electronegativity to participate effectively in hydrogen bonding, recent spectroscopic evidence indicates that the intramolecular hydrogen bond, NH···S, in *o*-aminothiophenol is, in fact, stronger than the corresponding hydrogen bond, NH···O, in *o*-aminophenol (Krueger, 1970). Thus, the anomalous reactivity profiles of the SH group of papain as well as the activity profile of the enzyme can be understood in terms of hydrogen bonding at the catalytic site. It may be expected that similar considerations will be useful in understanding reactivity profiles of other SH enzymes.

Nature of the Modified Enzyme. In addition to extending generalizations of the reactivity of α-halo acids with papain and provoking an explanation to account for this phenomenon, the present study demonstrates what our previous study (Yankeelov and Jolley, 1972) suggested. Specifically, α-bromo-β-(5-imidazolyl)propionic acid has potential for both exploring the chemical reactivity of sulfhydryl groups at active sites and specifically introducing imidazole groups into proteins.

Evaluation of geometry requirements for catalytic groups in enzymes is a challenging problem which has been approached from a number of points of view (Jencks, 1969; Cramer and Mackensen, 1966; VanderJagt *et al.*, 1970; Klotz *et al.*, 1971; Morawitz, 1970) and a controversy exists as to the criticality of alignment of groups during catalytic action (Storm and Koshland, 1970; Bruice *et al.*, 1971). The present work represents a first attempt at changing a proteolytic enzyme containing an SH group and an imidazole at its catalytic site to an enzyme containing two imidazoles. The disposition of the two imidazoles may to a first approximation resemble histidines-12 and -119 of ribonuclease A (Heinrikson *et al.*, 1965). Papain is particularly well suited to this study because its tertiary structure is unusually stable, being resistant to heat, organic solvents, and 8 M urea (Arnon, 1970). Introduction of an imidazole at its active site, accordingly, is unlikely to result in a serious conformational change. In the present study the catalytic potential of the modified enzyme was explored in a preliminary way with respect to 2′:3′-cytidine monophosphate and ribonucleic acid as potential substrates. No catalytic activity was detected. However, this is not totally unexpected since the binding sites of papain are not likely to accommodate these substrates. In order to adequately test this and related modified sites of papain for catalytic behavior, it may be necessary to design and incorporate phosphate ester or related linkages into peptide substrates that are known to be bound at appropriate subsites (Berger and Schecter, 1970).

[3] The hydrogen bond of Asn-175 to N-3 of His-159 (Wolthers *et al.*, 1970) would be expected to increase the pK of this imidazole rather than decrease it.

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