Ionization of a Nitrophenol-Containing Reporter Group at the Active Site of Papain†

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ABSTRACT: In order to obtain more information about the nature of the environment around the active site in papain, a derivative of papain (papain-R) was prepared in which the thiol group at the active site of papain was alkylated with 2-bromoacetamido-4-nitrophenol (**1**). The equivalent yields of 2-amino-4-nitrophenol and *S*-carboxymethylcysteine obtained on acid hydrolysis of papain-R established the thiol group of Cys-25 as the only site of reaction of **1** with papain. The apparent second-order rate constant for the reaction of **1** with papain at 25° is 20 sec^{-1} M^{-1} at pH 4.62 and 15 sec^{-1} M^{-1} at pH 7.20. At pH 4.62, **1** reacts 3200 times faster with the thiol group in papain than with the thiol group in L-cysteine. The pK of the nitrophenol-containing reporter group at the active site of papain is 0.87 unit higher than expected from studies of the ionization of a simple model compound, 2-β-hydroxyethylthioacetamido-4-nitrophenol (**2**). However, the absorbance maximum of the nitrophenolate anion in both papain-R and **2** appears at 410 nm. Since an apolar environment increases both the pK and the position of the absorbance maximum of the reporter group, it was concluded that the perturbation in pK of the reporter group at the active site of papain is caused by the reporter group being in a negatively charged, rather than in an apolar environment. Deprotonation of a second ionizable group on the protein perturbs the spectrum and/or the pK of the reporter group. The pK of this second group is about 10, when the reporter group is deprotonated. By setting reasonable limits on the magnitude of the spectral perturbation, it was possible to show that the pK of this second group might be as low as 7.21 when the reporter group is protonated and that deprotonation of the second group may cause the pK of the reporter group to rise from a value of about 6.8 to a value which may be as high as 9.58.

The catalytic activity of papain is tightly linked to the state of ionization of two groups with apparent pK's of about 4.1 and 8.3. The evidence implicating these groups in the catalytic cycle has been recently reviewed by Glazer and Smith (1971). Both groups, one in the protonated and the other in the deprotonated form, appear to be required for the acylation step in the catalytic pathway. In this step, a thiol ester is formed between the acyl portion of the substrate and the active site thiol group of Cys-25. Hydrolysis of the acyl enzyme completes the catalytic cycle. Usually, hydrolysis of the acyl enzyme appears dependent on the group with a pK of 4.1 being deprotonated. The group with the pK of 8.3 has been associated with the thiol group of Cys-25. This assignment is based mainly on the following observations: (a) The pK = 8.3 ionization is not seen in the deacylation step where ionization of the thiol group is not possible. (b) The heat of ionization of the pK = 8.3 group (Smith and Parker, 1958) is similar to that expected for organic thiols. (c) The thiol group is required for activity. (d) The pH dependence of the rate of alkylation of the thiol group by uncharged alkylating agents, *e.g.*, chloroacetamide (Chaiken and Smith, 1969), indicates the thiol group of Cys-25 has a pK of between 8 and 9.

The identity of the group having a pK of about 4.1 is less certain. Chemical studies (Husain and Lowe, 1968a,b) and X-ray crystallographic analysis (Drenth *et al.*, 1970) indicate a close proximity between His-159 and Cys-25. X-Ray crystallographic analysis (Drenth *et al.*, 1970) puts the N-1 of the imidazolyl ring of His-159 3.4 Å from the sulfur atom of Cys-25. Certainly, His-159 is close enough to the active site to be directly involved in catalysis. However, assignment of a pK of 4.1 to an imidazolyl group requires that it be in a special microenvironment in order to account for the perturbation of 2–3 units in its pK. A positively charged environment or an apolar environment in the active site region could lead to a drop in pK by destabilizing the solvated imidiazolium ion. The carboxylate side chain of Asp-158 is 7.5 Å from the active site thiol group (Drenth *et al.*, 1970). This carboxylate group, rather than His-159, may be responsible for the dependence of catalytic activity on a group with a pK of 4.3. This carboxylate group has been proposed to influence the mode of substrate binding and promote the removal of the product of deacylation which contains an anionic carboxylate group (Drenth *et al.*, 1971).

With the goal of mapping the microenvironment at the active site of papain in solution, we undertook this study of a reporter group covalently linked to Cys-25. This work describes the ionization of papain which was alkylated at Cys-25 with 2-bromoacetamido-4-nitrophenol (**1**), and how the properties of this derivative (papain-R)[1] indicate the existence of an area of high negative charge density in the region of the active site.

Materials and Methods

2-Bromoacetamido-4-nitrophenol (**1**) obtained from Pierce Chemical Co., Rockford, Ill., was recrystallized from 95% ethanol, mp 212–213° dec cor, lit. (Hille and Koshland, 1967) mp 212–213°.

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[1] Abbreviations used are: papain-R, papain alkylated at Cys-25 by reacting it with 2-bromoacetamido-4-nitrophenol; BzArgOEt, *N*-α-benzoyl-L-arginine ethyl ester.

2-β-Hydroxyethylthioacetamido-4-nitrophenol (**2**) was prepared by stirring 2.0 g (7.3 mmol) of **1** with 50 ml (700 mmol) of β-mercaptoethanol (from Matheson Coleman and Bell, Norwood, Ohio) in 350 ml of 1.0 N NaOH. After 2 hr in the dark at room temperature, the solution was brought to pH 2 with concentrated HCl and cooled in an ice bath. The resulting precipitate was filtered, dried, and recrystallized three times from ethyl acetate–petroleum ether, mp 186–187° cor. *Anal.* Calcd for $C_{10}H_{12}N_2O_5S$: C, 44.11; H, 4.44; N, 10.29; S, 11.77. Found: C, 44.39; H, 4.47; N, 10.39; S, 11.85.

Papain (*EC 3.4.4.10*), twice crystallized in suspension in 0.05 M acetate buffer (pH 4.5), was obtained from Worthinton Biochemical Corp., Freehold, N. J. The papain was further purified by affinity chromatography (Blumberg *et al.*, 1970). The pure papain was stored in water as inactive mercuripapain, which was prepared by adding a 10% excess of $HgCl_2$ to the papain-containing eluent from the affinity column.

Papain S-Alkylated with 2-Acetamido-4-nitrophenol (*Papain-R*) was prepared by reacting pure papain with **1** at pH 5.5. One part of 0.25 M citrate buffer was added to nine parts of a solution of 80 mg (3.4 μmol) of mercuripapain in water to give a final pH of 5.5. The concentration of papain was adjusted to about 5.3 mg/ml (230 μM) by ultrafiltration using a Diaflow ultrafilter equipped with a PM-10 membrane. The protein solution (15 ml) was stirred for 15 min with 12 mg (100 μmol) of L-cysteine (free base from Cyclo Chemical Co., Los Angeles, Calif.) and 6 mg (21 μmol) of Na_2EDTA, (from Aldrich Chemical Co., Inc., Milwaukee, Wis.) and then 3.6 mg (13.1 μmol) of **1** was added to the solution. The mixture was protected from the light, stirred for 4 hr, and then another 12 mg of L-cysteine, 6 mg of Na_2EDTA, and 3.6 mg of **1** were added to the solution. The reaction mixture was stirred for 24 hr. At this point, the enzymic activity of papain was less than 0.4% of the activity of native papain. The reaction mixture containing papain-R was subjected to gel filtration on a 2.5 × 40 cm column of Sephadex G-25 equilibrated and eluted with 0.05 M NaCl. Papain-R which is eluted in the void volume was concentrated and diluted with water on the Diaflow ultrafilter. This process was repeated several times until the concentration of salt was negligible, and the desired concentration of papain-R was obtained. The solution of papain-R was stored at 4°.

N-α-Benzoyl-L-arginine Ethyl Ester Hydrochloride (*BzArg-OEt-HCl*) (Aldrich analyzed) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Tris (Enzyme and Buffer Grade) and *S*-carboxymethyl-L-cysteine were obtained from Schwarz/Mann, Orangeburg, N. Y. Triethylamine (from Eastman Organic Chemicals) was treated with phenyl isocyanate and redistilled. Triethylamine hydrochloride was prepared by slowly neutralizing a cold aqueous solution of triethylamine with cold concentrated hydrochloric acid. The water was removed under reduced pressure on a rotary evaporator and the remaining triethylamine hydrochloride was recrystallized from absolute ethanol. The distilled water supplied to the laboratory was run through a demineralizer and redistilled in an all-glass still. All other chemicals used were Fisher, Mallinckrodt, or Baker-Adamson analytical reagents.

Measurements of pH were made using a Radiometer Model 4b pH meter which was standardized with a 1:1 phosphate NBS primary standard solution (Bates, 1964). The response of the glass electrode was checked with another NBS primary standard solution (borax) or phthalate. Any nonideality in the glass electrode response was corrected with the temperature compensator.

Amino acid analyses were performed by the method of Spackman *et al.* (1958) except that after development of the color, the absorbance at 570 nm was measured continuously on a Gilford Model 2000 multiple sample absorbance recorder with 0.5- and 1-cm flow-through cells. Areas under peaks were measured with a compensating polar planimeter sold by Gelman Instruments Co., Ann Arbor, Mich. The amino acid analyzer was calibrated with an 18 amino acid standard solution from Calbiochem, *S*-carboxymethyl-L-cysteine, and norleucine.

Determination of the Site and Number of Reporter Groups in Papain-R. Approximately 5 mg of papain and papain-R were hydrolyzed separately in 6 N HCl containing 250 nmol of norleucine as an internal standard. The solutions were held at 110° for 22 hr in evacuated sealed tubes. After hydrolysis, the acid was removed and the hydrolysate taken up in 4 ml of distilled water. An aliquot of the hydrolysate was acidified (by diluting the solution 1 part to 1.1 parts with 1 N HCl) and subjected to amino acid analysis. The amount of *S*-carboxymethylcysteine obtained from papain-R served to establish the extent of attachment of reporter groups to Cys, since the amido linkage in the reporter group is hydrolyzed in 6 N HCl. The method of Hille and Koshland (1967) was used to determine the number of reporter groups per mole of protein. This method is based on the observation that acid hydrolysis releases 2-amino-4-nitrophenol from the reporter group. This compound is easily determined from the contribution it makes to the absorbance at 445 nm (ϵ_{R445} 1.35×10^4 cm^{-1} M^{-1}) in 0.5 N NaOH (Hille and Koshland, 1967). Thus, 0.5 ml of the hydrolysate in water was added to 0.5 ml of 1.0 N NaOH and its absorbance at 445 nm was determined. The moles of reporter group incorporated per mole of papain-R (RI) was determined using the relationship

$$\mathrm{RI} = \frac{A_{445} - \epsilon_{p445}[\text{papain-R}]}{[\text{papain-R}]1.35 \times 10^4} = \frac{A_{445}}{[\text{papain-R}]1.35 \times 10^4} - \frac{\epsilon_{p445}}{1.35 \times 10^4} \quad (1)$$

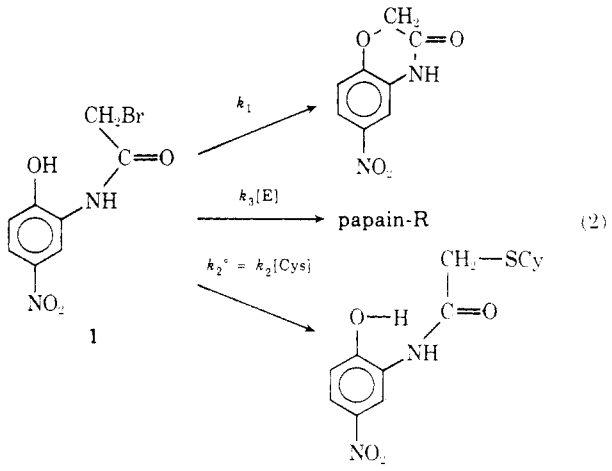
The concentration of papain-R was determined from amino acid analysis. The molar absorptivity of acid-hydrolyzed papain (containing no reporter group) in 0.5 N NaOH was determined from the absorbance of a known concentration of acid hydrolyzed papain. This molar absorptivity was $(4 \pm 2) \times 10^2$ cm^{-1} M^{-1}. The uncertainty in ϵ_{p445} had no significant effect on the value of RI estimated from eq 1, since $A_{445}/[\text{papain-R}]$ (1.35×10^4) was always much greater than $4 \times 10^2/1.35 \times 10^4$. Measured values of A_{445} were always between 0.2 and 0.3 absorbance unit for papain-R.

Buffers used for activity measurements, kinetic studies, and spectral studies were prepared by mixing a solution 0.05 M in the acidic form of the buffer with a solution 0.05 M in the basic form of the buffer until the desired pH was obtained. The solutions containing the acidic and basic forms of the buffer were adjusted to Γ/2 of 0.15 M with KCl prior to mixing. The buffers used were: HOAc–NaOAc (pH 3.4–5.8), NaH_2PO_4–Na_2HPO_4 (pH 6.0–7.8), Tris-HCl–Tris (pH 7.8–9.1), $NaHCO_3$–Na_2CO_3 (pH 9.5–11), triethylamine–HCl–triethylamine (pH 9.8–10.8), KOH (pH 12).

Assays of the catalytic activity of papain were performed by following the papain-catalyzed hydrolysis of BzArgOEt spectrophotometrically using a Gilford Model 240 recording spectrophotometer. Sample and reference chambers were maintained at 25 ± 0.1° with water from a constant-tempera-

ture circulator. For routine assays, 3.0 ml of pH 4.50 acetate buffer was allowed to equilibrate in a cuvet in the thermoregulated cell compartment; to this was added 0.100 ml of 15.5 mM BzArgOEt with mixing followed by 0.100 ml of enzyme solution after which time, the linear time dependence of absorbance at 255 nm was recorded for at least 5 min. The concentrations of papain were determined from the absorbancies at 280 nm measured on a Model 240 Gilford spectrophotometer, using a value of 5.77×10^4 cm^{-1} M^{-1} for the molar absorptivity of papain, based on a value of $E_{1\%}^{1\,cm} = 24.7$ (Bender *et al.*, 1966; Glazer and Smith, 1961) and a molecular weight of 23,350 (Wolthers *et al.*, 1970).

Kinetics of Reaction of Papain with **1**. Equation 2 depicts the pathway for decomposition of **1** in aqueous solutions



(2)

containing papain (E). The rate constants k_1 and k_2 were determined from studies of the decomposition of **1** in the absence of papain. The rate of decomposition of **1** was related to the loss of yellow color accompanying the cyclization of **1** to its benzoxazine derivative, and k_1 and k_2^0 were obtained from the first-order approach of the absorbance (A) at 410 nm to its final value (A_∞). The slopes of linear plots of ln ($A - A_\infty$) *vs.* time gave k_1 when neither cysteine nor papain was present. With cysteine present and papain absent the slopes of these plots were equal to $k_1 + k_2^0$. The reactions depicted in eq 2 were studied in 10% ethanol at pH values of 4.62, 5.63, 6.52, and 7.20. Buffered solutions were prepared as already described and were made 5 mM in cysteine (free base) and 0.5 mM in Na_2EDTA. The cysteine and EDTA were omitted for determinations of k_1. To nine parts of buffer solution, was added 1 ml of water or 1 ml of 100–200 μM mercuripapain in water. This solution (nine parts) was mixed with one part of **1** in absolute ethanol. The final concentrations were 4.5–144 mM **1**, 9–18 μM papain (when present), 4.05 mM L-cysteine, 0.405 mM EDTA, 10% ethanol, $\Gamma/2 = 0.12$. From pH 5.63 to pH 7.20, the conditions were such that the rates of the side reactions were comparable to the rate of formation of papain-R. Thus, after all the **1** was depleted, only a fraction of the enzymically active papain was transformed to enzymically inactive papain-R.

Since

$$-\mathrm{d[E]}/\mathrm{d}t = k_3[\mathbf{1}][\mathrm{E}] \tag{3}$$

and

$$-\frac{\mathrm{d[1]}}{\mathrm{d}t} = (k_1 + k_2^0)[\mathbf{1}] + k_3[\mathbf{1}][\mathrm{E}] \tag{4}$$

$$\frac{\mathrm{d[1]}}{\mathrm{d[E]}} = \frac{k_1 + k_2^0}{k_3}\frac{1}{[\mathrm{E}]} + 1 \tag{5}$$

Equation 6 is obtained on integrating (5) with the boundary

$$[\mathbf{1}^0] + [\mathrm{E}_\infty] - [\mathrm{E}^0] = \frac{k_1 + k_2^0}{k_3}\ln\frac{[\mathrm{E}^0]}{[\mathrm{E}_\infty]} \tag{6}$$

conditions [1] = [$\mathbf{1}^0$], [E] = [E^0] at $t = 0$; [1] = 0, [E] = [E_∞] at $t = \infty$; and [Cys] $\gg$ [$\mathbf{1}^0$] or [E^0], so that k_2^0 is constant during the time of the reaction. Values of $(k_1 + k_2^0)/k_3$ were obtained from the slopes of linear plots of [$\mathbf{1}^0$] + [E_∞] − [E^0] *vs.* ln ([E^0]/[E_∞]). The initial concentration of papain was obtained from its absorbance at 280 nm (ϵ_{280} 5.77×10^4 cm^{-1} M^{-1}). The final concentration of enzymically active papain ([E_∞]) was obtained from the relationship, [E_∞] = [E^0]V_∞/V^0, where V^0 and V_∞ represent initial and final enzymic activities toward BzArgOEt. Since **1** decomposed rapidly in solution the time required to obtain constant values of E_∞ was less than the time required for significant loss of the catalytic activity by processes other than reaction with [**1**] (*e.g.*, autolysis). Controls in which papain was incubated under the reaction conditions in the absence of **1** showed that less than 2% was lost during the time used to obtain the constant values of E_∞ when reaction with **1** was judged complete. The rate of loss of activity of papain in the absence of **1** appeared to be a function of the concentration of papain. Under the conditions used for the kinetic studies, incubation of 18 μM papain for 5 hr at pH 4.62–7.62 in the absence of **1** never resulted in the loss of more than 2% of the catalytic activity of the enzyme. However, at pH 5.5, 203 μM papain (the conditions for the preparation of papain-R) a 7–8% loss of catalytic activity occurred when papain was incubated for 4 hr in the absence of **1**. Using the separately determined values of k_1 and k_2^0, k_3 was evaluated from the ratio $(k_1 + k_2^0)/k_3$.

At pH 4.62, the rate of the side reactions was much slower than reaction with papain. Under these conditions

$$-\mathrm{d[E]}/\mathrm{d}t = k_3[\mathrm{E}]([\mathbf{1}^0] - [\mathrm{E}^0] + [\mathrm{E}]) \tag{7}$$

Integration of eq 7 with the boundary conditions [E] = [E^0], [**1**] = [$\mathbf{1}^0$] at $t = 0$; [E] = [E_∞], [**1**] = 0 at $t = \infty$; and [E^0] $\neq$ [$\mathbf{1}^0$], yields eq 8. Slopes of the linear plots of $-\ln$ ([E]/([$\mathbf{1}^0$] +

$$-\ln\frac{[\mathrm{E}]}{[\mathbf{1}^0] + [\mathrm{E}] - [\mathrm{E}^0]} = ([\mathbf{1}^0] - [\mathrm{E}^0])k_3t + \ln\frac{[\mathbf{1}^0]}{[\mathrm{E}^0]} \tag{8}$$

[E] − [E^0])) *vs.* t were divided by ([$\mathbf{1}^0$] − [E^0]) to obtain k_3.

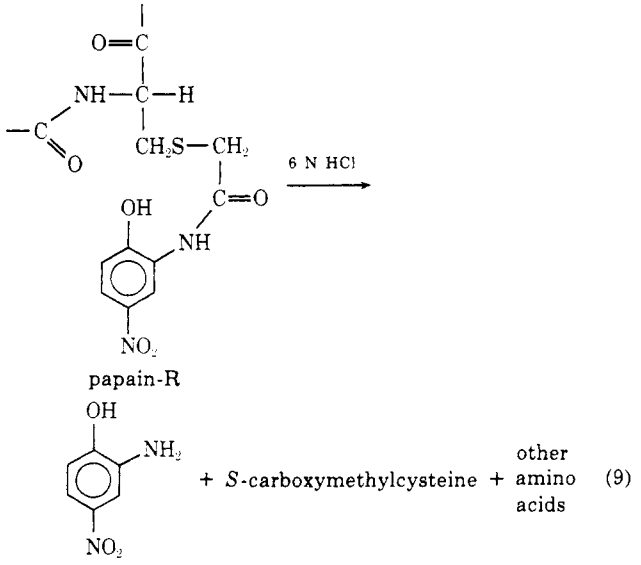
Spectral Studies. The pH dependence of the absorbance spectrum of papain-R and of the model compound, **2**, were determined at 25° with a Cary 118 spectrometer equipped with thermostated cell compartments. The recorder response was set at 0.01 absorbance unit per inch. Spectra were determined at several pH values by adding 0.100 ml of a water solution of either 399 μM papain-R or 155 μM **2** to 3.00 ml of buffer in a 1-cm path length quartz cell. The reference solution was in a matched cell and contained 3.00 ml of buffer plus 0.1 ml of water. Titration curves for papain-R at 320 nm were corrected for the small pH-dependent absorbance the protein makes to the absorbance of the reporter group by subtracting the absorbance of an equivalent concentration of pure papain from the absorbance of papain-R. This correction was negligible for the titration curves obtained at higher wavelengths.

Results

Characterization of the Reaction between Papain and 2-Bromoacetamido-4-nitrophenol (1). Reaction with **1** inactivates papain. The 4-hr treatment of papain with **1** in the preparation of papain-R resulted in a 99.5% loss in catalytic activity, whereas standing 4 hr in the absence of **1** causes a less than 8% loss in enzymic activity. Reversible oxidation of the thiol group at the active site of papain (*e.g.*, to a disulfide) is an unlikely cause of the loss in catalytic ability, since incubating with cysteine (50–80 mM) prior to assaying for enzymic activity did not restore any catalytic activity to papain which had been inactivated by **1**.

The papain used in these experiments was prepared by removing mercuric ion from the thiol group of mercuripapain with a large excess of cysteine and EDTA. When mercuripapain was treated for 4 hr with **1** as described above, but in the absence of cysteine or EDTA, more than 92% of the treated mercuripapain could still be converted to fully active papain.

The thiol group of Cys-25, the only thiol group in papain, is a likely site of reaction of papain with **1**. As depicted in eq 9,



(9)

acid hydrolysis of papain-R should yield equivalent amounts of 2-amino-4-nitrophenol and *S*-carboxymethylcysteine, if **1** alkylated only Cys-25.

Spectrophotometric determination of 2-amino-4-nitrophenol in the acid hydrolysate indicated that 0.89 ± 0.1[2] reporter groups are incorporated per molecule of papain-R. The conclusion that all of the reporter groups are indeed attached to Cys-25 was verified by amino acid analysis which indicated 1.04 ± 0.1[2] molecules of *S*-carboxymethylcysteine per molecule of hydrolyzed papain-R. No peak at the position of *S*-carboxymethylcysteine was detected on amino acid analysis of papain which had not been treated with **1**.

Kinetics of the Reaction of Papain with **1**. Rate constants for reaction of **1** with the thiol group at the active site of papain at four different pH values are listed in Table I along with the rate constants for the competing side reactions depicted in eq 2, namely cyclization of **1** (k_1) and reaction of **1** with cysteine (k_2). From pH 5.63 to 7.20, conditions for these kinetic studies were such that the side reactions were comparable in rate to the reaction of **1** with papain, so that all the papain did not react with **1** before the concentration of **1** decayed to zero. The concentration of papain remaining at the end of the reaction is related to the rate constants for the three reactions depicted in eq 2 and the initial concentrations of **1** ([**1**^{0}]) and papain ([E^0]). If the reaction between papain and **1** is second order (first order with respect to the concentration of papain and **1**), eq 6 predicts that plots of [**1**^{0}] + [E$_\infty$] − [E^0] *vs.* ln ([E^0]/[E$_\infty$]) should be linear and pass through the origin. This expectation is realized (Figure 1). Values of $(k_1 + k_2^0)/k_3$ were evaluated from the slopes of these plots. Values of k_3 were determined from this ratio using values of $k_1 + k_2^0$ obtained from spectrophotometric observations of the decomposition of **1** in the absence of papain. At pH 4.62, the side reactions were much slower than reaction of papain with **1**. Under these conditions, eq 8 was used to evaluate k_3 from second-order plots like the one shown in Figure 2. As expected for a second-order process, the value of k_3 was independent of the initial concentration of **1** (see Table I).

The observed compliance of the rate of reaction of **1** to a second-order rate law indicates that if **1** formed a complex with papain prior to alkylating Cys-25, only a small fraction of the papain would be complexed with **1** in the range of concentrations used to study this reaction. Reaction through a complex having a dissociation constant of 10^{-3} M would cause a 10% deviation from the first-order dependence of the

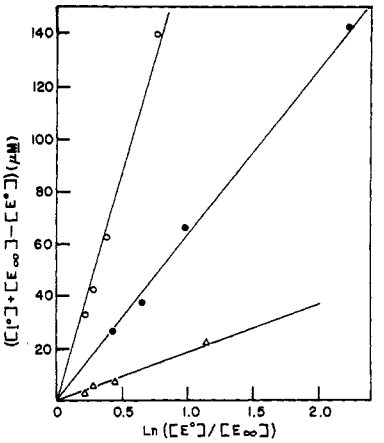


FIGURE 1: Determination of the ratio $(k_1 + k_2^0)/k_3$, pH 5.63; (△) pH 6.52, (●) pH 7.20, (○) in 4.05 mM L-cysteine, 0.405 mM EDTA, Γ/2 = 0.12, 10% ethanol, 25°, [E^0] = 9–18 μM.

TABLE I: pH Dependence of Rate Constants for the Decomposition of **1** in 10% Ethanol, Γ/2 = 0.12, at 25°.

pH	$10^4 k_1$ (sec^{-1})	$10^2 k_2^{a,b}$ (sec^{-1} M^{-1})	$k_3^{a,c}$ (sec^{-1} M^{-1})
4.62	0.48	0.62	19.3,[d] 20.2,[e] 19.8[f]
5.63	2.5	3.1	20.3[g]
6.52	6.4	14.7	19.8[h]
7.20	8.0	42	15.1[h]

[a] 4.05 mM Cys, initial concentration as are all of the following concentrations. [b] 144 μM **1**. [c] 9–18 μM papain. [d] 65 μM **1**. [e] 45 μM **1**. [f] 40 μM **1**. [g] 7–34 μM **1**. [h] 34–144 μM **1**. The superscripts *g* and *h* refer to the concentration ranges used to determine the ratio $(k_1 + k_2^0)/k_3$.

[2] Average of three different preparations of papain-R.

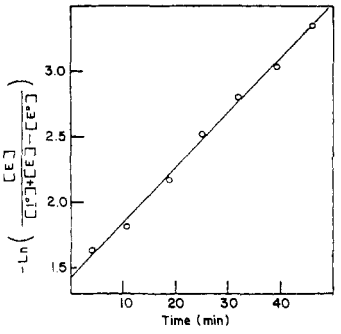


FIGURE 2: Determination of k_3 at pH 4.62; in 4.05 mM L-cysteine, 0.405 mM EDTA, $\Gamma/2$ = 0.12, 10% ethanol, 25°, $[E^0]$ = 10 μM, $[\mathbf{1}^0]$ = 45 μM.

rate with respect to the concentration of **1** at 100 μM **1**. This 10% deviation would be reflected in the plots of Figure 1. The limited solubility of **1** prevented determination of whether reaction with papain proceeds *via* a complex having a dissociation constant much larger than 10^{-3} M.

Examination of Table I reveals that the observed rate constants for the cyclization of **1** and the reaction of **1** with cysteine increase with increasing pH, whereas the rate constant for the reaction of **1** with papain is almost independent of pH. The increase in the rate constant (k_1) for cyclization with increasing pH has been observed previously and is attributed to cyclization occurring through the phenolate anion (Hille and Koshland, 1967). The increase in k_2 with increasing pH is not unexpected and is undoubtedly a consequence of an increase in the fraction of cysteine containing a thiolate anion as the pH is increased. However, the pH dependence of this reaction is perturbed by the ionization of **1**. Ionization of **1** also makes it difficult to compare the pH–rate profile for the reaction of papain with **1** to pH–rate profiles obtained for reaction of papain with other alkylating agents.

Comparison of the values of k_2 and k_3 in Table I shows that **1** reacts much more efficiently with the thiol group in papain than with the thiol group in cysteine. The 3200-fold difference in rates of alkylation of papain and cysteine seen at pH 4.62 cannot be attributed to a lower concentration of thiol anion of cysteine at pH 4.62. The pK for ionization of the thiol group from zwitterionic cysteine is 8.53 (Benesch and Benesch, 1955), whereas the pK of the thiol group at the active site of

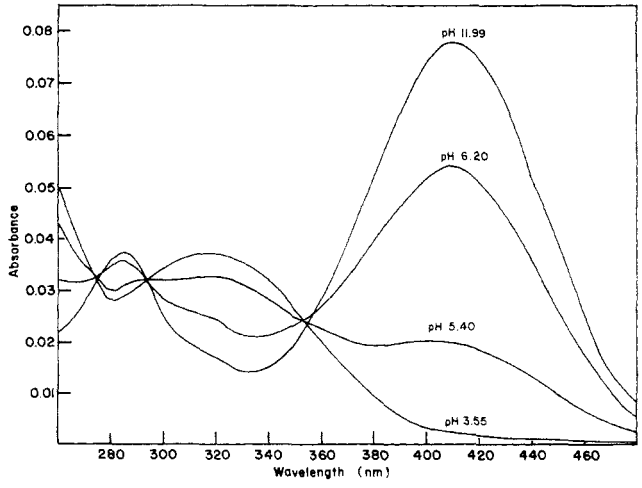


FIGURE 3: Dependence of the spectrum of model compound **2** on pH. At 5.00 μM **2**, 25°, $\Gamma/2$ = 0.145.

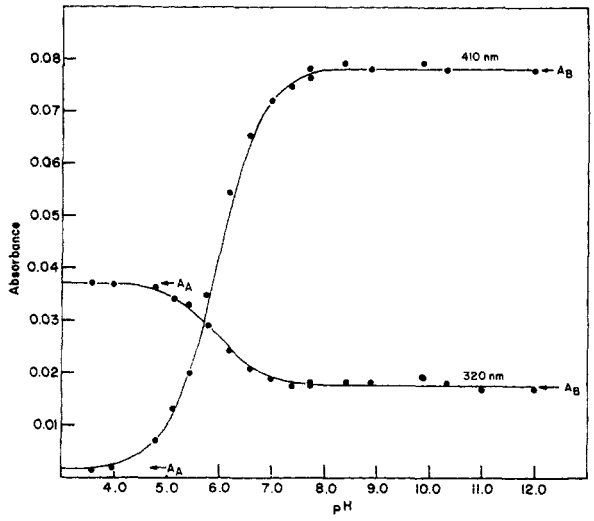
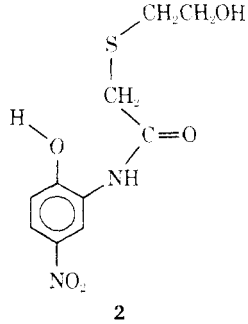


FIGURE 4: Spectrophotometric titration of 5.00 μM **2** at 320 and 410 nm. Conditions were the same as in Figure 3. Solid lines calculated from eq 10 with $K = 10^{-5.92}$, A_A = 0.0018 and A_B = 0.0784 at 410 nm, and A_A = 0.0370 and A_B = 0.0176 at 320 nm.

papain appears to be between 8 and 9. Thus, certain groups on papain must somehow facilitate alkylation of the active site thiol group by **1**. Large rate enhancements in acidic solutions also have been reported for alkylation of papain by halo acids (Chaiken and Smith, 1969; Wallenfels and Eisele, 1968; Sluyterman, 1968). At pH 6, chloroacetate alkylates the thiol group in papain 32,000 times more rapidly than it alkylates the thiol group in cysteine (Sluyterman, 1968).

Spectral Properties of the Reporter Group. Figure 3 depicts the pH dependence of the uv spectrum of **2**, in which the reporter group is attached to a simple thiol. The anionic form



2

of this model compound exhibits absorbance maxima at 285 and 410 nm. The plots in Figure 4 of absorbance (A) of **2** *vs.* pH have the form of a titration curve. An acid dissociation constant (K) was evaluated for **2** by interpreting a plot of A_{410} *vs.* $(A_{410} - A_A)[H^+]$ according to eq 10. With **2** at 5.00

$$A = A_B - (A - A_A)[H^+]/K \qquad (10)$$

μM, a value of 0.0018 was used for A_A, the absorbance of the protonated form of **2**. Values of 0.0784 for A_B, the absorbance of the anionic form of **2**, and $10^{-5.92}$ for K were obtained from the intercept and the reciprocal of the slope of the plot in Figure 5. The fit of the data to a titration curve expected for an acid with pK = 5.92 is illustrated by the solid lines in Figure 4. The change in molar absorptivity accompanying ionization of this derivative of nitrophenol is 15,320 cm^{-1} M^{-1} at 410 nm. Figure 6 illustrates the effect making the medium

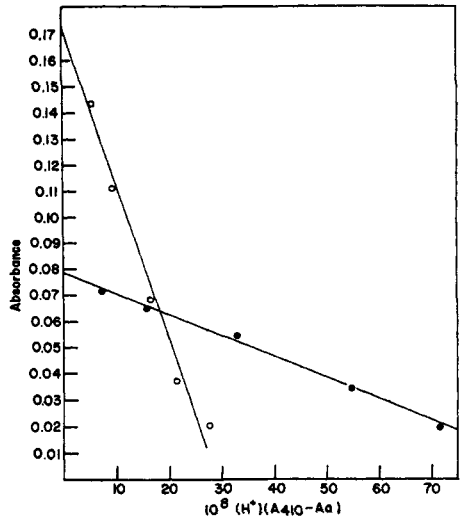
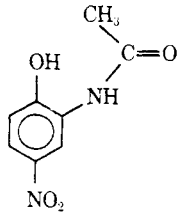


FIGURE 5: Determination of the ionization constant of the reporter group in **2** at 5.00 μM **2**: (●) and in papain-R at 12.9 μM papain-R; (○) using eq 10. A_A was set at 0.0018 and 0.0030 for **2** and papain-R, respectively.

less polar has on the absorbance spectrum of the acidic and basic forms of **2**. Dioxane (33%) causes a red shift, with the absorbance maximum for the nitrophenolate anion shifting from 410 to 420 nm. The uv spectrum and the pK of the reporter group in **2** and in 2-acetamido-4-nitrophenol (**3**) are very similar suggesting that the sulfur atom in **2** has little effect on the reporter group. Compound **3** has an absorption



3

maximum at 410 nm which exhibits a red shift in 33% dioxane (Hille and Koshland, 1967). The pK of 6.12 for **3** (in 0.05 M phosphate ions (Hille and Koshland, 1967)) is very close to the value of 5.92 reported here for the pK of **2**.

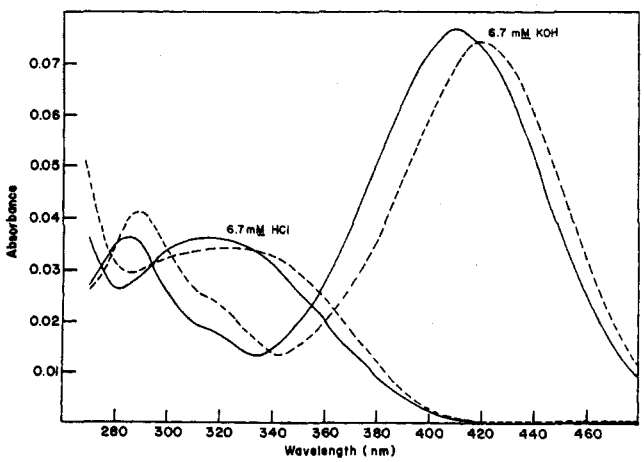


FIGURE 6: Effect of dioxane on the spectrum of the reporter group in **2**: (- - -) 33% dioxane; (—) water. $\Gamma/2$ = 0.0965, 5.00 μM **2**.

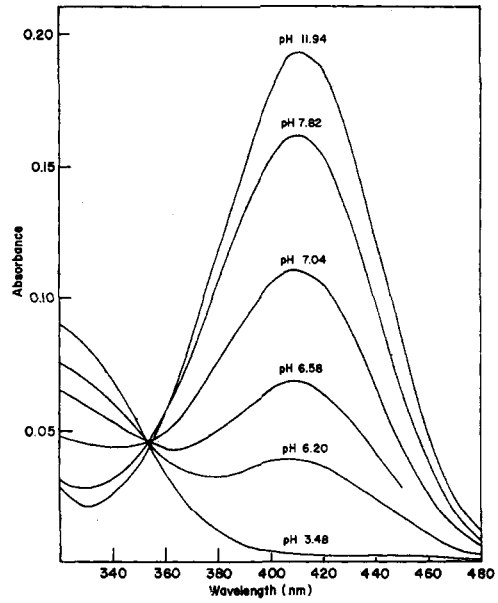


FIGURE 7: Dependence of the spectrum of papain-R on pH. At 12.9 μM papain-R, 25°, $\Gamma/2$ = 0.145.

Figure 7 depicts the pH dependence of the uv spectrum of the reporter group when it is linked to the thiol group at the active site of papain. Interestingly, the reporter groups in papain-R and in **2** exhibit similar spectra with an isosbestic point around 353 nm and an absorbance maximum of 410 nm for the nitrophenolate anion. Papain-R exhibits a fairly sharp isosbestic point below pH 8. Above this pH value, only small deviations from the isosbestic point become apparent. The dependence of the absorbance at 410 nm on pH illustrated in Figure 8 is consistent with two ionizing groups influencing the molar absorptivity of the reporter group. At 12.9 μM papain-R, values of 0.173 for A_B and $10^{-6.79}$ for K_I, the equilibrium constant for the first ionization, were obtained using eq 10 to interpret a plot of A_{410} *vs.* $(A_{410} - A_A)[H^+]$ at values of $[H^+] > 10^{-8}$ M and with A_A set at 0.003 (Figure 5).

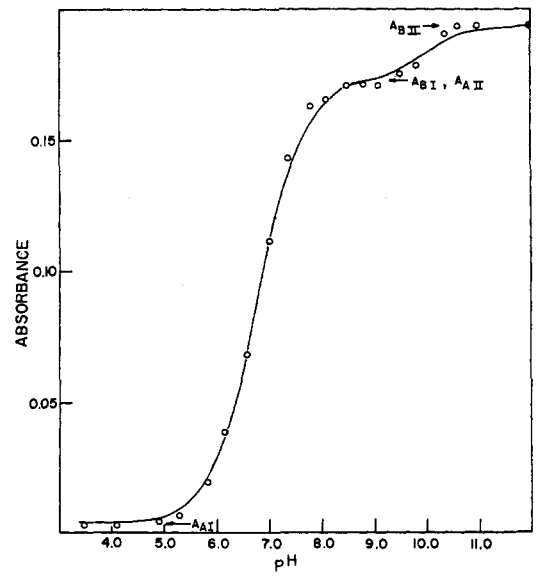


FIGURE 8: Spectrophotometric titration curve for 12.9 μM papain-R at 410 nm. Conditions were the same as in Figure 7. Solid line is a calculated titration curve for two acidic groups pK_I = 6.79, pK_{II} = 10. With A_{AI} = 0.003, A_{BI} = A_{AII} = 0.173, and A_{BII} = 0.194.

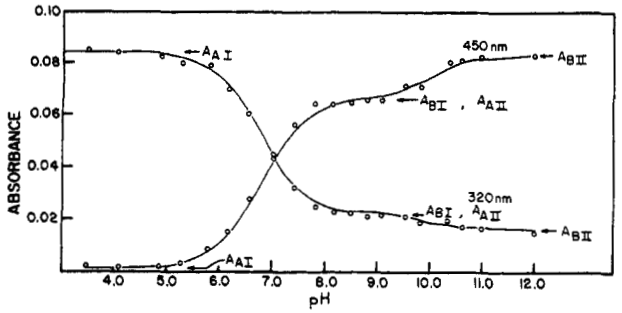


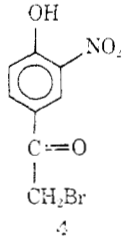
FIGURE 9: Spectrophotometric titration curves for 12.9 μM papain-R at 320 and 450 nm. Solid lines are calculated titration curves for two acidic groups, $pK_I = 6.79$, $pK_{II} = 10$, with $A_{AI} = 0.084$, $A_{BI} = A_{AII} = 0.022$, and $A_{BII} = 0.016$ at 320 nm, and $A_{AI} = 0.001$, $A_{BI} = A_{AII} = 0.066$, and $A_{BII} = 0.083$ at 450 nm.

Because of the small change in absorbance associated with the transition occurring above pH 8, the pK associated with this transition was estimated from the pH at which the transition is half complete ($pK_{II} = 10$, Figure 8). This high pH transition was completely reversible. Mixing papain in 0.05 M phosphate buffer (pH 6.20) with an equal volume of 0.01 M KOH–0.14 M KCl gave a spectrum identical with that obtained by mixing papain incubated in 0.01 M KOH–0.14 M KCl with an equal volume of 0.05 M phosphate buffer (pH 6.20). The final pH of the mixtures was 6.57. The high pH transition cannot be attributed to specific interactions between papain-R and the buffer. The values of A_{410} plotted in Figure 8 changed by less than 1% on going from carbonate buffer to triethylamine buffer at pH values of 9.8 and 10.8. The value of A_{410} also remained unchanged on going from phosphate buffer to Tris buffer at pH 7.8.

The fit of the data to a titration curve expected for two acidic groups $pK_I = 6.79$, $pK_{II} = 10.0$ $A_{AI} = 0.003$, $A_{BI} = A_{AII} = 0.173$, $A_{BII} = 0.194$ at 12.9 μM papain is illustrated by the solid line in Figure 8. The changes in molar absorptivity (at 410 nm) accompanying the first and second ionization are 13,200 and 1,630 cm^{-1} M^{-1}, respectively. Figure 9 depicts the dependence of absorbance at 320 and 450 nm on pH. Figures 8 and 9 illustrate the observation that although the relative magnitudes associated with the first and second ionization vary somewhat with wavelength the pK for the first and second ionization is independent of wavelength.

Discussion

The idea of using a reporter group to obtain information about the active site of papain was first presented by Furlanetto and Kaiser (1970) in their studies of papain which had been alkylated at its thiol group by reacting it with α-bromo-4-hydroxyl-3-nitroacetophenone (**4**). Spectrophotometric titration of papain which had been alkylated with **4** yielded a simple titration curve, which showed that the pK of the phenolic hydroxyl group had increased by 0.3 unit upon reaction with papain (Furlanetto and Kaiser, 1970). The reporter group in this derivative of papain cannot assume a conformation in which the phenolic oxygen atom is close to the sulfur atom of Cys-25. In all reasonable conformations of the reporter group in papain-R, the distance between the phenolic oxygen atom and the sulfur atom of Cys-25 is smaller than the corresponding distance in the derivative produced by alkylating papain with **4**. This fact coupled with the observation that the pK of the reporter group in papain-R exhibits a larger perturbation than that exhibited by the previously studied derivative suggests that the factors responsible for the perturbation of the pK of the reporter group in papain-R are also influencing the environment close to the sulfur atom of Cys-25.

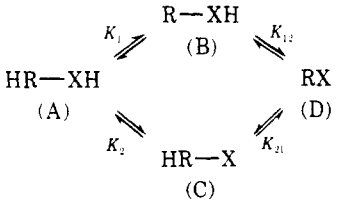


4

Perturbation of the pK of an ionizable group on a protein is usually ascribed to the polarity or charge of the group's environment. The pK of the reporter at the active site of papain is 0.87 unit higher than that of model compound **2**. It is unlikely that this perturbation in pK is caused by the phenolic group being in an apolar environment, since such an environment would be expected to produce a pronounced red shift in the absorbance spectrum. The 33% dioxane which causes a 10-nm red shift in the absorbance maximum of the nitrophenolate anion only causes a 0.4 unit increase in the pK of **3** (Hille and Koshland, 1967).

Thus, the 0.87 unit increase in the pK in the absence of a red shift in the spectrum indicates that the reporter group in papain-R is occupying a region which has a net negative charge. The β-carboxylate group of Asp-158 may be responsible for this negative charge. This carboxylate group forms part of the wall of the active site groove and is within 7.5 Å of the sulfur atom of Cys-25 (Drenth *et al.*, 1971). The finding of a negative charge density at the active site of papain is consistent with previous observations that ionization of the carboxyl group in carboxylic acid inhibitors of papain lowers the affinity of these inhibitors for papain (Berger and Schechter, 1970).

The spectrophotometric titration of papain-R reflects the ionization of a second group having an apparent pK of about 10 (Figure 8). Such secondary ionizations are almost always ascribed to the ionization of some group on the protein causing a change in the molar absorptivity of the reporter group. Using this reasoning one would conclude that deprotonation of a group with a pK of about 10 results in an increase in the molar absorptivity of the nitrophenolate anion. This change in molar absorptivity may be a consequence of either direct interaction of a nearby ionizable group with the reporter group or a change in environment induced by reversible changes in conformation which may accompany deprotonation of a distant ionizable group. If the perturbation in molar absorptivity is ascribed to a direct interaction with a nearby ionizable group, it immediately becomes apparent that the ionized reporter group may perturb the pK of the second group (and *vice versa*). Such perturbations would contribute to the secondary ionization and complicate the interpretation of the titration curve. In fact, a change in pK in one group induced by ionization of the other group can explain the secondary ionization without invoking perturbation of the molar absorptivity of the reporter group. Consider the equilibria in eq 11. If the molar absorptivities of B and D are identical, the absorbance will be a linear function of R^-. The apparent constant for the first ionization (K_I) will be $K_1 + K_2$ and the apparent constant for the second ionization (K_{II}) will be $K_1K_{12}/(K_1 + K_2)$. The fraction of the total



(11)

change in absorbance occupied by the first ionization is equal to the ratio $K_1/(K_1 + K_2)$. Applying these relationships and the identity $K_1K_{12} = K_2K_{21}$ to the curve in Figure 8 yields the result: $pK_1 = 6.84$, $pK_2 = 7.74$, $pK_{21} = 9.04$, $pK_{12} = 9.95$. It should be noted that the assumption that a second ionizable group interacts with the reporter group does not alter the conclusion that the pK of the reporter group on the protein is perturbed to higher pK values (compare pK_1 and pK_{21} with the pK of the model compound). If this analysis is correct and the secondary ionization is caused only by perturbations in pK induced by ionization of the two groups, the fraction of the titration curve occupied by the first ionization should be independent of wavelength. The fraction of the titration curve occupied by the first ionization was 0.79, 0.89, and 0.91 at 450, 410, and 320 nm, respectively. If the differences between these fractions are real, the dependence on wavelength of the fraction of the titration curve occupied by the first ionization indicates that the second group must perturb the spectrum of the reporter group. This fraction should be independent of wavelength if the molar absorptivity of the reporter group were independent of the state of ionization of group X in eq 11.

The possibility that ionization of one group simultaneously induces changes in the molar absorptivity and pK of the other group makes it difficult to relate the pK's for the first and second ionizations to the microscopic equilibrium constants of eq 11. Exact determination of these equilibrium constants from the titration curve would be possible if the molar absorptivities of A, B, C, and D were known. Equation 12

$$\frac{K_1}{K_1 + K_2} = f_I \frac{\epsilon_D - \epsilon_A}{\epsilon_B - \epsilon_C} - \frac{\epsilon_C - \epsilon_A}{\epsilon_B - \epsilon_C} \qquad (12)$$

relates the ratio $K_1/(K_1 + K_2)$ to these molar absorptivities and the fraction (f_I) of the total absorbance change, at a given wavelength, which is caused by the first ionization. Equation 12 can be used to establish limits for the ratio $K_1/(K_1 + K_2)$ and thereby establish limits for the microscopic ionization constants.[3] The molar absorptivities of the doubly protonated (ϵ_A) and the unprotonated (ϵ_D) species can be measured so that we need only estimate ϵ_C and ϵ_B. Ionization of X is responsible for the difference between ϵ_A and ϵ_C as well as the difference between ϵ_B and ϵ_D. Since a goal of this analysis is to determine the value of $K_1/(K_1 + K_2)$ to within the narrowest limits, one should apply eq 12 at a wavelength where the uncertainties in the values $(\epsilon_D - \epsilon_A)/(\epsilon_B - \epsilon_C)$ and $(\epsilon_C - \epsilon_A)/(\epsilon_B - \epsilon_C)$ are a minimum. For evaluating $K_1/(K_1 + K_2)$ in this work, f_I was measured at 410 nm. At 410 nm, the absorbance maximum for B, distortions and shifts in the spectrum caused by ionization of X should have the least effect on the ratio ϵ_D/ϵ_B. It is reasonable to assume that $0.7 < \epsilon_D/\epsilon_B < 1.3$ at 410 nm. This 30% upper limit for the perturbation of the molar absorptivity of the nitrophenolate anion is three times the 10% spectral perturbation seen at 410 nm in 33% dioxane. At 410 nm, $\epsilon_D \gg \epsilon_A$, so that the assumption $\epsilon_B \gg \epsilon_C$ is reasonable. With these assumptions, eq 12 reduces to

$$\frac{K_1}{K_1 + K_2} = f_I \frac{\epsilon_D}{\epsilon_B} \qquad (13)$$

Substituting the measured value of 0.89 for f_I and the assumed limits of ϵ_D/ϵ_B into eq 13 leads to the conclusion that $0.623 < K_1/(K_1 + K_2) < 1$. Thus, the values of the microscopic constants in eq 11 become $6.79 < pK_1 < 7.00$, $pK_2 > 7.21$, $9.79 < pK_{12} < 10$, $pK_{21} < 9.58$.

Thus, the pK of XH might be as low as 7.21 when the reporter group is uncharged. It has already been shown that if ionization of X does not alter the molar absorptivity of the reporter group at 410 nm, the pK of the second group would be fixed at 7.74 when the reporter group is uncharged and when the reporter group becomes negatively charged, the pK of this second group would increase to 9.95. These findings are consistent with the second group being His-159. The imidazolium and β-carboxylate groups of His-159 and Asp-158 are within 6.75 Å of each other (Drenth *et al.*, 1970). If the charge in the environment of the reporter group is indeed determined only by His-159 and Asp-158, the net negative charge density near the reporter group might be attributed to the reporter group being closer to the negative charge of Asp-158 than to the positive charge of His-159.

Conclusions

The reporter group at the active site of papain is in an environment with a net negative charge. Deprotonation of a second group on the protein perturbs the spectrum and/or pK of the reporter group. The pK of this group is around 10 when the reporter group is deprotonated. When the reporter group is protonated, the pK of this group may be as low as 7.21. Deprotonation of this second cationic group may cause the pK of the reporter group to rise from a value of about 6.8 to a value which may be as high as 9.58.

Acknowledgment

The authors are happy to acknowledge valuable discussions of this work with Dr. M. J. Hunter.

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[3] It should be noted that even though ionization of X perturbs the molar absorptivity of the reporter group, the apparent ionization constants for the first and second ionization are still related to the microscopic constants by the relationships, $K_I = K_1 + K_2$ and $K_{II} = K_1K_{12}/(K_1 + K_2)$ so that all the microscopic constants are fixed once the ratio $K_1/(K_1 + K_2)$ is established.

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The Preparation of a Chemically Cross-Linked Complex of the Basic Pancreatic Trypsin Inhibitor with Trypsin†

Tsun-Wen Wang and Beatrice Kassell*

ABSTRACT: The complex of the basic trypsin inhibitor of bovine pancreas with trypsin has been cross-linked chemically with dimethyl adipimidate. Both inter- and intramolecular cross-linking occur. In the monomeric complex, two to three pairs of lysine residues are cross-linked and about five lysine residues are monofunctionally substituted. Other amino acid residues are not affected. The cross-linked complex is not dissociated at pH 2 or during gel electrophoresis in the presence of sodium dodecyl sulfate. A variety of reaction conditions were studied to obtain the maximum yield of nonpolymerized cross-linked complex.

Studies of the mechanism of action of the basic pancreatic trypsin inhibitor with trypsin, based on X-ray (Huber *et al.*, 1971a,b; Stroud *et al.*, 1971; Blow *et al.*, 1972) and chemical (Chauvet and Acher, 1967; Kress and Laskowski, 1968; Fritz *et al.*, 1969) data, have shown that lysine-15 of the inhibitor binds to the active site serine-183 of trypsin. This binding alone does not account for the K_i of 10^{-11} M (Green, 1963; Pütter, 1967) to 6×10^{-14} M (Vincent and Lazdunski, 1972) or for the total free energy of 14 (Rigbi, 1971) to over 19 kcal/mol (Vincent and Lazdunski, 1972). The association of the inhibitor with trypsinogen (Dlouhá and Keil, 1969), with TLCK[1]-β-trypsin (Imhoff and Keil-Dlouhá, 1971) and with anhydrotrypsin (Ako *et al.*, 1972) and the fact that guanidinated or amidinated inhibitors, with lysine-15 modified, still inhibit trypsin (Kassell and Chow, 1966; Fritz *et al.*, 1969) indicate that other forces are important in the binding. From the X-ray studies, hydrogen bonds and van der Waal's contacts are known to occur in the region near lysine-15 of the inhibitor and the active site of trypsin (*e.g.*, Blow *et al.*, 1972).

The inhibitor complexes with trypsin over the broad range of pH from 3 to 10. Free trypsin undergoes conformational changes as the pH is decreased or increased (*e.g.*, Lazdunski and Delaage, 1965, 1967). The inhibitor's conformation is stable between pH 2 and 10 (Vincent *et al.*, 1971; Karplus *et al.*, 1973). Therefore, the surfaces of contact between the inhibitor and trypsin may change in the complex as the pH is changed, particularly at the extremes of pH where the association constant is decreased by several orders of magnitude (Green and Work, 1953; Vogel *et al.*, 1968). On the other hand, the trypsin may be stabilized by complex formation, as it is by a synthetic inhibitor (Bechet and D'Albis, 1969). The X-ray studies, for which the crystals were prepared at pH 7 (Rühlmann *et al.*, 1971), cannot detect the conformational changes that may occur.

We are approaching the elucidation of sites of contact by cross-linking the complex chemically with reagents of different specificities, chain lengths, and pH of reaction. This should help in understanding the alterations in the structure of the complex that may occur. The reagent dimethyl adipimidate, DMA, which is specific for lysine residues, was first studied by Hartman and Wold (1967); they were successful in cross-linking ribonuclease. The present paper describes the preparation of the first cross-linked inhibitor–trypsin complex and includes a study of the conditions required to apply the DMA reaction to this complex.

Materials and Methods

Bovine trypsin from Novo Industri A/S, Copenhagen, was purified further by chromatography on SE-Sephadex C-25 (Papaioannou and Liener, 1968). The basic pancreatic trypsin inhibitor was the same as that described earlier (Kassell *et al.*, 1963), except that CM-cellulose was used for the final puri-

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[1] Abbreviations used are: DMA, dimethyl adipimidate; TLCK-, tosyllysine chloromethyl ketone derivative of; the complex, the basic pancreatic trypsin inhibitor–trypsin complex.