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Potentiometric Determination of Ionizations at the Active Site of Papain†

Sidney D. Lewis, Frederick A. Johnson, and Jules A. Shafer*

ABSTRACT: The ionization behavior of groups at the active site of papain was determined from the pH dependence of the difference in proton content of papain and the methylthio derivative of the thiol group at the active site of papain (papain-S-SCH_3). This difference in proton content was determined directly by two independent methods. One method involved potentiometric measurements of the protons released on demethylthiolation of papain-S-SCH_3 with dithiothreitol, as a function of pH. The other method involved analogous measurements of the protons released on methylthiolation of papain with methyl methanethiolsulfonate. The methylthio pH-difference titrations generated by these measurements indicate that ionization of the thiol group at the active site of papain is linked to the ionization of His-159. The pK of the thiol group changes from 3.3 to 7.6 on deprotonation of His-159 at 29 °C, $\Gamma/2$ 0.05. Similarly, the pK of His-159 shifts from 4.3 to 8.5 when the active site thiol group is deprotonated. The microscopic ionization constants determined in this work for Cys-25 and His-159 indicate the equilibrium constant for transfer of a proton from Cys-25 to His-159 is 8–12, and that in the physiological pH range the active site thiol group exists mainly as a thiol anion.

Recently, much discussion has centered around the relationship between the pH dependence of the catalytic efficiency of papain and the state of ionization of the thiol and imidazoyl groups at the active site of this enzyme (Chaiken and Smith, 1969; Polgar, 1973, 1974; Shipton et al., 1975; Drenth et al., 1975; Lowe, 1976). At the heart of this controversy is a failure to resolve an ambiguity which always arises when one attempts to determine a microscopic ionization constant of a group on a polybasic molecule from the pH dependence of some spectral or kinetic property. This ambiguity occurs because neighboring ionizations could perturb the pK of the group in question as well as the response factor being used to measure the degree of ionization of the group under study. A relationship which illustrates such an ambiguity is given in the Appendix. One way around the ambiguity is to determine directly the pH dependence of the proton content of the group in question from a potentiometric pH-difference titration between the polybasic substance and a derivative of the substance, wherein the ionization of the group under study is blocked by a small group which does not perturb other ionizations. For example, pH-difference titrations, wherein one potentiometrically deter-

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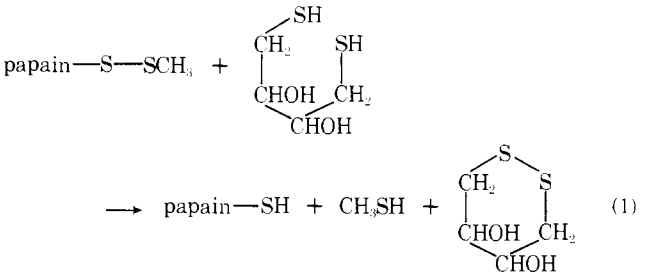
mines differences in proton content of papain and a derivative of papain in which the sulfur atom of Cys-25 is covalently linked to a small uncharged group, should yield information about the ionization behavior of the active site thiol group and the effects of this ionization on other ionizations. pH difference titration curves can be generated from the separate titration curves of a protein and its derivative. For example, Parsons and Raftery (1972) successfully determined the ionization behavior of the carboxyl groups at the active site of lysozyme from the difference in the potentiometric titration curves of lysozyme and the β-ethyl ester derivative of Asp-52 in lysozyme. The general application of this approach, however, has some limitations. Significant errors in such curves could arise if one of the samples had a small amount of ionizable impurity. In the case of a proteolytic enzyme, this ionizable impurity might be a product of autolysis which did not alter the activity of the enzyme. Furthermore, since the pH-difference titrations are generated by subtracting away the effects of most of the ionizable groups on the protein they are the result of small differences between large numbers. Thus, the larger the number of ionizable groups per protein molecule (larger protein molecules), the larger the molar concentration needed to determine an accurate pH-difference titration. Finally, the accuracy of the method relies on being able to match precisely the molar concentrations of the two samples.

These limitations are removed in this work, wherein we *directly* determine differences in proton content between papain and the methylthio derivative of Cys-25 on a *single* sample of protein. Differences in proton content are determined directly by two independent methods. One method involves potentiometric measurements of the protons released in reaction 1 at different pH values. The other method involves analogous measurements of the protons released in reaction 2.



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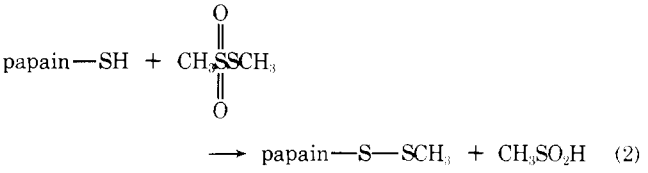

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Experimental Section

Materials

Carbonate-free potassium hydroxide analytical concentrate was from J. T. Baker Chemical Co. DTT[1] was from Calbiochem. Methyl methanethiolsulfonate was generously supplied to us by Dr. George Kenyon, Department of Pharmaceutical Chemistry, University of California, San Francisco. *N*-α-Benzoyl-L-arginine ethyl ester was from Aldrich Chemical Co. 2-Mercaptoethanol was from Matheson Coleman and Bell. *N*-α-Benzoyl-DL-arginine *p*-nitroanilide was from Schwarz/Mann. Crude dried papaya latex, iodoacetamide, Nbs_2, and reagent grade Tris were from Sigma Chemical Co. The distilled water supplied to the laboratory was passed through a Barnstead demineralizer, redistilled in an all-glass still, and used for the preparation of all solutions.

Papain was isolated from crude dried papaya latex and purified by affinity chromatography by the method of Burke et al. (1974). The pure protein was stored as the mercuri derivative which was regenerated just prior to use by the addition of 11 mM 2-mercaptoethanol and 20 mM EDTA. The activated papain was subjected to a repetition of the affinity chromatography step used in the purification procedure in order to separate it from the other components in the activation mixture.

Papain-S-SCH_3, the *S*-methylthio derivative of Cys-25 of papain, was prepared by a modification of the method of Smith et al. (1975). A 20% molar excess of 0.1 M methyl methanethiolsulfonate in water was added to a solution of 50 μM papain at room temperature which had been adjusted to pH 7.8 with KOH. The absence of catalytic activity (<1%) after addition of methyl methanethiolsulfonate indicated the reaction was complete. In order to separate papain-S-SCH_3 from the other components in the reaction mixture, the mixture was subjected to either ultrafiltration in a 400-ml Amicon Diaflow apparatus equipped with a PM 10 membrane (Lot 451) using five cycles of tenfold dilution (with 1 mM KCl) and concentration or by passing the reaction mixture through a 1 × 14 cm column of mixed bed resin (AG 501-X-8, control 3274) from Bio-Rad. Solutions resulting from these two procedures were concentrated to 200–250 μM by ultrafiltration. These two methods of preparation of papain-S-SCH_3 gave samples which were indistinguishable with respect to their behavior in the potentiometric titrations. The specific catalytic activity of the papain regenerated from the papain-S-SCH_3 by treatment with excess thiol (20 mM 2-mercaptoethanol at pH 4.5) was within experimental error (±5%) of pure papain.

Papain-S-CH_2CONH_2, the *S*-carboxamidomethyl derivative of Cys-25 was prepared by adding a fourfold excess of solid iodoacetamide to an aqueous solution of 50 μM papain at 4 °C which had been adjusted to pH 7.8 with KOH. The absence of catalytic activity (<1%) at the end of 1 h indicated the reaction was complete. The reaction mixture was protected from the light and subjected to ultrafiltration using ten cycles of tenfold dilution (with 5 mM KCl) and concentration. The resulting solution was concentrated to 200–250 μM by ultrafiltration. Treatment of purified papain-S-CH_2CONH_2 with excess thiol did not generate catalytically active protein. Acid hydrolysis (6 N HCl, 108 °C, 20 h) followed by amino acid analysis showed 1 mol of *S*-carboxymethylcysteine per mol of papain.

Methods

Concentrations of papain and derivatives of papain were determined from the absorbance at 280 nm using a molar absorptivity of 5.77×10^4 cm^{-1} M^{-1} (Skalski et al., 1973). Using this value for the molar absorptivity of papain, the thiol titer of the papain used in this work was 0.96 as determined from the increase in absorbance at 420 nm when 1 mM Nbs_2 and 0.05 mM papain were mixed at pH 7.2.

The catalytic activity of papain was routinely assayed with 100 μl of 15 mM *N*-α-benzoyl-L-arginine ethyl ester in 3 ml of pH 4.5, 0.05 M acetate buffer. After thermal equilibration

[1] Abbreviations used are: DTT, dithiothreitol, Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); papain-S-SCH_3, the *S*-methanethio derivative of Cys-25 of papain; papain-S-CH_2CONH_2, the *S*-carboxamidomethyl derivative of Cys-25 of papain; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid disodium salt; NBS, National Bureau of Standards.

at 25 °C of the substrate solution in the thermostated cell compartment of a Gilford 240 recording spectrophotometer, 100 μl of a protein solution was added and the rate of change of absorbance at 255 nm was monitored.

Measurements of pH for potentiometric titrations were made using a Radiometer PHM 4c pH meter, and pH measurements for the fluorometric titrations were made with a Radiometer Model TTT 1 meter equipped with a PHA 630Ta Scale expander. The pH meters were standardized at the appropriate temperature with a 1:1 phosphate–NBS primary standard solution (Bates, 1964), and either phthalate or tetraborate standards depending on the pH range of interest.

Potentiometric Determination of the Protons Released in Reactions 1 and 2. Samples were prepared by diluting an aliquot of a solution of papain-S-SCH_3, papain-S-CH_2CONH_2, or papain with an appropriate volume of KCl solution containing a small amount of HCl or KOH so that the desired initial pH, Γ/2, and protein concentration (50–120 μM) were obtained. The sample (2 ml) in the titration vessel was aspirated with stirring until bumping became a problem, whereupon the titration vessel was connected to a Sargent-Welch S-30072-15 combination glass electrode.

The titration vessel was a 16 × 125 mm Pyrex test tube with an ~22-mm bulge blown into it ~25 mm from the bottom of the tube. A rubber sleeve consisting of rubber tubing formed an air-tight seal between the body of the electrode and the titration vessel. Four Teflon tubing lines (0.031 in. i.d. × 0.063 in. o.d.) extended into the titration vessel through small holes in the sleeve. A Teflon line which carried the titrant from a 0.200 μl/div syringe microburet (Model No. SB22, Micrometric Instrument Co., Cleveland, Ohio) extended into the sample solution. This line was sealed at its end and a pin hole, for the delivery of the titrant, was made in the side of the tubing near the seal. Another line extending into the sample solution was used for delivery of reagent solutions, reagent blanks, standard HCl solutions and KCl blanks, from a Lang-Levy 50-μl pipet which was connected at its tip to the delivery line. Solutions were forced into the titration vessel with a syringe connected to the 50-μl pipet. After additions, a sample from the titration vessel was drawn up the delivery line twice to rinse out any remaining solutions. The third line which was used for delivery of nitrogen extended to just above the sample solution. The fourth line was a vent which extended only to the top of the titration vessel. Constant temperature (±0.2 °C) was maintained by immersing the titration vessel in a thermostated water bath. Prepurified nitrogen was passed through a soda lime tube and was bubbled through a KCl solution at the same Γ/2 and temperature as the sample prior to entering the titration vessel.

The 2-ml sample in the titration vessel was stirred using a 3 × 10 mm magnetic stirring bar and allowed to equilibrate thermally, with the nitrogen blowing over the solution to remove carbon dioxide. When the rate of drift in pH due to loss of carbon dioxide became negligible (10–30 min), 50 μl of reagent (DTT in reaction 1 and methyl methanethiolsulfonate in reaction 2) in a KCl solution at the Γ/2 of the sample was added. After 5 min (reaction 1) or 1 min (reaction 2), the pH was titrated back to its value just prior to addition of the reagent using 0.01 M KOH at the Γ/2 of the sample. When excess reagent was added, a second aliquot of reagent was added which was equal in volume to the first aliquot, but which contained less reagent by a molar amount equal to the amount of protein present in the sample. After 5 (reaction 1) or 1 min (reaction 2), the solution was titrated back to its original pH value, when the pH decreased. Otherwise, the pH increase was simply noted. At this point, 50 μl of a KCl solution at the Γ/2 of the sample was added. When the pH decreased, the solution was back-titrated immediately with the KOH. Otherwise, the pH increase was noted. An HCl standard solution (50 μl containing 200 nmol of HCl at the Γ/2 of the sample) was then added to the sample and the solution immediately back-titrated in order to determine the exact titer of the KOH. When the second addition of reagent or the addition of KCl produced an increase in pH, the volume of KOH titrant required to produce the increase in pH observed in these instances was determined during the back-titration of the HCl standard. After each run the electrode was rinsed extensively with water in order to remove excess reagent, which if left on the electrode would cause the next sample to react prematurely.

The total amount of protons released in a reaction (ΔH) was calculated from eq 3 and 4

$$\Delta H = C_{OH}(V_r - V_{rb}) \quad (3)$$

$$C_{OH} = 200/(V_s - V_{sb}) \quad (4)$$

where V_r, V_{rb}, V_s, and V_{sb} are the volumes of KOH required to back-titrate the reagent, a reagent blank, the 200 nmol of HCl standard, and a blank for the HCl standard, respectively; C_{OH} is the concentration of the KOH titrant. When an addition produced an increase in pH, the negative of the volume of KOH titrant which would be required to produce the same increase in pH as the addition was set equal to the corresponding value of V in eq 3 and 4. The value of C_{OH} as determined from eq 4 at the end of each run was always within a few percent of the expected value.

For reaction 1 in the pH range 4.0–9.6 and reaction 2 over the entire pH range, the reagent blanks were obtained from the volume of KOH (V_{rb}) required to back-titrate the second addition of reagent. In this pH range for reaction 1 and concentration of DTT used (<12 mM), when papain-S-SCH_3 was replaced by papain-S-CH_2CONH_2, addition of DTT produced no proton release above that observed when DTT was added to a KCl solution. The use of a molar excess of 0.2 or less of DTT over papain-S-SCH_3 at pH >8 kept the blank due to ionization of excess thiol below 20% of the observed proton release. In some experiments, papain-S-SCH_3 was activated by a molar concentration of DTT which was less than the molar concentration of papain-S-SCH_3. In these runs, the volume of KOH required to back-titrate the KCl blank was used as the reagent blank. This KCl blank was also used to determine the blank correction for the HCl standard. The KCl blank correction for the HCl standard became significant below pH 4.

The stock solution of methyl methanethiolsulfonate was checked for the presence of acid and for its reactivity toward papain-S-CH_2CONH_2. In the pH range 3–7, addition of methyl methanethiolsulfonate in a KCl solution to solutions of KCl with or without papain-S-CH_2CONH_2 produced a negligible amount of protons (<0.02 proton per molecule of methyl methanethiolsulfonate) above that observed when these solutions were diluted with solutions of KCl containing no reagent. Thus, the use of the second addition of methyl methanethiolsulfonate as a reagent blank in reaction 2 appears justified. At pH values above 8.3, hydrolysis of the excess methyl methanethiolsulfonate occurs during the determination. The protons released by hydrolysis, however, do not appear in the value of ΔH when the second addition of reagent is used to determine the reagent blank. Furthermore, use of only a 5% excess of methyl methanethiolsulfonate in alkaline solutions limited the protons released on hydrolysis of excess reagent to

less than 0.05. It should be noted that in every case the reagent reacts with papain much faster than it hydrolyzes. Thus the 5% excess of methyl methanethiolsulfonate was sufficient to inactivate more than 99% of the papain.

It is realized that a proper blank correction requires that the volume of the sample prior to the addition of reagent or standard equals the volume of the sample prior to the addition of the blank. Similarly, final volumes of the sample after back-titration should be equal for the reagent or standard and the blank. The errors due to the inequality in volumes were determined and found to be negligible at the highest and lowest pH values where the blank corrections are greatest.

Below pH 4 high concentrations of DTT were used so that reaction 1 would proceed rapidly. In many of these runs, the thiol reagent had a nonspecific effect on the protein. Different amounts of protons were taken up or liberated when a high concentration of DTT was added to KCl solutions with and without papain-S-CH_2CONH_2. For studies of reaction 1 below pH 4, the blank was determined from the volume (V_{rb}) of titrant required for back-titration of pH decreases produced on the first addition of thiol reagent to a blank containing papain-S-CH_2CONH_2 in place of papain-S-SCH_3. When this addition produced a pH increase (lowest pH values), the negative of the volume of KOH required to produce the same pH increase was used for V_{rb} in eq 3 and 4. For the reported data, the proton changes accompanying the second addition of thiol reagent to papain-S-SCH_3 and papain-S-CH_2CONH_2 were within a few percent of each other. This observation indicates that the blank correction used for eliminating nonspecific effects of the DDT is valid.[2]

Determination of the Rate of Proton Release in Reaction 1. In one study the rate of activation of papain-S-SCH_3 was slowed down (by using 1 mM DTT at pH 4) so that the time dependence of the release of protons could be determined. After the first addition of DTT, the pH dependence as a function of time was noted for 30 min and the sample was back-titrated to relate the observed pH changes to amounts of titrant. The second aliquot of DTT was added and the pH observed as a function of time to establish the blank drift. After 30 min the blank was back-titrated and then the titer of the KOH was established in the usual manner with the standard HCl. The amount of protons released at given time periods was obtained from the difference between the values for the protons released by the first and second addition of thiol at comparable times after each addition. The correction for the time dependence of the blank was small. If the blank was assumed to be constant at its initial value, the reported value for the amount of protons released in the reaction would have been altered by less than 10%.

Correlation of Proton Release with Regeneration of Catalytic Activity in the Reaction of Papain-S-SCH_3 with Thiol. For these studies, assays for catalytic activity were performed using an Aminco-Morrow stopped-flow apparatus attached to a Beckman monochromator equipped with a logarithmic photometer. Since the time scale of the rate assay was the order of several seconds, a strip chart recorder (Heath EU-208) was used to monitor the change in absorbance. Solutions (4 ml) of papain-S-SCH_3 were adjusted to the concentration, pH, and $\Gamma/2$ of a potentiometric run. After thermal equilibration of the solution (at the temperature of the potentiometric run), thiol was added and the solution was transferred to the drive syringe of the stopped-flow apparatus, which was thermostated at the temperature of the potentiometric run. At appropriate times portions of the protein solution were shot against *N*-α-benzoyl-DL-arginine *p*-nitroanilide (0.1 mM) in buffer at the pH and $\Gamma/2$ of the potentiometric run. In addition to measuring the rate of regeneration of catalytic activity, these assays were used to ascertain that at each pH value the time allowed for thiol to react with papain was sufficient to convert over 90% of the papain-S-SCH_3 to active papain. These assays also were used to measure the extent of regeneration of active papain when less than a stoichiometric amount of thiol was added to the papain-S-SCH_3.

Fluorometric Titrations. The pH dependence of the fluorescence (excitation 280 nm, emission 350 nm) of buffered solutions of papain-S-SCH_3 (4 μM) was determined using a ratio fluorometer equipped with Schoeffel GM100 monochromators for excitation and emission. The instrument was built by Dr. David Ballou and Mr. Gordon Ford of this department. Constant temperature $\pm$0.2 °C was maintained by circulating water from a thermostated bath through the cell holder. When the temperature was below 20 °C, dry air was blown into the cell compartment to prevent fogging of the cuvette windows. Buffers were made using acetic acid–sodium acetate (pH 3–5.6), sodium monophosphate–potassium diphosphate (pH 5.9–8.0), Tris-HCl (pH 8.0–9.0), and sodium bicarbonate–sodium carbonate (pH 9.2–10). KCl was used where necessary to ensure constant ional concentration. All buffers and protein solutions were filtered through 0.45-μm Millipore filters into clean dry vials before use in fluorometric measurements. The pH dependence of the relative fluorescence intensity (I_{350}) was fitted using the weighted, least-squares method of Wilkinson (1961) to give the best K_a for the quenching group.

Results

Stoichiometry of the Reactivation of Papain-S-SCH_3 by DTT. DTT was chosen for the reactivation of papain-S-SCH_3, so that cyclization of the mixed disulfide intermediate would drive reaction 1 to completion. Since the equilibrium constant for reduction of cystine by DTT to cysteine and the oxidized cyclic form of DTT is about 1.3×10^4 (Cleland, 1964), papain-S-SCH_3 was expected to be fully regenerated with DTT without using a large excess of thiol. This consideration is important at high pH values where the blank due to the ionization of the excess thiol would decrease the accuracy of the titration. It was anticipated that the regeneration reaction would require 1 mol of DTT per mol of papain reactivated. However, at pH 9.2 it was observed that 0.8 mol of DTT regenerated more than 0.95 mol of papain. This result indicated that the methanethiol produced in reaction 1 could at least partially convert papain-S-SCH_3 to papain and methyl disulfide, when the concentration of DTT was limiting.

The Proton Content of Papain and Papain-S-SCH_3 from the Protons Released in Reactions 1 and 2. Equation 5 relates the difference in the proton content of unmodified papain (h_u) and papain-S-SCH_3 (h_m) to the protons released per molecule of papain reactivated ($\Delta h_{r1}'$) in reaction 1.[3]

$$\Delta h_{r1} \equiv \Delta h_{r1}' - t = 1 - (h_u - h_m) \qquad (5)$$

where t represents the contribution of the ionization of methanethiol to the protons released. A derivation of eq 5 is

[2] The validity of this correction also rests on the assumption that ionization of an amount of thiol reagent equivalent to the molar amount of protein-S-SCH_3 produces a negligible amount of protons. This assumption is certainly true below pH 4.

[3] $\Delta h_{r1}'$ and $\Delta h_{r2}'$ were determined by dividing the amount of protons released (ΔH eq 4 and 5) by the total amount of protein reacted.

given in the Appendix. The value of t was negligible at pH <8.3 and was less than 0.15 at the highest pH value used for reaction 1.[4] The relationship between the protons released in reaction 2 ($\Delta h_{r2}'$) and the proton content of papain and papain-S-SCH_3 is given by eq 6[3]

$$\Delta h_{r2} \equiv \Delta h_{r2}' + h_s = h_u - h_m \quad (6)$$

where h_s is the proton content of methylsulfinic acid produced in reaction 2.[5] The value of h_s was negligible above pH 4 and less than 0.15 at the lowest pH value used for reaction 2. Comparison of eq 5 and 6 indicates that the relationship of eq 7 should hold

$$\Delta h_{r2} = 1 - \Delta h_{r1} \quad (7)$$

The fact that this relationship is seen for the values of Δh_{r1} and Δh_{r2} determined in this work (Figure 1) is a strong indication that both measurements are valid. Furthermore, the agreement between the two methods also means that the concentration of papain used to calculate values of Δh_r from the amount of protons released is correct. Thus, the possibility of an error in $h_u - h_m$ because of an error in the absolute concentration of papain can be excluded.

Possible Complications due to Proteolysis and Other Reactions. Because papain is a proteolytic enzyme, it was necessary to exclude the possibility that papain-catalyzed proteolysis introduced an error in the measured value of Δh_r. The time course of regeneration of enzymatic activity was monitored at pH 4 and found to parallel proton release (Figure 2). Furthermore, the protons released were independent of the initial concentration of papain-S-SCH_3 (Figure 1). At pH values greater than 8, the correlation between proton release and active papain regenerated was tested by using less than an equivalent of thiol to regenerate papain partially. When papain was regenerated with less than an equivalent of thiol, the degrees of completion as measured by the protons released and the activity regenerated were within 10% of each other.

Under certain conditions proteolysis became significant. After reactivation of papain-S-SCH_3 was complete at alkaline pH values, there was a slow continual rate of proton release due to autolysis of the activated papain. Since values of $\Delta h_{r1}'$ were obtained by subtracting from the protons released during the 5 min used for activation, the protons released due to a blank incubated with the activated papain for 5 min (see the Methods section), autolysis should not significantly affect the reported values of $\Delta h_{r1}'$. Furthermore, the correction due to autolysis was always less than 30% of the total proton count observed during activation. Proteolysis also became a problem at about pH 3, where the rate of proton uptake due to proteolysis was comparable to the rate of reaction 1. Reaction 2 which yielded an inert product and which proceeded more rapidly than reaction 1 was the method of choice for determining $h_u - h_m$ at the extreme ends of the pH range where the rate of proton uptake or release due to proteolysis was highest.

It is unlikely that reduction of intrachain disulfide bonds or protein denaturation contributes to the proton count observed during the activation reaction. At each pH value above pH 4, for which we report values of Δh_r, the addition of an equivalent concentration of activating thiol to papain-S-CH_2CONH_2

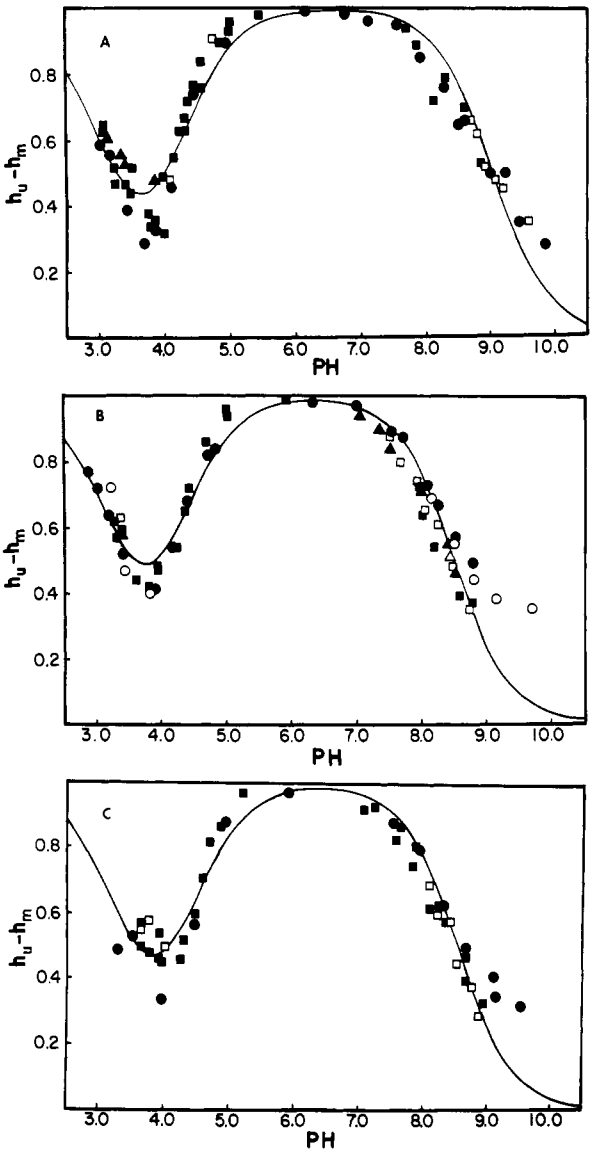


FIGURE 1: pH dependence of $h_u - h_m$. (A) 15 °C, Γ/2 0.05; (B) 29 °C, Γ/2 0.05; (C) 29 °C, Γ/2 0.15. Squares indicate demethylthiolation with dithiothreitol (reaction 1), circles indicate methylthiolation (reaction 2), and triangles indicate use of 2-mercaptoethanol for demethylthiolation of papain-S-S-CH_3. Open symbols indicate 50 μM protein and closed symbols indicate 100 μM protein. In the pH range 7-9.6, a 0.8-fold (B and C) or a 1.2-fold (A) molar excess of dithiothreitol over papain-S-SCH_3 was used for demethylthiolation. In the pH range 3.1-7.0, the DTT concentration was 1-184 mM. The higher DTT concentrations were used at the lower pH values. Several square symbols clustered around a single pH value represent runs with a varying DTT concentration. Methylthiolation was performed with 1.05-fold (pH 7-9.8) or a 2-fold (pH 2.9-7.0) molar excess of methyl methanethiolsulfonate. A 3000-fold (pH 3-4) or 2- to 10-fold (pH 7.0-8.5) molar excess of 2-mercaptoethanol was used for demethylthiolation.

[4] The value of t was estimated from the relationship $t = [K_t/([H] + K_t)][(\text{methanethiol})_f/(\text{papain-SH})_f]$, where K_t is the ionization constant of methanethiol and the subscripts f and i (see below) denote total molar amounts after and before activation. To calculate $(\text{methanethiol})_f$, it was assumed that reduction of oxidized dithiothreitol (DTT) by methanethiol was negligible so that when $(\text{DTT})_i > (\text{papain-S-SCH}_3)_i$, $(\text{methanethiol})_f = (\text{papain-SH})_f$ and when $(\text{DTT})_i < (\text{papain-S-SCH}_3)_i$, $(\text{methanethiol})_f = 2(\text{DTT})_i - (\text{papain-SH})_f$. The term $K_t/([H] + K_t)$ was determined using pK values of 10.24 (29 °C) and 10.46 (15 °C) for methanethiol. These pK values were estimated from the pK of 10.3 for methanethiol at 25 °C (Kreevoy et al., 1964), assuming that the value of 6.42 kcal/mol for the heat of ionization of ethanethiol (Irving et al., 1964) is the same as that for methanethiol. The term $K_t/([H] + K_t)$ is negligible below pH 8.3 and less than 0.15 at the highest pH values used for reaction 1.

[5] The value of h_s was determined from the relationship $h_s = [H]/([H] + K_s)$, where K_s, the ionization constant of methanesulfinic acid, was determined by titrating the methanesulfinic acid formed when 0.05 M methyl methanethiolsulfonate reacted with 0.075 M 2-mercaptoethanol. The values of pK's obtained were: 2.13 (Γ/2 0.05, 29 °C), 2.12 (Γ/2 0.15, 29 °C), 2.09 (Γ/2 0.05, 15 °C).

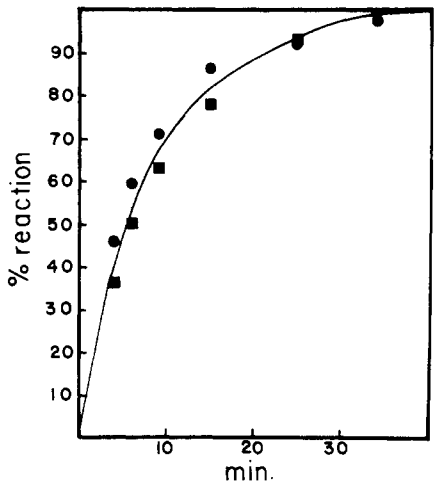


FIGURE 2: Time dependence of the regeneration of catalytic activity (●) and proton liberation (■) in the demethylthiolation of 100 μM papain with 1 mM DTT at pH 4.0, 15 °C, Γ/2, 0.05. The percent reaction was calculated from the ratio of the value observed at a given time to its final value.

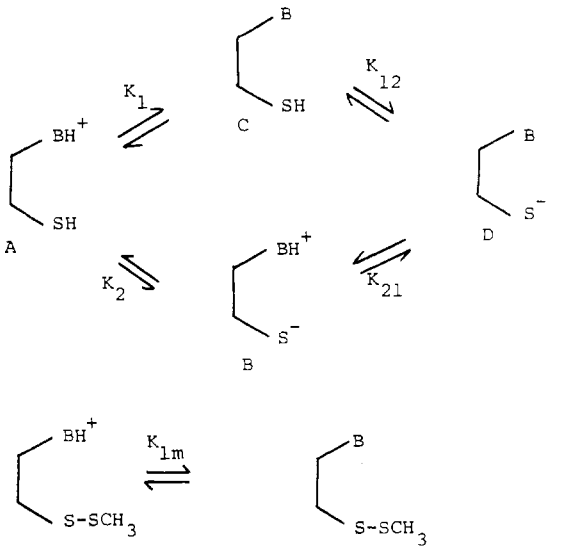


FIGURE 3: Ionization scheme for the thiol group and another group at the active site of papain.

yielded no proton count above that expected for the dilution of the thiol. Furthermore, equivalent concentrations of excess 2-mercaptoethanol and excess DTT, two thiols which should have widely differing reduction potential (Cleland, 1964), yielded the same values of Δh_r (Figure 1). Below pH 4 where higher concentrations of DTT were used, blanks containing papain-S-CH_2CONH_2 were determined to correct for reactions (between DTT and protein) other than reaction 1. For all the pH values used, over 90% of the catalytic activity of papain was regenerated in reaction 1 and over 99% of the catalytic activity of papain was lost in reaction 2. Treatment of the papain-S-SCH_3 produced in reaction 2 with excess thiol regenerated over 95% of the catalytic activity of papain. Subjecting papain to the incubation conditions at the highest pH values used for reaction 2 resulted in no loss in catalytic activity when the papain was assayed in the absence of added thiol.

It is unlikely that aggregation of the protein in the sample introduced an error in $h_u - h_m$ since the value of $h_u - h_m$ was independent of the protein concentration. It should be noted, however, that in alkaline solution the concentration of papain was close to its saturation level. Below pH 8.3, all determinations were made with clear solutions. Above this pH value, some of the solutions of papain-S-SCH_3 used for reaction 1 were faintly cloudy. If these solutions were allowed to stand for a few hours, they would develop a precipitate.

The pH Dependence of $h_u - h_m$. If there were no effect of the thiol group on other ionizations, the pH dependence of $h_u - h_m$ would reflect only the ionization of the thiol of Cys-25 (eq 8)

$$h_u - h_m = \frac{1}{1 + \frac{K_2}{[H]}} \tag{8}$$

where K_2 represents the dissociation constant of the thiol group of Cys-25. The marked deviation from eq 8 of the pH dependence of $h_u - h_m$ (Figure 1) clearly indicates that the thiol group at the active site of papain perturbs the pK of at least one other ionizable group in papain. If we assume that the ionization of the thiol group perturbs the pK of only one other group and vice versa (see Figure 3), eq 9 is obtained for the pH dependence $h_u - h_m$

$$h_u - h_m = \frac{\frac{2[H]}{K_I} + 1}{1 + \frac{[H]}{K_I} + \frac{K_{II}}{[H]}} - \frac{1}{1 + \frac{K_{1m}}{[H]}} \tag{9}$$

where K_I and K_{II} are the macroscopic ionization constants for removal of a proton from the doubly protonated form (A) and the singly protonated forms (B + C), respectively. These constants are related to the microscopic constants of Figure 3 by eq 10 and 11 (Edsall and Wyman, 1958)

$$K_I = K_1 + K_2 \tag{10}$$

$$\frac{1}{K_{II}} = \frac{1}{K_{12}} + \frac{1}{K_{21}} \tag{11}$$

Simms (1926) has shown that the relationship of eq 12 holds for the first term of eq 9.

$$\frac{\frac{2[H]}{K_I} + 1}{1 + \frac{[H]}{K_I} + \frac{K_{II}}{[H]}} = \frac{1}{1 + \frac{G_1}{[H]}} + \frac{1}{1 + \frac{G_2}{[H]}} \tag{12}$$

where G_1 and G_2 are titration constants and are related to K_I and K_{II} by eq 13 and 14.

$$K_I = G_1 + G_2 \tag{13}$$

$$\frac{1}{K_{II}} = \frac{1}{G_1} + \frac{1}{G_2} \tag{14}$$

Substituting eq 12 into eq 9, we get eq 15.

$$h_u - h_m = \frac{1}{1 + \frac{G_1}{[H]}} + \frac{1}{1 + \frac{G_2}{[H]}} - \frac{1}{1 + \frac{K_{1m}}{[H]}} \tag{15}$$

The solid lines in Figure 1 indicate the best fit of the observed pH dependence of $h_u - h_m$ to eq 15. Values of pK_{1m}, pG_1, pG_2, pK_I, and pK_{II} which gave the best fit to eq 15 are listed in Table I. Deviations from eq 15 of the measured values of $h_u - h_m$ may be a consequence of the thiol group affecting more than one ionization. For example, the positive deviations from eq 15 seen at high pH values can be accounted for by a slightly lower value for pG_2 and an upward perturbation in apparent pK of a group from about 9.5 in papain-S-SCH_3 to about 10

TABLE I: Parameters for Ionizations at the Active Site of Papain.

	Γ/2 0.05 15 °C	Γ/2 0.05 29 °C	Γ/2 0.15 29 °C
pG_1 or pK_I[a]	3.09 ± 0.04	3.29 ± 0.05	3.34 ± 0.07
pG_2 or pK_{II}[a]	9.04 ± 0.04	8.52 ± 0.03	8.60 ± 0.04
pK_{1m}	4.19 ± 0.04	4.26 ± 0.05	4.35 ± 0.06
pK_1[b]	4.19	4.26	4.35
pK_2[b]	3.13	3.34	3.38
pK_{12}[b]	7.94	7.55	7.59
pK_{21}[b]	9.00	8.47	8.56

[a] The difference in pG_1 and pG_2 indicates (eq 13 and 14) $pG_1 \approx pK_I$ and $pG_2 \approx pK_{II}$. Values of pG_1, pG_2, and pK_{1m} and the accompanying standard deviations were obtained by a least-squares fitting of the pH dependence of $h_u - h_m$ to eq 15 with no constraint on the variations of the parameters. The fitting of the data was done by a modification of the Chifit program of Bevington (1969). The modified program was written by Dr. S. L. Hsu of The University of Michigan Physics Department. [b] Microscopic ionization constant defined in Figure 3 and calculated assuming $pK_{1m} = pK_1$.

in papain. Such a perturbation could be caused by the extra negative charge (from the thiol anion) in papain at high pH values. Interestingly, other workers have shown that an ionization with an apparent pK of about 10 affects the environment at the active site of papain (Brubacher and Bender, 1966; Hinkle and Kirsch, 1970, 1971; Lewis and Shafer, 1974). Deviations from the solid lines in Figure 1 also could be caused by small perturbations in several groups. Additional pH-difference titrations with other derivatives of papain may reveal the source of these deviations.

Fluorometric Titrations. Lowe and Whitworth (1974) presented evidence showing that His-159 is responsible for the quenching of fluorescence of Trp-177 in papain-S-SCH_2CH_2OH, and that the pK of this group as determined by fluorometric titration is 4.2. Similar fluorometric determination (Figure 4) of the pK of papain-S-SCH_3 yielded pK's of 4.1 (Γ/2 0.05, 15 and 29 °C) and 4.3 (Γ/2 0.15, 15 and 29 °C).

Discussion

Effects of the Active Site Thiol Group on Neighboring Ionizations. The N-1 of His-159 is the ionizable group closest to the sulfur atom of Cys-25 (Drenth et al., 1970, 1971). Therefore, it is the most likely group to undergo a perturbation in its ionization constant in the transition between papain-S-SCH_3 and papain. The similarity between the fluorometrically determined pK values of His-159 in papain-S-SCH_3 and K_{1m} is consistent with this view that K_{1m}, the ionization constant in papain-S-SCH_3 which is altered in the transition to papain, is the acid dissociation constant of the protonated N-1 of His-159.

Assuming that the ionization constant for the imidazolium ion adjacent to an un-ionized thiol group (K_1) is equal to the dissociation constant for the imidazolium ion adjacent to the *S*-methylthio group (K_{1m}), eq 10 and 11, and the identity $K_1K_{12} = K_2K_{21}$, can be used to calculate the microscopic equilibrium constants of Figure 3 from K_I and K_{II}. These constants are listed in Table I. If the ionization scheme depicted in Figure 3 obtains, the pK of the thiol group at the active site of papain would shift from 7.94 to 3.13 (at 15 °C) when the neighboring imidazole group is protonated. If a unit-positive charge (4.8×10^{-10} esu) is localized on the N-1 of His-159, one can assess the feasibility of a neighboring positive charge

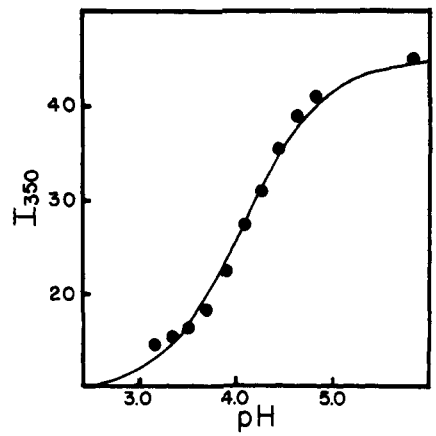
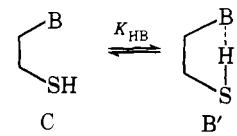


FIGURE 4: pH dependence of the fluorescence intensity (I_{350}) of 4 μM papain-S-SCH_3 at 29 °C, Γ/2 0.05.

on His-159 perturbing the pK of the thiol group 4.81 units downward at 15 °C. Setting the electrostatic potential for two point charges equal to the difference in free energies for dissociation, we obtain

$$RT \ln \frac{K_2}{K_{12}} = \frac{q^2N}{Dr} \qquad (16)$$

where q is the electronic charge, N Avogadro's number, and r the distance between the charges. Setting r to 3.4×10^{-8} cm, the distance between the S of Cys-25 and the N-1 of His-159, and solving eq 16 for the dielectric constant D, one obtains a value of 15 for the effective dielectric constant in the region between the thiol group and the N-1 of His-159. Since the value of the effective dielectric constant for intramolecular charge–charge interactions of organic molecules in aqueous solution can be much less than 15 (Westheimer and Kirkwood, 1938), the positive charge on His-159 could account entirely for the thiol group at the active site of papain having a pK of ~3.1. Other factors such as the positive charge on the protein and the microenvironment might also contribute to the low pK's observed for the thiol and imidazoyl groups at the active site of papain. Also, more complex ionization schemes which involve pK perturbations of additional groups in the transition between papain and papain-S-SCH_3 can be devised to account for the pH dependence of $h_u - h_m$ without requiring an abnormally low pK for the thiol group of Cys-25. Consideration of such complex schemes, however, should await observations which are not adequately accounted for in terms of the simple ionization scheme of Figure 3. The values for pK_1 and pK_2 listed in Table I indicate that the equilibrium constant for the prototropic shift C ⇌ B is 12 (K_2/K_1). Thus in the physiological pH range, about 90% of the papain contains a thiolate–imidazolium ion pair at the active site if the ionization scheme depicted in Figure 3 obtains. It should be pointed out, however, that the pH dependence of $h_u - h_m$ by itself does not require that protomer B exists as an ion pair. Replacement of B in Figure 3 with the hydrogen-bonded intermediate B′ also would yield the observed pH dependence of $h_u - h_m$



(17)

If papain ionized through B′ and C rather than through B and C, K_{HB} would have to be about 12 to account for the pH dependence of $h_u - h_m$. Studies of the proton nuclear magnetic

resonance spectra of the C-2 H of His-159 of papain should indicate whether His-159 is protonated in the pH range 3–7, and thus reveal whether the imidazoyl group of His-159 and the thiol group of Cys-25 exist as an ion pair or as a hydrogen-bonded pair. The existence of an ion pair is consistent with kinetic and spectrophotometric studies of Polgar (1973, 1974) and the studies of zinc binding by Drenth et al. (1975) which indicated the existence of the thiolate anion at low pH values. However, the pH-difference titrations reported in this work indicate a substantially different pK value (~3.2, see Table I) for the active-site thiol group than the value of 4 deduced from the pH dependence of rate constants and changes in absorbance associated with the alkylation of papain and the pH dependence of the binding of zinc ion. Although further work is needed to identify the source of this discrepancy, a perturbation in molar absorptivity, reactivity, and affinity for zinc ion of the thiol group by another group in addition to His-159 could account for the difference in the pK values obtained using these different methods.

Relationship between Ionizations at the Active Site and the pH Dependence of the Catalytic Efficiency of Papain. If B, C, or B′ (see Figure 3 and eq 17) were the catalytically active form of papain, and if His-159 and Cys-25 were the only ionizable species involved in determining the catalytic efficiency of the enzyme, plots of k_{cat}/K_M vs. pH should be bell shaped and determined by eq 18.

$$\frac{k_{cat}}{K_M} = \frac{\frac{k_{cat}^\circ}{K_M^\circ}}{1 + \frac{[H]}{K_I} + \frac{K_{II}}{[H]}} \tag{18}$$

where k_{cat}° and K_M° are the intrinsic pH-independent constants. Papain-catalyzed reactions usually give rise to bell-shaped plots of k_{cat}/K_M vs. pH predicted by eq 18. These plots yield values of K_{II} consistent with those determined from pH-difference titrations. The values of the microscopic constants listed in Table I indicate that K_{II} is a good approximation of the ionization constant of the imidazolium ion of His-159 when it is adjacent to the thiol anion of Cys-25. Interestingly, the kinetically determined values of K_I are usually about 4.1 and differ substantially from the values of about 3.2 determined from pH-difference titrations. This discrepancy indicates that a third ionization with a pK of about 4 may be involved in regulating the catalytic activity of papain, assuming that the values of K_I determined from plots of k_{cat}/K_M vs. pH are not perturbed by kinetic constants. If this situation obtains, the activity of the enzyme should also depend on an ionization with a pK of about 3.2, in addition to the pK dependence normally reported. The pK 3.2 dependence could have easily been missed since most k_{cat}/K_M–pH profiles for papain have not been determined below pH 3.5. Observations of Sluyterman and Wijdenes (1973), Bendall and Lowe (1976), and preliminary studies in this laboratory indicate that the acid limb of the k_{cat}/K_M–pH profile for the papain-catalyzed hydrolyses of α-*N*-benzoyl-DL- and -L-arginine *p*-nitroanilide indeed depends on the ionization of two acidic groups.

Conclusions

Measurement of the protons released on introduction and removal of a methylthio group at the active site of papain yields information about the ionization behavior of the thiol group at the active site of papain and other groups whose ionization is affected by the thiol group. Similar methylthio pH-difference titrations may be useful in determining the ionization behavior of thiol groups in other proteins. The methylthio pH-difference titrations presented in this study indicate that ionization of the thiol group at the active site of papain is linked to the ionization of His-159. At 29 °C, $\Gamma/2$ 0.05, the pK of the thiol group changes from 3.3 to 7.6 on deprotonation of His-159. Similarly, the pK of His-159 shifts from 4.3 to 8.5 when the active site thiol group is deprotonated. Microscopic ionization constants determined in this study indicate that in the physiological pH range, ~90% of the papain exists in a form wherein the active site contains a thiol anion and an imidazolium cation.

Acknowledgment

The authors are happy to acknowledge excellent technical assistance from Marlene A. DeSmet during the early stages of this work. It is also a pleasure for the authors to acknowledge helpful discussions of this work with Dr. Rowena G. Matthews.

Appendix: Ambiguities Associated with pH Dependencies Generated by Linked Ionizations

Equation 19 describes the pH dependence of a property (R) which is a function of the linked ionization depicted in Figure 3.

$$R = \frac{\frac{R_A[H]}{K_I} + \frac{R_B K_2 + R_C K_1}{K_1 + K_2} + \frac{R_D K_{II}}{[H]}}{\frac{[H]}{K_I} + 1 + \frac{K_{II}}{[H]}} \tag{19}$$

where R_A, R_B, R_C, R_D are the values of R for species A, B, C, and D, respectively.

Whereas K_I and K_{II} can be determined by fitting the pH dependence of R to equation 19, the ratio K_1/K_2, which would allow calculation of the ionization constants for the individual species in Figure 1, cannot be obtained without knowledge of the value of R_B and R_C. Furthermore, the possibility of a perturbation of the values of R_A, R_B, R_C, and R_D by a third group in the protein complicates the fitting of the pH dependence of R to eq 19.

Derivation of Equation 5. Equation 20 relates the amount of DTT consumed in reaction 1, $(DTT)_i - (DTT)_f$, to the amount of papain and methanethiol produced.

$$(DTT)_i - (DTT)_f = \frac{(\text{papain-SH})_f + (\text{methanethiol})_f}{2} \tag{20}$$

Since the DTT added was fully protonated (i.e., the pH of the DTT solution was not adjusted to the pH of the run prior to its addition), each molecule of DTT consumed produces two protons. Thus, the amount of protons released in reaction 2 (above the blank) per molecule of papain reactivated is given by eq 21.

$$\Delta h_{rl}' = \frac{(\text{papain-SH})_f + (\text{methanethiol})_f}{(\text{papain-SH})_f} - (h_u - h_m) - \frac{[H]}{[H] + K_t}\frac{(\text{methanethiol})_f}{(\text{papain-SH})_f} \tag{21}$$

where K_t is the ionization constant of methanethiol. Substituting the definition

$$t \equiv \frac{K_t}{[H] + K_t}\frac{(\text{methanethiol})_f}{(\text{papain-SH})_f} \tag{22}$$

into eq 21 and rearranging, one obtains

$$\Delta h_{rl}' - t = 1 - (h_u - h_m) \tag{5}$$

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Fluorescence Energy-Transfer Measurements between the Calcium Binding Site and the Specificity Pocket of Bovine Trypsin Using Lanthanide Probes†

Dennis W. Darnall,*‡ Floyd Abbott, Joseph E. Gomez, and Edward R. Birnbaum

ABSTRACT: Using fluorescence energy-transfer experiments we have measured the distance between the specificity pocket and the calcium ion binding site of bovine pancreatic trypsin. Proflavin and thionine were used to block the specificity site, whereas various lanthanide ions were substituted for the calcium. It was then possible to choose various donor–acceptor pairs which exhibited suitable energy transfer. We have calculated the distance between proflavin and Nd(III), Pr(III), and Ho(III) to be 10.9, 10.3, and 10.3 Å, respectively. This agrees very well with the value of approximately 10 Å we obtained between the methyl protons of *p*-toluamidine (a competitive inhibitor) and Gd(III) using nuclear magnetic resonance techniques (Abbott, F., Gomez, J. E., Birnbaum, E. R., and Darnall, D. W. (1975), *Biochemistry 14,* 4935). This is strong evidence that, in solution, the calcium binding site is composed of the side chains of Ser-190 and Asp-194.

It has been established for some time that bovine pancreatic trypsin binds one calcium ion (Delaage and Lazdunski, 1967). Although the presence of calcium has no apparent effect upon the activity of trypsin, the metal ion does retard denaturation and degradation of the protein by autolysis (Green and Neurath, 1953; Delaage and Lazdunski, 1967; Gabel and Kasche, 1973; Lazdunski and Delaage, 1965). The exact nature of the single trypsin calcium ion binding site has been the subject of several investigations. Chemical modification and titration of carbonyl side chains on trypsin indicate that at least one carboxyl group is involved in calcium ion coordination to the enzyme (Abita and Lazdunski, 1969; Delaage and Lazdunski, 1967; Duke et al., 1952). It was earlier proposed that the cal-

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