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The Activation of Papain and Ficin by Phosphorothioate*

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ABSTRACT: Phosphorothioate (PSH) was found to activate papain and ficin in an equimolar ratio. One molecule of PSH is reversibly incorporated into the protein molecule with the formation of one free sulfhydryl group, which is directly related to the enzymic activity of papain and ficin, as was demonstrated by the reaction of iodoacetic acid with the above proteins. The equilibrium constant, K, for the activation of papain was evaluated from (a) the change in activity of papain upon the addition of small quantities of PSH, and (b) the simultaneous decrease in fluorescence intensity. The value obtained by the two methods was found to be 6.0×10^6 l./mole at 23°. The value of the enthalpy change accompanying the reaction, ΔH, was derived from activation curves at various temperatures. $\Delta H = -9.8$ kcal/mole. The decay of fluorescence of papain after a rapid addition of PSH is of the order of seconds, and therefore the kinetics of the interaction between papain and the activator could be evaluated from fluorescence decay curves. The kinetic studies showed that the reaction between papain and PSH is a second-order reaction with a rate constant $k_1 = 3.2 \times 10^3$ sec^{-1} mole^{-1} l. This is consistent with the observation that 1 mole of PSH reacts with 1 mole of papain. The rate constant of the decomposition of the papain–PS complex, k_{-1}, as calculated from the equation $K = (k_1/k_{-1})$, is equal to 5.3×10^{-4} sec^{-1}, which corresponds to a half-life of 21 min for the isolated papain–PS complex. The half-life of the isolated papain–PS complex, obtained after gel filtration, was found to be 28 min, which is in good agreement with the value obtained from the fluorescence studies. The equilibrium constant, K, for the reaction between PSH and ficin was found to be $K = 6.5 \times 10^6$ l./mole at 23°.

It is now accepted that papain and ficin possess a single reactive sulfhydryl group, which is essential for activity, and which probably participates in the catalytic action of the enzyme (Smith and Kimmel, 1960; Smith, 1958b). Furthermore, it is generally agreed that papain and ficin are activated by reduction (Smith, 1958a; Finkle and Smith, 1958). The study presented here was prompted by the fact (Neumann, 1965) that papain and ficin are activated by phosphorothioate in a 1:1 molar ratio. Phosphorothioate was shown in previous papers to be a reducing agent (Neumann *et al.*, 1965) and to cleave disulfide bonds in small model molecules (Neumann, 1965) and in proteins (Neumann *et al.*, 1964). In this work we studied the mode of activation of papain and ficin by phosphorothioate.

Experimental Section

Materials

Papain and Ficin. Twice-crystallized-papain and ficin prepared by the Worthington Biochemical Corp. were used. Every batch used was checked for activity in the absence of activator. Only batches that showed

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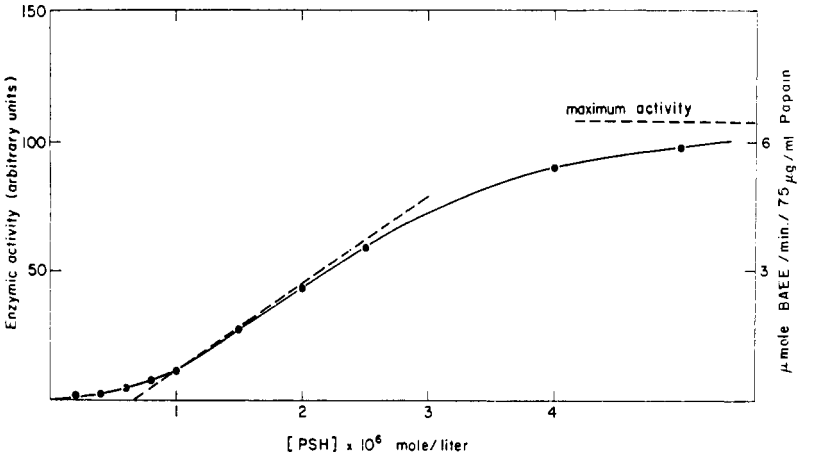

FIGURE 1: The enzymic activity of 75 μg/ml of papain as a function of PSH concentration at 28°. For assay conditions see Experimental Section.

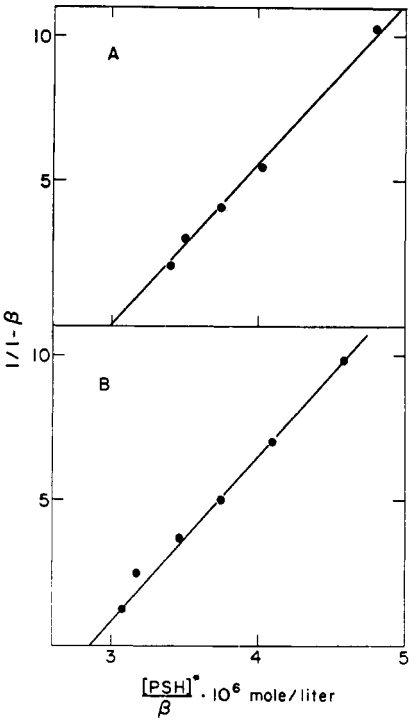

FIGURE 2: Plot of $1/(1-\beta)$ *vs.* $[PSH]^*/\beta$. $[PSH]^*$ and β were derived (A) from the enzymic activity curve given in Figure 1, and (B) from the fluorescence intensity curve given in Figure 3.

no activity under our experimental conditions were used. BAL[1] was purchased from Organic Research Chemicals Ltd., Poyle Estate, Bucks, England. Sephadex G-25 was purchased from Pharmacia, Uppsala, Sweden. Iodoacetic acid was obtained from Eastman Organic Chemicals, Rochester, N. Y. PMB was purchased from Sigma Chemical Co., St. Louis, Mo. Phosphorous trichloride labeled with ^{32}P, radioactive sulfur ^{35}S, and ^{14}C-labeled iodoacetic acid were obtained from The Radiochemical Centre, Amersham, Bucks, England. BAEE was purchased from Mann Research Laboratories Inc., New York, N. Y.

Preparation of Trisodium Phosphorothioate. Trisodium phosphorothioate was prepared from thiophosphoryl chloride (Yasuda and Lambert, 1954; Knotz, 1949) and sodium hydroxide by the modification of Akerfeldt (1960). Two radioactive preparations were synthesized, one labeled with ^{35}S (sp act. 30 c/mole) and the other with ^{32}P (sp act. 112 c/mole).

Methods

Measurements of Enzymatic Activities. Esterase activity of papain and ficin was assayed by determining the rate of hydrolysis of BAEE using a Radiometer Model TTT-1a autotitrator with 0.1 N NaOH as titrant. The rate of hydrolysis was calculated from the amount of NaOH consumed per minute. The reaction mixture was 10 ml in volume containing 0.045 μg/ml of enzyme, 0.05 M NaCl, and 0.1 M BAEE. The 10^{-3} M activator solution was added from a microsyringe in 2–10-μl portions. Temperature control (±0.1°) was provided. The reaction was carried out under nitrogen atmosphere.

Gel Filtration. Sephadex G-25 in a column (1.5 × 45 cm) was used to separate the labeled papain preparations. Acetic acid (0.1 M) was used as eluent, 3.5-ml fractions being collected. Protein concentrations of the solutions of papain were calculated from their absorption at 280 mμ using the molar absorption ε 51,000 as given by Glaser and Smith (1961). For the estimation of the amount of phosphorothioate or carboxymethyl residue bonded to the protein, 0.1 ml eluates were plated on planchets, dried, and counted for 1–5 min in a Nuclear-Chicago gas-flow counter equipped with an automatic sample changer and digital

[1] Abbreviations used: BAL, 2,3-dimercaptopropanol; PMB, sodium *p*-hydroxymercuribenzoate; BAEE, benzoyl-L-arginine ethyl ester; PSH, phosphorothioate.

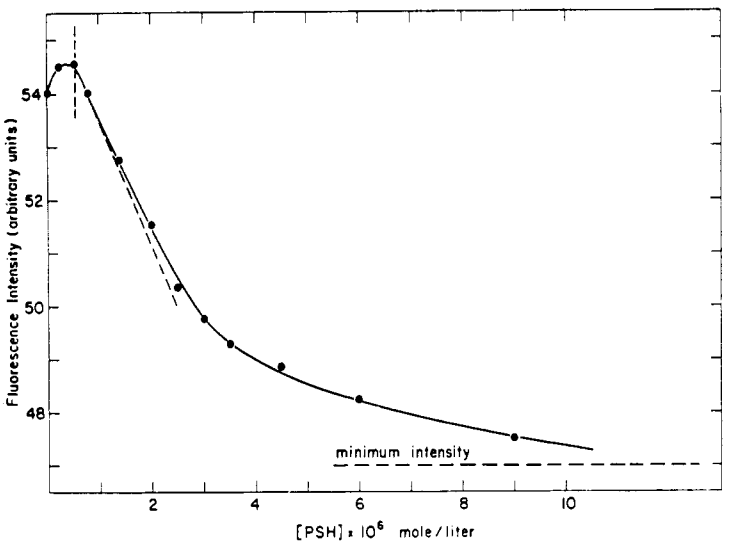

FIGURE 3: The fluorescence intensity of 75 μg/ml of papain as a function of PSH concentration at 23°. For experimental conditions see Experimental Section.

recorder. Concentrations of the radioactive material were calculated by comparison with known standards prepared, plated, and counted together with the unknown solutions. The optical densities of the solutions were measured with a Beckman DU spectrophotometer, using quartz curvets with a light path of 10 mm.

Titration of the Sulfhydryl Groups. The free sulfhydryl groups of the proteins produced upon activation of papain and ficin by phosphorothioate were assayed by titration with PMB (Boyer, 1954). PMB was added stepwise to the activated papain solution and the change in activity and optical density at 250 mμ was measured after each addition. The free sulfhydryl group of the activated papain or ficin was also estimated from the amount of iodoacetic acid required to inactivate the respective enzyme in the presence of activator and 0.1 M substrate.

Amino Acid Analysis. For amino acid analysis, activated and alkylated papain was subjected to hydrolysis with 6 N HCl for 18 hr at 110°. A Spinco Model 120B amino acid analyzer (Spachman *et al.*, 1958) was used for all determinations.

Fluorescence intensity at 345 mμ was measured with an Aminco Keirs spectrophosphorimeter using an excitation wavelength of 285 mμ. To avoid errors due to changes in intensity in the light source, the fluorescence intensity of each sample was compared to that of a standard solution of tryptophan.

Fluorescence decay curves were obtained with a Tektronix 502A oscilloscope attached to the above Aminco Keirs spectrophosphorimeter.

Results and Discussion

The Activation of Papain by Phosphorothioate. The hydrolysis of BAEE catalyzed by papain and ficin was followed by a recording autotitrator as described under Methods. First the activity of papain and ficin in the absence of the activator was checked for several minutes and then 2–10-μl portions of 10^{-3} M PSH were added at 10-min intervals. The quantities of PSH added were chosen so that no more than 10% activation would result in the enzyme. To achieve maximum activity, the experiment was completed by adding a 100-fold excess of PSH. Rates of hydrolysis, in slope units, were calculated and plotted *vs.* the amount of PSH present in the reaction mixture. A typical example of such an activity curve at 28° in which 75 μg/ml of papain was used, is given in Figure 1. We assume that the upward concave part of the curve at low concentration of PSH (<10^{-6} mole/l.) is due to the presence of another species which has a much higher affinity for

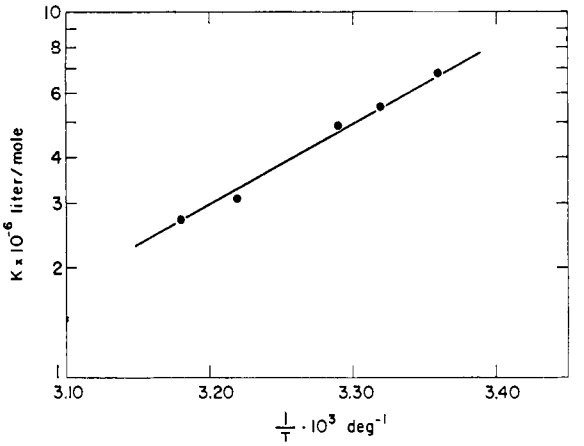

FIGURE 4: The variation of the equilibrium constant of the papain–PSH system, K, with the absolute temperature, T, plotted as log K *vs.* $1/T$.

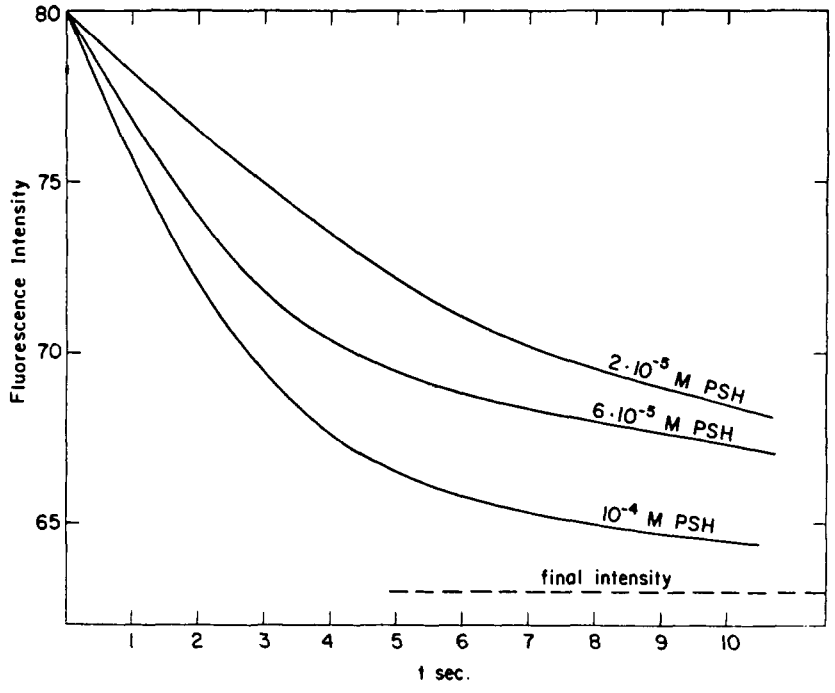

FIGURE 5: The decrease with time of the fluorescence intensity of papain (20 μg/ml) after a fast addition of excess of PSH at pH 4.8 (Tris-acetate buffer 0.05 M) and 23°.

PSH than papain has. Using this assumption, all activation curves obtained are characteristic to a mole per mole interaction between the enzyme and activator. The extrapolation of the curve to zero gives a value of 6.5×10^{-7} mole/l., which is the amount of PSH bonded to this species, and is, therefore, unavailable for activation. The effective concentration of the activator [PSH]* in this assay is, therefore, [PSH]* = [PSH] -6.5×10^{-7}. The type of activation shown in Figure 1 suggests a mole per mole interaction between the inactive enzyme, E, and the activator, A, having a relatively high equilibrium constant, K, given by eq 1.

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \tag{1}$$

$$K = \frac{k_1}{k_{-1}} = \frac{[EA]}{[E][A]} \tag{2}$$

[E], [A], and [EA] are the molar concentrations of inactive enzyme, activator, and activated enzyme, respectively. Introduction of the factor β, defined here as the ratio of the active enzyme concentration to the total enzyme concentration, into eq 2 leads to

$$K = \frac{\beta}{(1 - \beta)(a - \beta e)} \tag{3}$$

where a and e are the total concentrations of the activator and the enzyme, respectively. Equation 3 may be converted into a more useful form (eq 4).

$$\frac{1}{1 - \beta} = K\frac{a}{\beta} - Ke \tag{4}$$

A plot of $1/(1 - \beta)$ *vs.* a/β should yield a straight line at constant enzyme concentration with a slope of K. Equation 4 is a general expression for every mole per mole interaction and is useful in cases in which the concentration of the binding protein is uncertain, such as in the case of papain.

The data presented in Figure 1 are replotted in Figure 2A according to eq 4. β is equal to the ratio of the apparent activity to the maximum activity. The slope of the line in Figure 2 is equal to the equilibrium constant, K, and its value is $K_{28°} = 5.8 \times 10^6$ l./mole. It is interesting to note that the molar concentration of pure papain in the above system, as derived from the intercept of this line, is 3×10^{-6} mole/l., which shows that the enzyme batch used was 75% pure.

The activation of ficin with PSH is similar to that of papain. One equivalent of PSH yields about 90% activation. From the activity curve of ficin, using the same technique as was applied for papain, the equilibrium constant was found to be $K = 6.5 \times 10^6$ l./mole at 23°.

Change in fluorescence intensity upon activation of papain was observed. The percentage of quenching was found to depend on pH, and at pH 5.8 (Tris-acetate buffer 0.1 M) the fluorescence intensity of the fully activated papain was found to be 86% of that of its nonactive precursor. The change in the fluorescence intensity of 75 μg/ml of papain with PSH concentration at pH 5.8 and 23° is shown in Figure 3. The slight increase

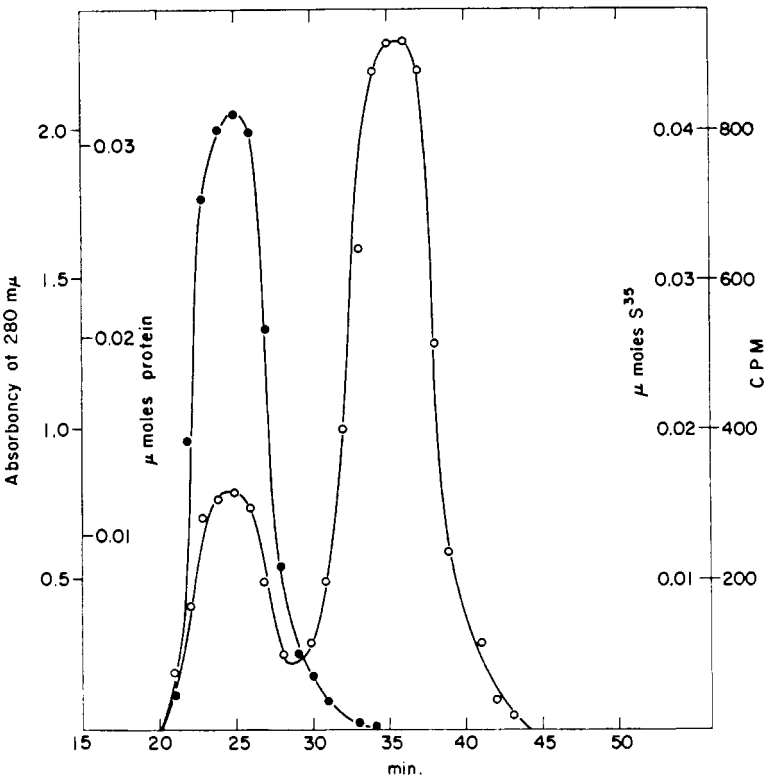

FIGURE 6: A typical gel filtration pattern of a mixture of papain (2 μmoles) and $P^{35}SH$ (4 μmoles). For details see text.

in fluorescence intensity at low concentrations of PSH may be due to the species which binds PSH as mentioned above, and which itself acts as a quencher toward papain. Extrapolation to maximum intensity shows that the amount of unavailable PSH is 6.5×10^{-7} mole/l., which is identical with that found from the activity curve (see Figure 1). The replot of Figure 3, according to eq 4 in analogy to the enzymic activity method, is given in Figure 2B. The values of K and e as derived from the slope and the intercept of this line are $K_{23°} = 6.0 \times 10^{6}$ l./mole and $e = 3 \times 10^{-6}$ mole/l. The addition of PSH to ficin only slightly decreases the fluorescence intensity of this protein, and therefore the fluorescence technique described above cannot be applied for this enzyme.

In order to obtain more information about the type of reaction between PSH and papain during the activation process we used the enzymatic activity method to obtain the K values for different temperatures in the range 24–41°. The enthalpy of binding, ΔH, was then derived from the well-known thermodynamic equation

$$\frac{\mathrm{d} \ln K}{\mathrm{d}\frac{1}{T}} = -\frac{\Delta H}{R} \qquad (5)$$

where T is the absolute temperature. The plot of log K *vs.* $1/T$ for the papain–PSH system is given in Figure 4. The ΔH value obtained from the slope is $\Delta H = -9.8$ kcal/mole. This relatively high value of ΔH may be due to a cleavage of a high-energy bond by which the cysteine residue is bound to an unknown group, X, in the native papain molecule.

$$\begin{matrix} \mathrm{X} \\ | \\ \mathrm{S} \end{matrix} + \mathrm{PSH} \rightleftharpoons \begin{matrix} \mathrm{X\text{-}SP} \\ \\ \mathrm{SH} \end{matrix} \qquad (6)$$

The presence of a high-energy bond in papain before activation was postulated by Smith (1958b).

Kinetics of the Papain–PSH Reaction. The time of decrease in fluorescence intensity of papain after rapid addition of PSH was found to be several seconds. Thus from the change in fluorescence with time, at various concentrations of PSH and enzyme, the kinetics of the interaction between papain and PSH could be evaluated.

We have recorded the fluorescence decay curves of several systems at pH 4.8 (Tris-acetate buffer 0.05 M) and 23°, in which a 50–100-fold excess of PSH was rapidly introduced into a dilute solution of papain. Three typical decay curves are shown in Figure 5. In all cases first-order decay curves were obtained in which the rate constants were found to be proportional to PSH

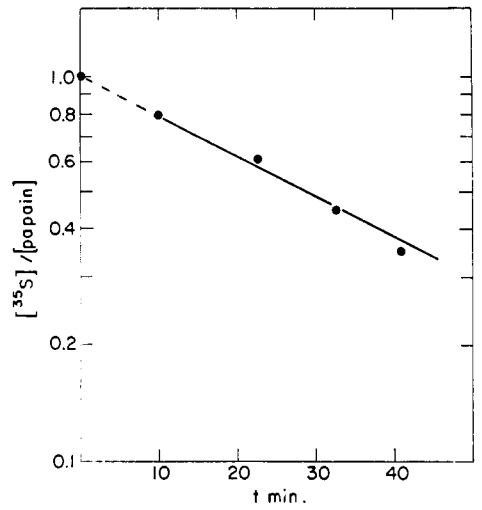

FIGURE 7: The variation of the molar ratio P^{35}SH/papain with the isolation time, t, as obtained by gel filtration of a mixture of papain (2 μmoles) and P^{35}SH (4 μmoles) using a set of Sephadex G-25 columns of different lengths.

concentration. Different concentrations of papain at constant concentration of PSH gave the same rate constants. Thus the reaction between papain and PSH is first order with respect to each of the reactants. Division of the pseudo-first-order rate constants obtained from the fluorescence decay curves by the corresponding concentration of PSH gave the second-order rate constant value $k_1 = 3.2 \times 10^3$ sec^{-1} mole^{-1} l. for all experiments carried out. The first-order rate constant k_{-1} of the decomposition of the papain–PS complex can be evaluated from the relation between the equilibrium constant K and the rate constants of formation k_1 and decomposition k_{-1} of the enzyme–activator complex $K = k_1/k_{-1}$. Substituting $K = 6.0 \times 10^6$ l./mole^{-1} and $k_1 = 3.2 \times 10^3$ sec^{-1} mole^{-1} l. gives $k_{-1} = 5.3 \times 10^{-4}$ sec^{-1} which corresponds to a half-life of 21 min for the isolated papain–PS complex. It was possible to measure k_{-1} directly, and the measured value was in satisfactory agreement with the calculated one (see next paragraph).

Reversibility of the PSH–Papain Reaction. The activation of papain by PSH was studied further to ascertain whether PSH was bound to the activated enzyme species. Papain (2 μmoles) was activated by PSH (4.0 μmoles). PSH was labeled in one set of experiments with ^{35}S and in the other set with ^{32}P. The reaction mixture, 2.5 ml in volume, was transferred to Sephadex columns (G-25, crude, 1.5 cm in diameter) of various lengths (60, 45, 30, and 20 cm), equilibrated with 0.1 M acetic acid. Elution was performed at room temperature using the same solvent. The velocity of flow was kept constant (1 ml/min), thus the time required for the elution of papain was proportional to the length of the column. The amount of PSH bound to the protein was evaluated from optical density and radioactivity measurements, as described in the Experimental Section. A typical gel filtration pattern is given in Figure 6. However, the amount of PSH bound to the protein varied with the length of the column, *i.e.*, with time required for its elution. The semilogarithmic representation of the variation of the molar ratio of bound PSH:papain with the isolation time, t, gives a straight line (Figure 7) from whose slope the first-order rate constant $k_{-1} = 4.1 \times 10^4$ sec^{-1} was obtained. This corresponded to a half-life of 28 min for the isolated papain–PS complex. This agrees well with the values obtained from the fluorescence studies. The higher value of k_{-1} obtained by this method can be explained by the fact that the separation of the papain–PS complex from the reactants is not complete. The same results were obtained both with ^{32}P- and ^{35}S-labeled PSH.

Inhibition of PSH-Activated Papain and Ficin by Idoacetic Acid and by p-Mercuribenzoate. The inhibition of papain and ficin by typical sulfhydryl reagents such as iodoacetic acid, PMB, and *N*-ethylmaleimide has been studied and reported (Light, 1964). It was, therefore, important to show that during the activation process with PSH also one sulfhydryl group per mole of enzyme is liberated, and that this sulfhydryl group is directly involved in the enzyme activity of papain and ficin. Because the activation of these enzymes with PSH occurs at an equimolar concentration of enzyme and activator, the properties of their sulfhydryl group could be studied directly.

The inhibition of papain and ficin by iodoacetic acid was followed by an autotitrator. Various amounts of iodoacetic acid were added to 10 ml of fully active papain solution of the following composition: 0.1 M BAEE, 0.05 M NaCl, 4×10^{-6} M papain or ficin, and 4×10^{-6} M PSH. Prior to the addition of iodoacetic acid, the reaction was allowed to proceed for 5 min, and then 5-μl portions of 10^{-3} M iodoacetic acid were added from a microsyringe. Upon the addition of 40 μl of iodoacetic acid (a total of 4×10^{-6} M) the reaction stopped completely for both enzymes. From these experiments we concluded that the activator PSH produced a new sulfhydryl group on the enzymes, which is directly related to their catalytic properties.

The inhibition of papain and ficin by PMB was also measured. The reaction of PMB with the PSH-activated papain (or ficin) provided more information about the "thiol" group of phosphorothioate present in the activated papain. In solution, PMB reacts with sulfhydryl groups. This reaction can be followed in two ways: (a) spectrophotometrically, and (b) by the change in enzymatic activity of a sulfhydryl enzyme (1 mole of PMB inactivates 1 mole of papain or ficin) (Light, 1964). To a reaction mixture with the same composition as was used in the inhibition of papain and ficin by iodoacetic acid, 10^{-3} M PMB was added in 5-μl portions and the change in the proteolytic activity of the enzyme was followed by an autotitrator as described in the Experimental Section. In another set of experiments the substrate was omitted from the reaction mixture, and the change in optical density was measured upon each addition of PMB. The ratio between proteolytic

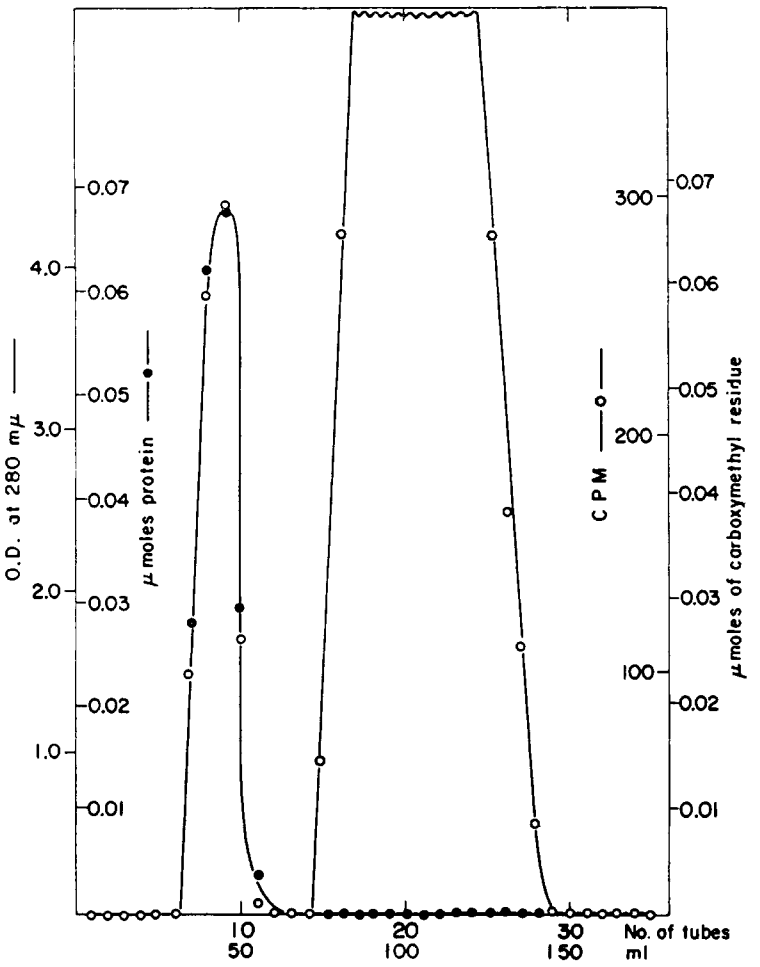

FIGURE 8: Gel filtration pattern of PSH-activated papain after treatment with ^{14}C-labeled iodoacetic acid. For details see text.

activity and the number of reactive thiol groups per mole of protein (as estimated spectrophotometrically with PMB) was found to be 1:3.2. This agrees well with the finding (Neumann, 1965) that 2 equiv of PMB react with one molecule of the type SPO_3. The consumption of the third equivalent of PMB is due to the free sulfhydryl group on the enzyme.

Binding of ^{14}C-Labeled Carboxymethyl Residue to the Activated Papain. Iodoacetic acid labeled with ^{14}C was added at pH 8.0 to PSH-activated papain. The pH of the reaction mixture was kept constant with an autotitrator. After an incubation period of 1 hr the reaction mixture was transferred to a Sephadex G-25 column (1.5 × 4.6 cm), eluted with Tris-acetated buffer, and 5.0-ml fractions were collected. It was found (Figure 8) that the radioactive ^{35}S is in 1:1 molar ratio to the protein. Thus it appears that the inhibition of 1 mole of activated papain by 1 mole of iodoacetic acid, and the binding of 1 mole of carboxymethyl residue, are related to the enzymic activity of the protein. Amino acid analysis of the ^{14}CM protein showed one carboxymethyl cysteine per mole of papain.

Conclusions

Previous studies have shown that PSH activates papain and ficin almost fully in equimolar ratio (Neumann, 1965). In this study it was demonstrated that the activated papain and ficin were fully inhibited by 1 equiv of iodoacetic acid. When excess ^{14}C-labeled iodoacetic acid was added to the active enzyme only 1 equiv of ^{14}C was found to be bound to the isolated proteins. Amino acid analysis of the above isolated proteins showed that the one carboxymethyl residue is bound to cysteine. These findings demonstrate that during activation of papain and ficin with PSH only one cysteine sulfhydryl group is liberated, a feature which is also reflected in the enzymic activity of these enzymes. The process of activation of papain and ficin by PSH is, therefore, suggested to be similar to that caused by other activators (Smith and Kimmel, 1960). On the basis of the above findings it was suggested that one molecule of PSH interacts with one molecule of the enzyme. Thus the kinetics and the thermodynamic constants of this interaction could be evaluated using relatively simple methods. The equilibrium

constant K of the enzyme–activator system as obtained from activation curves was found to be 6.0×10^6 and 6.5×10^6 l./mole at 23° for papain and ficin, respectively. Practically the same value of K for papain was obtained by using a fluorescence quenching method. From the dependence of the equilibrium constant on temperature the enthalpy of activation $\Delta H = -9.8$ kcal/mole was obtained. From fluorescence decay curves it was revealed that the interaction between papain and PSH is a second-order reaction with a rate constant of $k_1 = 3.2 \times 10^3$ sec^{-1} $mole^{-1}$ l. This is in accord with the proposition that one molecule of PSH interacts with one molecule of papain. The value of the first-order rate constant k_{-1} of the decomposition of the isolated papain–PS complex obtained from a series of gel filtrations was found to agree well with the value $k_{-1} = 5.3 \times 10^{-4}$ sec^{-1} $mole^{-1}$ l. obtained from the equation $K = k_1/k_{-1}$. The obtained value of k_{-1} corresponds to a half-life at 21 min for the isolated papain–PS complex.

It is of interest to note that the fluorescence quenching of papain during activation is much higher than that of ficin. This difference may be due to the fact that the residue next to the active cysteine in papain is a tryptophan residue (Light *et al.*, 1964), while in ficin it is a tyrosine residue (Wong and Liener, 1964).

The results obtained show that phosphorothioate is a highly specific reagent toward papain and ficin with the ability to modify the enzymes from the inactive to the active form as a result of only one chemical reaction. These properties of phosphorothioate may be very useful in studies of the mechanism of the activation of papain and ficin.

Acknowledgment

The authors with to thank Professor Ephraim Katchalski and Dr. Izchak Z. Steinberg for valuable discussions and assistance with this work.

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