THE MECHANISM OF THE ACTIVATION OF PAPAIN

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The predominant form of inactive papain prepared by the method of Kimmel and Smith (1) is shown to be a mixed disulfide formed between a sulfhydryl group on the enzyme and cysteine. Reduction or other nucleophilic cleavage of this bond frees the active thiol of papain and stoichiometric amounts of free cysteine or cysteine whose sulfur atom is covalently bound to the activating nucleophile. It is shown specifically that K ^{14}CN activation of papain produces ^{14}C labeled 2-iminothiazolidine-4-carboxylic acid (ITC) and a stoichiometric amount of free SH on the protein. Performic acid oxidation of inactive papain yields an amount of cysteic acid approximately equivalent to the quantity of ITC obtained in the cyanide activation experiment.

Crystalline papain prepared by the method of Kimmel and Smith (1) is generally obtained in an inactive form. Activation of the enzyme is promoted by such reagents as thiols, cyanide (2,3), or sodium borohydride (4). The nature of the inactive form of the enzyme obtained by their procedure has not yet been established. Three suggestions which have been made are shown diagrammatically in Fig. 1 together with the presumed mechanisms of activation.

Form A has been supported by the experiments of Neumann et al (5) who showed that sodium phosphorothioate (Na_3PO_3S) activated the enzyme on a 1:1 molar ratio and became reversibly bound to it during the course of activation as demonstrated by Sephadex chromatography. Morihara (6) and earlier workers (7,8) have argued for structure B in which the active thiol is bound as an internal hemithioacetal from the fact that cyanide activated papain is particularly sensitive to inhibition by carbonyl reagents. Inactive form C is suggested by experiments of Sluyterman (9) who showed that the kinetics of activation of papain were virtually identical to those exhibited by an artificially prepared mixed disulfide of papain and cysteine. Glazer and Smith (4)

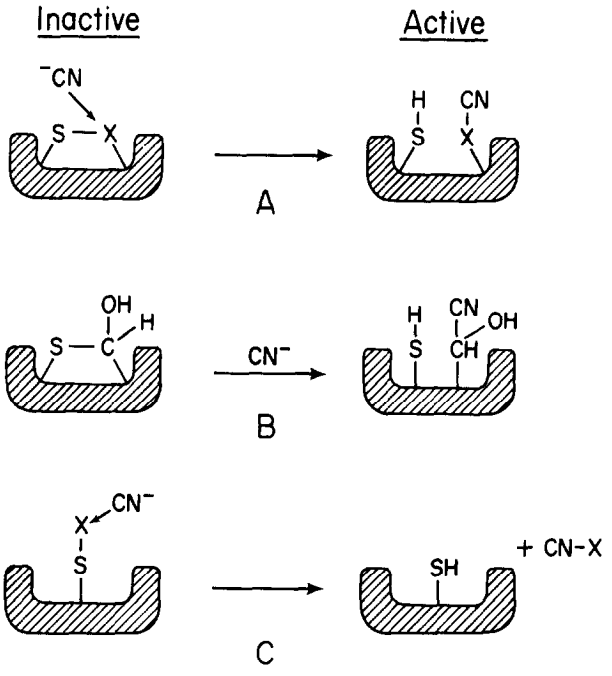

Figure 1. Previously suggested structures of inactive papain: A, (Ref. 5); B, (Ref. 6); and C, (Ref. 9), XH = cysteine or unidentified inactivating species. The mode of activation of the enzyme by cyanide suggested by Morihara (6) for structure B is shown together with those presumed for A and C.

did look for such a mixed disulfide by analyzing for cysteic acid after performic acid oxidation of the enzyme but found quantities that were generally considerably less than the thiol titer of the enzyme.

The results reported herein demonstrate that the predominant inactive form of papain is indeed a mixed disulfide formed from cysteine and the enzyme.

Methods and Materials--Papain (Worthington lots 8CA and 7DB. Both lots were inactive in the absence of added activator and had less than 0.07 moles titrable thiol per mole of protein) dialyzed extensively against distilled water to remove free cysteine or cystine was dissolved in 0.01 M EDTA-0.01 M sodium phosphate buffer at pH 6.8. Activation was carried out at 25°C for 4-5 hr with a final concentration of enzyme and $K^{14}CN$ of 3.6×10^{-4} M and 1.0×10^{-3} M respectively. The enzyme was assayed by the method of Kirsch and Igelström (10). Protein concentration was determined by using $\epsilon_{280} = 51{,}000$ (11).

Gel filtration was done on a 17 x 1 cm column of Sephadex G-10 with 0.01 M ammonium acetate pH 6.8 as the eluent. The flow rate was about 1 ml/min.

Thiol was titrated with Ellman's reagent (12).

Carbon-14 was counted in a toluene-ethanol scintillation solution (13) in a Packard Tricarb Model 3003 Liquid Scintillation Spectrometer.

Performic acid oxidation was done on about 0.4 μmoles of papain (lot 8CA) for 2,4 and 8 hrs (14). The partially soluble lyophillized residue containing oxidized papain and cysteic acid was extracted with pH 2.2 sodium citrate buffer (Beckman/Spinco recommended sample dilutor-Instruction Manual AIM-2, part 5) using a Potter-Elvehjem homogenizer fitted with a teflon pestle. Insoluble material was removed by centrifugation. Soluble protein was separated from cysteic acid using an Amicon Diaflo 1 ml capacity ultrafilter fitted with a 25mm UM-10 membrane. Cysteic acid was determined using a Beckman/Spinco Model 120 Amino Acid Analyzer fitted with an Infratronics Model CRS 10AB Digital Readout System and compared to known standards (15).

2-Iminothiazolidine-4-carboxylic acid was detected using the paper chromatography system of Bradham et al (16). Further identification and isolation of this compound was carried out by ion exchange chromatography on a 1 cm x 1 cm column of Bio Rad Agl-X8 (OH^-), 200-400 mesh. An aliquot of K ^{14}CN activated papain was put on the column in 1 M NH_4OH. 30 ml of distilled water was used to elute the protein and excess ammonia and then a NaCl gradient (0-0.15M) was applied to separate ITC from excess CN^-. ITC was eluted at 0.06 M and CN^- at 0.09 M NaCl.

Results and Discussion--Fig. 2 illustrates the gel filtration elution profile of a 0.5 ml aliquot of a papain solution activated as described in Materials and Methods. The last Peak (III) contains unreacted cyanide. Two new radioactivity containing peaks appear which are due to the cyanide reaction with protein. The first (I) and smaller one is coincident with the void volume and the protein peak. The middle one (II) corresponds to a new species

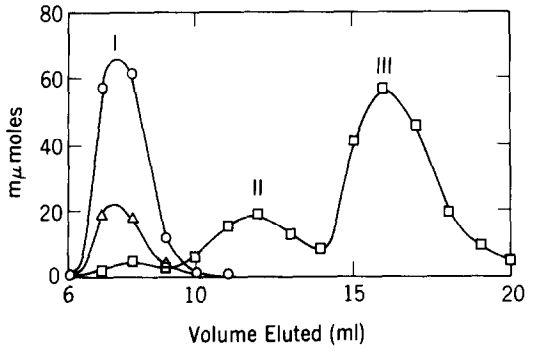

Figure 2. Separation of the products of the K ^{14}CN activation of papain by Sephadex G-10 chromatography as described in Materials and Methods; (○) protein, (□) carbon-14 and (△) thiol.

produced by the reaction of the protein with the activator. This latter material was lyophillized and subsequently identified as ITC by paper chromatography using the system of Bradham et al (16).

The identification was supported by ion exchange chromatography as described in Materials and Methods.

The most straightforward explanation of this result is shown diagrammatically in Fig. 3 where cyanide is shown to react with inactive papain to produce the active enzyme and β-thiocyanoalanine. The latter species cyclizes under the conditions of these experiments to give ITC (17).

Since peak II has more ^{14}C than peak I, the activating nucleophile must react preferentially with the sulfur atom on the labile cysteine. Attack of $^{14}CN^-$ at the other sulfur atom of the mixed disulfide would lead to inactive enzyme with the active site cysteine converted to ^{14}C-β-thiocyanoalanine.

Peak II contains about 50 mμmoles of ^{14}C while peak I has only 9 mμmoles. The titratable thiol in the protein peak is about 40 mμmoles, although there are over 100 mμmoles of protein based on the extinction coefficient. About 85% of the maximal CN^- induced catalytic activity was obtained in this experiment. It is thus evident from the graph that even if all the protein were active, the thiol titer would only be equal to approximately 0.5 mole/mole protein. It

therefore follows that not all the papain molecules are activatable. This conclusion has been drawn previously by others on the basis of different experiments (4,9,18). The unactivatable protein may represent products of partial autolysis.

The values shown in Table I demonstrate that the amounts of modified cysteine produced by cyanide cleavage are approximately equal to the thiol titer of the enzyme. Cysteic acid to protein ratios for 2, 4, and 8 hrs of performic acid oxidation were 0.45, 0.51, and 0.51. These latter values are only qualitative due to the incomplete separation from cysteic acid on the amino acid analyzer column of a small amount of contaminating material produced during the oxidation. Although the results of these experiments support the scheme given in Fig. 3, they cannot exclude a more complicated mechanism of activation in which the inactive papain is bound in mixed disulfide linkage to

Table I. Comparison of Thiol Titer and 2-Iminothiazolidine-4-carboxylic acid (ITC) Produced by the K ^{14}CN Activation of Papain.[a]

Papain Lot #	Thiol per mole protein %	ITC per mole protein %
8CA	40.5	52.5
	43.3	56.2
7DB	56.0	61.0

a. Based on protein concentration as determined from absorbance at 280 mμ and the maximum obtainable activity.

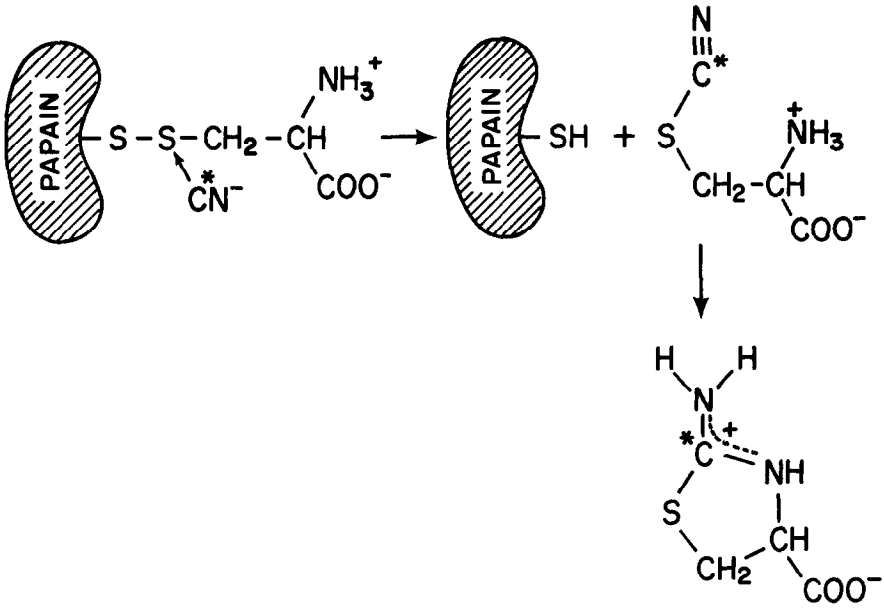

Figure 3. Reaction of cyanide with inactive papain.

labile cysteine through a thiol group different from that which eventually forms the active site. Activation would then proceed in two steps; release of external cysteine as shown in Fig. 3 followed by an internal disulfide exchange to form the active site. It has been shown that one of the internal dithio bridges in the enzyme can be broken without loss of activity (19), but it remains to be seen whether or not this property is related to the activation process.

The mixed disulfide form of inactive papain is analogous with that of a streptococcal protease, in which a volatile but as yet unidentified mercaptan is associated with the inactive form of the enzyme (20), and with the mixed disulfide formed from human plasma mercaptalbumin and cysteine (21).

Further experiments related to the reaction of papain with carbonyl reagents and the apparent binding of activators will be reported in a later communication.

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