The Activation of Papain and the Inhibition of the Active Enzyme by Carbonyl Reagents*

IRA B. KLEIN AND J. F. KIRSCH

From the Department of Biochemistry, University of California, Berkeley, California 94720

SUMMARY

The activation of papain with four different activators is not accompanied by the binding of any of them to the protein. These experiments, taken together with previously reported results, show that the inactive form of papain prepared by the method of Kimmel and Smith (J. Biol. Chem., 207, 575 (1954)) is a mixed disulfide formed between the active site sulfhydryl group of the protein and free cysteine.

The known inhibition of the activated enzyme by reagents having affinity for carbonyl groups has been investigated in order to determine whether an aldehyde residue which is intimately connected with the activation process is present on the enzyme, or if the observed inactivation of the protein by this class of reagents can be accounted for in some other manner. The following relevant observations were made. (a) Phenylhydrazine inactivates and binds to cyanide-activated papain but neither inactivates nor binds to cysteine- or borohydride-activated papain in the presence of excess activator. (b) The cysteine-activated enzyme is also inhibited by phenylhydrazine when the cysteine to papain ratio is low. (c) This inhibition in the presence of cyanide or low concentrations of cysteine is readily reversible with excess cysteine which also releases the bound phenylhydrazine from the protein. (d) Treatment of the enzyme with either phenylhydrazine or hydrogen peroxide in the presence of K4CN results in the release of thiol equivalent to the amount of cyclized β-thiocyanatoalanine released from the protein (6). This compound is the expected product of the reaction of cyanide upon the nonprotein sulfur atom of the mixed disulfide. Cysteine can be oxidatively bound to active papain, and the kinetics of reactivation of this species are identical with those observed for the activation of native papain (7). The activation is apparently accomplished without a major conformational change as determined by fluorescence, ultraviolet difference spectroscopy, and circular dichroism (8).

Papain, when isolated by the method of Kimmel and Smith (1), is generally inactive and must be treated with either mercaptans (1–5), cyanide (2–4), or other reducing agents (4) in order to release the free active thiol and the concurrent proteolytic or esterase activity. The structure of the major fraction of inactive papain has recently been demonstrated to be a mixed disulfide of papain and cysteine (6, 7). The evidence in support of this model is as follows. Activation of papain with K4CN results in the release of thiol equivalent to the amount of cyclized β-thiocyanatoalanine released from the protein (6). This compound is the expected product of the reaction of cyanide upon the nonprotein sulfur atom of the mixed disulfide. Cysteine can be oxidatively bound to active papain, and the kinetics of reactivation of this species are identical with those observed for the activation of native papain (7). The activation is apparently accomplished without a major conformational change as determined by fluorescence, ultraviolet difference spectroscopy, and circular dichroism (8).

These recent findings are difficult to reconcile with other phenomena associated with activation which have been described in the literature. It has been reported for example that phosphorothionate ion (PO4S−) becomes reversibly bound to papain during the course of activation (5). It has also been argued, from the sensitivity of papain to carbonyl reagents (9–12), that an aldehyde moiety is present on the protein and that the inactive form is a hemithioacetal formed between the active site thiol residue and this aldehyde (9, 12).

The experiments reported herein were designed to determine the total extent of binding of activators during the course of activation, and to elucidate the mechanism of the inactivation of papain by carbonyl reagents.

EXPERIMENTAL PROCEDURE

Materials

Radiochemicals—35S-Thiophenol, 26.5 mCi per mmole, purchased from the Radiochemical Centre (Amersham, England),

* This research was supported by National Institutes of Health Grant GM12278 and National Science Foundation Grant GB4066.
was diluted with redistilled unlabeled compound obtained from Matheson, Coleman and Bell, East Rutherford, New Jersey, to a specific activity of 173 cpn per pmole. \(^{35}\)S-cysteine, 43.1 mCi per pmole, was obtained from Calbiochem and was diluted with Calbiochem D-glucose II monohydrate to 243 cpn per pmole. Activation with unlabeled cysteine was performed using either Nutritional Biochemical Corporation L-cysteine or the D-glucose II monohydrate from Calbiochem. \(^{14}\)C-Phenylhydrazine HCl, 126 mCi per pmole from Tracerlab, Richmond, California, was diluted with unlabeled compound obtained from Eastman (White Label) to a specific activity of from 50 to 100 cpn per pmole. Labeled P-chitotase\(^1\) was synthesized from \(^{35}\)PSCl\(_2\) (Volk Radiochemical Company, Irvine, California) by the method of Åkerfeldt (13) to a final specific activity of 167 cpn per pmole. This preparation was used within a week of synthesis, and the specific radioactivity was redetermined daily. \(^{14}\)C-Sodium acetate, 7.5 mCi per pmole, was purchased from Volk Radiochemical Company, and diluted with unlabeled compound (Eastman White Label) to 50 cpn per pmole.

Other Reagents—Toluene-acetamide was obtained from Calbiochem and recrystallized from 100% ethanol. Hydroxylamine HCl was purchased from Fisher Scientific Company, Pittsburgh, Pennsylvania; hydrogen peroxide as a 30% solution, "Superoxol," from Merck; potassium cyanate from Matheson, Coleman and Bell; 5,5'-dithiobis(2-nitrobenzoic acid) from Aldrich and benzaldehyde (White Label) from Eastman. NaBH\(_4\) was obtained from Metal Hydrides, Inc., Beverly, Massachusetts. Bio-Gel P-2 was purchased from BioRad Laboratories, and Sephadex G-10, G-25, and G-75 from Pharmacia Fine Chemicals. Z-glycine p-NP was prepared by the method of Bodanzky and du Vigneaud (14), m.p., 127.5-128°, literature 128°.

Four papain preparations were used in these experiments: Worthington Biochemicals, twice recrystallized enzyme, lot numbers 5629, 7DB, and SCA, and enzyme purified in this laboratory from dry papaya latex (Wallertstein Company, New York, New York) by the method of Kimmel and Smith (1), as modified by Masuda (15). This is referred to as IK(-cys). These Worthington Biochemical preparations had similar maximal velocities when assayed with Z-glycine p-NP, while IK(-cys) as modified by Masuda (15). This is referred to as IK(-cys).

These activation experiments were performed for 1 hour at pH 6.8 and 25° in 20 mM phosphate buffer containing 1 mM EDTA and 35 mM phosphate buffer. Reaction time was 2 hours. The \(^{35}\)P-P-thiophenol used to activate papain was 0.17 mM as was the enzyme. Activation proceeded for 1 hour in a 30 mM EDTA, 30 mM phosphate buffer, and then the solution was chromatographed as described in Table I. NaBH\(_4\) activation was done with 180 mM NaBH\(_4\) in 0.67 mM EDTA and 25 mM phosphate buffer at pH 7 and 0° for 45 min, similar to the procedure described by Glazer and Smith (4). Thiol titer was determined by the method of Eillman (22).

Inactivation Conditions—Inactivation reactions were usually performed for 1 hour at pH 6.8 and 25° in the dark at concentrations of papain ranging between 80 and 300 \(\mu\)M in the presence of 6 mM carbonyl reagents unless otherwise stated. Potassium cy-
TABLE I
Extent of binding of activators to various forms of papain

<table>
<thead>
<tr>
<th>Activator treated with</th>
<th>Amount of activator bound per mole/mole</th>
<th>Protein</th>
<th>Thiol released</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSCN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inactive papain (7DB)</td>
<td>0.04</td>
<td>0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxamidomethyl papain (7DR)</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine activated papain (activator free) (7DB)</td>
<td>0.09</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32P-P-thioate treatment of Inactive papain (7DB)</td>
<td>0.01</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3S-Cysteine treatment of Papain (IK-cys)</td>
<td>0.07</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Lot 7DB was purchased from Worthington, while IK-(cys) was isolated by the method of Kimmel and Smith (1) as modified by Masuda (15).

b Based on thiol determination by the method of Ellman (22).

 RESULTS

On Binding of Activators to Papain—The extent of binding of 3S-cysteine, KSCN, and 3P-P-thioate to papain is shown in Table I. After the incubation with the activator as described under "Methods," the mixtures were chromatographed on Sephadex G-10 or Bio-Gel P-2 to remove nonprotein-bound activator. It can be seen that neither of the two thiol activators nor cyanide was bound in quantities comparable with the enzyme thiol formed upon activation. The binding of 3S-thiophenol was also investigated on a sample of papain that had some activity in the absence of activator (lot 5629), and the thiol was observed to bind to an extent of less than 0.1 mole per mole of protein thiol formed while fully activating the enzyme. It has been shown before (6), and here again, that only about 0.1 mole of 14C-cyanide was bound during cyanide-mediated activation of papain per mole of protein thiol released. This could be due to a nonspecific reaction of cyanide with one of the three disulfide bridges on the papain molecule (22, 24) or to a "wrong side attack" at the active site which would release free cysteine disulfide bridges on the papain molecule (23, 24) or to a "wrong side attack" at the active site which would release free cysteine.

The fact that cyanide also binds to a similar extent to carboxamidomethyl- and cysteine-activated papain suggests that the former explanation is more probable. The cyanide bound to cyanide-activated Worthington papain was stable to rechromatography, probably indicating that it is in covalent linkage with the protein. The extent of binding of thiol activators to the enzyme was likewise small compared with the amount of protein-bound thiol released. Similar results using P-thioate and KCN were obtained with another preparation of papain (lot 8CA).

The binding ratio is presented in two ways in the table to illustrate a characteristic of papain which has been noted before (3, 4, 6, 7, 25); that is, that the usual preparations of fully activated papain contain considerably less than 1 mole of thiol per mole of protein, presumably indicating the presence of some irreversibly inactivated papain. As shown in Fig. 1, the activity of the enzyme is directly proportional to the titrable thiol on the protein irrespective of the mode of activation. This result is similar to the one obtained previously by Sanner and Pihl with no added activator (2). At the maximum rate of the Z-glycine p-NP assay of 1.0 min⁻¹ (micrograms of enzyme per ml)⁻¹, this particular batch of papain had 0.54 mole of —SH per mole of protein based on εSH = 51,000 (17). This rate of esterase activity is the highest that could be obtained on several batches of Worthington papain, and on papain isolated by the method of Kimmel and Smith (1) as modified by Masuda (15) under many different activating conditions.

In some of the experiments it was necessary to use low ratios of activator to protein so that all of the potential protein-bound thiol was not released. Since the amount of nonspecific binding of the activator was fairly constant, a small thiol titer resulted in a higher molar ratio of bound activator to thiol released as is seen by the difference between the amount of activator bound per mole of protein and per mole of thiol (Table I).

\[
\begin{align*}
\text{S-S-OH} & \text{OH} \quad \text{NH}^+ \quad \text{CN}^- \quad \text{COO}^- \\
\text{SCN} + \text{CYSTEINE} & \text{(1)}
\end{align*}
\]
The chromatography on Bio-Gel P-2 of 32P-thioate-activated papain is shown in Fig. 2. The elution profiles show that a small amount of radioactivity comes out with the void volumes of the columns and was taken to be protein bound. These elution profiles are similar to the one previously shown for 32C-cyanide-activated papain (6), except that Bio-Gel P-2 was used here instead of Sephadex G-10. Fig. 2B illustrates how small a amount of P-thioate is bound to papain, but, more interestingly, shows that a new 32P-containing compound is formed as a result of the reaction of P-thioate with inactive papain. The small thiol peak on the right of the graph corresponds to the elution of P-thioate when chromatographed alone.

The reaction of phenylhydrazine with papain

From the fact that cyanide-activated papain is particularly sensitive to inhibition by carbonyl reagents (10, 11), Morikara et al. (9, 12) have postulated that the inactive form of the enzyme has a structure in which the active thiol is bound as an internal hemithioacetal. The reactions of papain with this type of reagent were, therefore, investigated in order to attempt to find a satisfactory explanation for this phenomenon.

### Table 11

<table>
<thead>
<tr>
<th>Activator</th>
<th>Activator present when phenylhydrazine is added</th>
<th>Esterase activity</th>
<th>Phenyldiazine bound per protein after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, native, inactive papain</td>
<td>0.97 0.066 0.61 0.64</td>
<td>-</td>
<td>0.12</td>
</tr>
<tr>
<td>None, carboxamido-methyl derivative of papain</td>
<td>- 0.91 0.049 0.071 0.076</td>
<td>+</td>
<td>0.01</td>
</tr>
<tr>
<td>KCN</td>
<td>+ 0.80 0.61 0.79 0.08</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td>Cysteine</td>
<td>- 0.89 0.061 0.17 0.22</td>
<td>+</td>
<td>0.01</td>
</tr>
<tr>
<td>Sodium borohydride</td>
<td>- 0.75 0.045 0.57 0.46</td>
<td>-</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* After inactivation, an aliquot of the mixture was diluted into 12 ml of the standard assay solution to a final enzyme concentration of 0.3 to 0.7 μM. Two portions of this diluted solution were assayed immediately, and the remaining two portions were treated with 0.3 mM cysteine for 3 hours and then assayed to determine recoverable activity as described under "Methods."

* A 10-fold molar excess of cyanide over protein was added with the phenylhydrazine.

Having affinity for carbonyl groups which have been investigated, phenylhydrazine has been shown to be the most potent inhibitor of papain. A particularly puzzling aspect of the inactivation of papain by phenylhydrazine is that the inhibition is more pronounced when papain is activated by cyanide than when it is activated by cysteine (9, 12). The experiments reported in Table II confirm this observation, and provide a plausible explanation for it. Papain activated either by KCN, cysteine, or NaBH₄ was inactivated after the activator had been removed by gel filtration before treatment with phenylhydrazine. Under these conditions, very little radioactive phenylhydrazine was bound to the protein as a result of the inactivation process. When cyanide was used as the activator and was not removed prior to the addition of phenylhydrazine, papain was also inactivated by the phenylhydrazine. In this case about 1 mole of phenylhydrazine was bound per mole of active enzyme. If either cysteine or NaBH₄ were present during the phenylhydrazine treatment, there was no appreciable inactivation, nor did phenylhydrazine become bound to the protein. The inactivation medi-
 Activation and Inhibition of Papain

Vol. 244, No. 21

activated in the presence of cyanide was partly reversed by the addition of excess cysteine, and this reactivation was accompanied by the displacement of phenylhydrazine from the enzyme (Fig. 3). The inactivation caused by phenylhydrazine in the absence of activator cannot be reversed by cysteine. The requirement for cyanide for binding of phenylhydrazine and reversible inactivation was explored further by the experiments shown in Table III. The results demonstrate that, in addition to the presence of cyanide being required for the binding of $^4$C-phenylhydrazine to papain (Table II), either phenylhydrazine or hydrogen peroxide will couple $^4$C-cyanide to the enzyme. These experiments suggest that the mechanism of inactivation of papain in the presence of cyanide by these reagents is not due to a reaction of a carbonyl group on the enzyme, but rather to an oxidative coupling of cyanide to the active sulphydryl group of the enzyme converting the essential cysteine residue at position 25 in the primary sequence.

**Fig. 3.** Reversal of phenylhydrazine inactivation by cysteine.

Phenylhydrazine-inhibited cyanide-activated papain was prepared as described in Table II. The inactivated papain was chromatographed on Bio-Gel P-2. Two fractions containing 40 and 30 mM papain were treated with 0.3 mM cysteine for 15 hours, and 30 mM cysteine for 24 hours, respectively, at pH 6.8 and 25°C in 10 mM phosphate buffer. Rechromatography on Bio-Gel P-2 was done to determine activity recovered and the remaining bound phenylhydrazine as described under "Methods."

**Table III**

Oxidative coupling of $^4$C-cyanide to activated papain

The reactions were carried out at pH 6.8 and 25°C in 10 or 30 mM phosphate buffer containing 1 mM or 10 mM EDTA. Papain concentrations varied from 80 to 400 μM. Phenylhydrazine was about 6 mM, cyanide, 1.6 mM, and peroxide, 2 mM. Cyanide activation was done for 4 hours; cysteine activation, 45 min; and hydrogen peroxide and phenylhydrazine inactivation 30 and 60 min, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Esterase activity</th>
<th>$^4$CN bound per mole of protein before reactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After activation</td>
<td>After phenylhydrazine treatment</td>
</tr>
<tr>
<td>Carboxamidomethyl papain with $^4$C-cyanide and phenylhydrazine</td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>Cysteine-activated, cysteine-free papain* with $^4$C-cyanide and phenylhydrazine</td>
<td>0.76</td>
<td>0.070</td>
</tr>
<tr>
<td>$^4$C-Cyanide activation of papain followed by Phenylhydrazine</td>
<td>0.69</td>
<td>0.11</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>0.69</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Cysteine was separated from papain using a Bio-Gel P-2 column (1 X 15 cm), 0.01 M phosphate buffer, pH 6.8, at 25°C.

**Fig. 4.** The oxidative binding of cyanide to the active site of papain. Sephadex G-75 chromatography in 10 mM phosphate buffer, pH 6.8, of cyanide-activated papain (see "Methods"). A, half the activation mixture was inactivated with 20 mM phenylhydrazine. B, the other half was treated with 50 mM iodoacetamide which completely abolished the activity, and then followed by 20 mM phenylhydrazine. Protein (C) was determined using the optical density at 280 μm, and radioactivity (Δ) as described under "Methods."
TABLE IV
Hydroxylamine and semicarbazide reactions with cyanide activated papain

Papain (0.4 mM) was activated with 1.6 mM cyanide for 4 hours at 25° in 0.01 M phosphate buffer, and then incubated with 6 mM carbonyl reagent or 3 mM H₂O₂. The hydrogen peroxide inactivation was completed after 30 min. Semicarbazide and hydroxylamine were incubated with the enzyme for 2 hours unless otherwise indicated. All reactions were in 30 mM phosphate-30 mM EDTA, pH 6.8, at 25°.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (µM)</th>
<th>Concentration of Carbonyl Reagent (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14C-Semicarbazide reacted with cyanide-activated papain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hours</td>
<td>0.83</td>
<td>3x10⁻⁴</td>
</tr>
<tr>
<td>12 hours</td>
<td>0.71</td>
<td>3x10⁻⁶</td>
</tr>
<tr>
<td>14C-Semicarbazide reacted with peroxide-inactivated cyanide-activated papain</td>
<td>0.87</td>
<td>3x10⁻⁸</td>
</tr>
<tr>
<td>Hydroxylamine reacted with cyanide-activated papain</td>
<td>0.81</td>
<td>3x10⁻⁴</td>
</tr>
</tbody>
</table>

* This rate was determined 12 hours after the enzyme was initially activated, but without semicarbazide treatment. The original rate was 0.762.

FIG. 5. The oxidation of cysteine by carbonyl reagents. Cysteine (24 µM) was treated with semicarbazide (Δ), phenylhydrazine (O), or hydroxylamine (□) for 1 hour at 25°, pH 7.0 (10 mM phosphate, 1 mM EDTA). Thiol was determined by the method of Ellman (22). Cysteine which was not treated with carbonyl reagents showed less than 2% loss of thiol under these conditions.

TABLE VI
Cysteine protection of papain against phenylhydrazine inhibition

Papain was reacted with phenylhydrazine in the presence of increasing amounts of cysteine. The initial enzyme concentration was 0.5 mM in 2.5 mM phosphate buffer, pH 6.8. The protein was incubated 25 min at 25° with cysteine and then diluted to 24 µM before treatment with phenylhydrazine.

<table>
<thead>
<tr>
<th>Cysteine concentration</th>
<th>Treatment</th>
<th>Esterase activity</th>
<th>Recoverable with more activator*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>None</td>
<td>0.82</td>
<td>0.90</td>
</tr>
<tr>
<td>0.00</td>
<td>Phenylhydrazine 3 mM for 1 hour in dark</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>0.08</td>
<td>Phenylhydrazine 3 mM for 1 hour in dark</td>
<td>0.12</td>
<td>0.84</td>
</tr>
<tr>
<td>0.49</td>
<td>Phenylhydrazine 3 mM for 1 hour in dark</td>
<td>0.87</td>
<td>1.01</td>
</tr>
<tr>
<td>1.71</td>
<td>Phenylhydrazine 3 mM for 1 hour in dark</td>
<td>1.03</td>
<td>0.93</td>
</tr>
</tbody>
</table>

* Inactivation mixture (0.2 ml) was added to 12.17 ml of the standard assay solution described under "Methods" which contained a final concentration of 35 mM phosphate, pH 6.8, 1 mM EDTA, and 0.5 µM papain. One-half of this was assayed, and the remainder was made 0.3 mM in cysteine, incubated at 25° for 3 hours, and then assayed to determine the recoverable activity.

(23, 24) to β-thiocyanatoalanine. These reactions are summarized in Equation 2.

The proposed structure of the adduct produced by the addition of phenylhydrazine to the thiocyanate group is based on the stoichiometry of the binding of approximately 1 mole of cyanide.
per mole of phenylhydrazine, and is supported by analogous model reactions (see "Discussion"). The location of the bound cyanide at the active site cysteine is strongly suggested by the fact that alkylation at this position completely prevents the binding of cyanide caused by phenylhydrazine (Fig. 4).

The oxidation hypothesis was further tested by the experiments reported in Table IV, wherein it is shown that neither one of two other well known carbonyl reagents, semicarbazide or hydroxylamine, significantly inhibit cyanide-activated papain under these conditions, nor is $^{14}$C-semicarbazide bound to the enzyme to any appreciable extent. It is shown below that these compounds are poor oxidizing agents as well. Hydroxylamine, moreover, does not cause as much $^{14}$C-cyanide to bind to papain as does phenylhydrazine or hydrogen peroxide.

Benzaldehyde has been reported to provide partial protection against the phenylhydrazine-induced inactivation of the enzyme (11). This result is confirmed by some of the experiments shown in Table V, where it is observed in addition, that benzaldehyde itself causes some inactivation of the enzyme. The protection by benzaldehyde need not, however, reflect a competition between this aldehyde and a similar group on the enzyme, but only the complexation of the phenylhydrazine presumably through the formation of the benzaldehyde phenylhydrazone. The inactivation caused by the benzaldehyde itself was not further investigated, but may be due to the formation of a hemithioacetal between the active site thiol group and the added aldehyde. It should be further noted that benzaldehyde was only able to moderate the phenylhydrazine-induced inhibition when it was added with the phenylhydrazine; benzaldehyde added after treatment with phenylhydrazine was totally ineffective in reversing the inhibition of the enzyme.

The oxidative inactivation hypothesis was further tested by the model experiments shown in Fig. 5 where phenylhydrazine, but neither semicarbazide nor hydroxylamine, is shown to completely oxidize cysteine at a concentration of 0.03 M. This observation, along with the experiments reported in Table VI, provides a plausible explanation for the fact that phenylhydrazine does not inactivate cysteine-activated papain in the presence of excess cysteine; i.e., sufficient cysteine remains after treatment with phenylhydrazine to either prevent oxidation of the enzyme or to reactivate the oxidized papain. Papain itself is oxidized in 1 hour at a phenylhydrazine concentration of one-tenth the amount necessary to oxidize cysteine.

Sluyterman (26) has shown that cyanate ion inactivates papain by carbamylation at the active site thiol. The possibility that the inactivation of the enzyme promoted by oxidizing agents in the presence of cyanide is due to carbamylation by cyanate formed by the oxidation of cyanide was excluded by the following two observations. (a) The extent of $^{14}$C-phenylhydrazine binding to carbamylated papain prepared from activator-free papain, which was inactivated by 20-fold molar excess of potassium cyanate was only 0.15 mole per mole of protein as opposed to an average of 0.65 mole per mole of protein when cyanide itself was present; and (b) incubation of the enzyme with cyanate after it was inactivated with hydrogen peroxide in the presence of $^{4}$C-cyanide resulted in the formation of 2-iminothiazolidine-4-carboxylic acid, presumably by the mechanism shown in Equation 3.

This process is the reverse of the mechanism of activation of papain described previously (6). This product could not have arisen from $S$-carbamylated papain, and its appearance proves that the cysteine used in the reactivation experiments must attack the thiocyanato moiety at the carbon rather than the sulfur atom, since 2-iminothiazolidine-4-carboxylic acid, rather than CN$^-$, was released.

**Discussion**

The radioactively labeled mercaptans, $^{3}$S-thiophenol, $^{3}$S-cysteine, and $^{32}$P-P-thioate, along with K$^{14}$CN did not bind to the protein in sufficient quantity to account for the amount of active thiol released on activation of the enzyme by these reagents. These results do not support models that require that the activation process consists of a noncatalytic cleavage of a simple intramolecular bond of the active thiol of papain.

Although the evidence discussed above for an inactive form of papain in which there is a mixed disulfide formed between a sulphydryl group on the enzyme and cysteine (6, 7) is convincing, there are experiments suggesting other inactive forms of papain that must be explained. Some of these have led to proposals in which the sulphydryl group which, after activation, becomes the catalytic nucleophile of the enzyme, is actually bound intramolecularly to some group on the polypeptide chain (5, 9, 12). It has been reported in one instance that the binding of the activator, P-thioate, was proportional to the increase in the catalytic activity of the enzyme; the conclusion being based upon observed changes in fluorescence of the protein upon activation, and on the binding of the activator to the enzyme as shown by Sephadex chromatography in 0.1 M acetic acid. In contrast to the former result, Barel and Glazer (8) have concluded that the activation of papain has little effect on the ultraviolet circular dichroism or the extent of perturbation of the enzyme's aromatic chromophores. The experiments we have just presented show that no activator, including P-thioate, binds to papain under the activation and assay conditions described in this paper.

We have no definite explanation for the differences between our results and those in the literature (5) with respect to the apparent binding of P-thioate. This reagent is known to attack thioleth bridges in proteins (27) and such a reaction might have occurred in acetic acid with denatured protein. There is no doubt however that papain activity is not dependent upon the continued presence of the activator (Table I) (2, 6, 8, 21).

Although the inhibition of papain by carbonyl reagents has been interpreted in terms of a reaction of this reagent with a critical aldehyde moiety on the enzyme, the experiments described above show that the mode of inhibition of papain by phenylhydrazine is due to the ability of this compound to oxidize...
papain rather than to the formation of an enzyme-bound phenylhydrazone. This conclusion is further supported by the observation that carbonyl reagents such as semicarbazide and hydroxylamine, which do not have the ability to oxidize cysteine at low concentrations (Fig. 5), are not effective inhibitors of the enzyme (Table IV).

The proposed structure of the phenylhydrazine-thiocyanato papain adduct shown in Equation 2 is, to a certain extent, speculative, but it is supported by the fact that phenylhydrazine will not bind to the protein unless cyanide is also present, and that these two compounds are bound in approximately equal stoichiometry (Tables II and III). The structure is, moreover, chemically reasonable since the thiocyanato carbon atom is known to form adducts under mild conditions with nitrogen nucleophiles as occurs in the cyclizations of $\beta$-thiocyanatoalanine to form 2-iminothiazolidine-4-carboxylic acid (28) and 2-aminothiocyanatobenzene to form 2-aminobenaothiazole (29). The fact that there is more cyanide bound when peroxide rather than phenylhydrazine inactivates CN$^{-}$-activated papain may be because a similar mechanism is operating in which a second mole of cyanide attacks the carbon atom of the initially formed thioacyanato group; a reaction which is, to a certain extent, analogous with the dimerization of HCN (30).

The treatment of activator free papain with oxidizing agents leads to an inactive form of the enzyme that cannot be reactivated with cysteine. The nature of this species is unknown, but it presumably arises through the conversion of the active site cysteine residue to a sulfinic or a sulfonic acid. In the presence of activating nucleophiles, the intermediate papain sulfenium ion is trapped (Equation 2), preventing further irreversible oxidation.

Acknowledgments—We are grateful to Dr. W. Dixon Riley for the gift of Tracerlab $^{14}$C-phenylhydrazine, and we wish to thank Mrs. Patricia Hinkle for preparing the carboxamidomethylated derivative of papain.

REFERENCES
The Activation of Papain and the Inhibition of the Active Enzyme by Carbonyl Reagents
Ira B. Klein and J. F. Kirsch

_J. Biol. Chem._ 1969, 244:5928-5935.

Access the most updated version of this article at [http://www.jbc.org/content/244/21/5928](http://www.jbc.org/content/244/21/5928)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/244/21/5928.full.html#ref-list-1](http://www.jbc.org/content/244/21/5928.full.html#ref-list-1)