Site-Specific Inactivation of Papain by Ascorbic Acid in the Presence of Cupric Ions

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The mechanism of inactivation of papain (EC 3.4.22.2) by ascorbic acid (AsA) in the presence of cupric ions (Cu^{2+}) was investigated. The aerobic combination of Cu^{2+} and AsA resulted in an irreversible loss of enzyme activity. The inactivation was found to be an apparent first order reaction. The prior mixing of Cu^{2+} and AsA caused the complete disappearance of the inactivation. The addition of iron ions led to significant suppression against the inactivation. Cu^{2+} was bound to the enzyme in a molar ratio of 1:1. At lower concentrations of Cu^{2+} (molar ratio of enzyme to Cu^{2+} of 1: <1), the extent of inactivation showed the same dependence against the extent of oxidation of AsA. The rate of inactivation increased as the concentration of AsA was increased. Saturation kinetics were observed with respect to the concentration of AsA. Changes in the concentration of Cu^{2+} had no effect on the dissociation constant of the enzyme-AsA complex (K_1), though the rate constant of inactivation (k_2) showed a linear relationship with the concentration of Cu^{2+} . At various pH values tested, no change of k_2 was found, whereas the value of K_1 increased when the pH became lower. At higher concentrations of Cu^{2+} , the rate of inactivation fell beyond a certain concentration of AsA.

The present results suggest that both Cu^{2+} and AsA bind to the enzyme to form a ternary complex and that free radicals are site-specifically formed and react preferentially with the enzyme, at the site of their formation, impairing its activity.

Keywords site-specific inactivation; cupric ion-ascorbic acid system; papain; ternary complex; saturation kinetics

Introduction

The autoxidation observed in many biomolecules, such as ascorbic acid (AsA), catecholamines, or thiols, takes place following the addition of catalytic transition metal ions.¹⁾ In these biomolecules, the mechanism of biological damage caused by the autoxidation of AsA with transition metal ions has been investigated in a wide variety of enzymes.^{2–10)}

AsA has been reported to inactivate thiol enzymes, urease²⁾ and β -amylase,³⁾ by reducing cupric (Cu^{2+}) to cuprous ion (Cu^+) with the subsequent formation of a Cu^+ -mercaptide complex. Orr suggested that free radicals, known to be generated during the autoxidation of AsA in the presence of Cu^{2+} , are responsible for the inactivation of catalase⁴⁾ and the most likely candidates are hydroxyl and superoxide radicals.⁵⁾ Similarly, AsA in the presence of transition metal ions (Cu^{2+} , Fe^{2+} , and Fe^{3+}) has also been reported to inactivate acetyl-Co A hydrolase by generating free radicals.⁶⁾

Furthermore, it was suggested that the inactivation of glutamine synthetase by an Fe^{3+} -AsA system involves two steps: first, ferric ions bind to one or more of several metal binding sites on the enzyme; second, the redox reaction between AsA and the enzyme-bound Fe^{3+} generates site-specifically free radicals at the iron binding site(s), causing its inactivation.⁷⁾ In addition, the inactivation of acetylcholine esterase by AsA in the presence of Cu^{2+} also was explained in terms of the same notion of a site-specific mechanism.⁸⁾ However, the relation between the rate of inactivation and the dissipation of AsA is not unequivocally pointed out. In the meantime, it has been found that many enzymes are readily inactivated by metal-catalyzed oxidation (MCO) systems,⁹⁾ including the AsA/metal ions/ O_2 system, and that most of the enzymes that are highly sensitive to modification by MCO systems require metal ions for enzyme activity.¹⁰⁾ Although AsA thus inactivates many enzymes in the presence of the various

metal ions, there remains some uncertainty as to the details of the inactivation of enzymes.

The present paper deals with the inactivation of papain (EC 3.4.22.2) by AsA in the presence of Cu^{2+} in order to better understand the mechanism. This enzyme is a relatively small single-peptide protein and contains an active thiol group, and its activity is independent of metal ions.¹¹⁾ Experimental evidence suggests that AsA, as well as Cu^{2+} , also binds to the enzyme at a locus near a vital site for the catalytic function of the enzyme to form a ternary complex, and the redox reaction between Cu^{2+} and AsA in the locus generates site-specifically free radicals, causing the inactivation.

Materials and Methods

Materials Papain (2 × crystallized) was obtained from Sigma Chemical Co., St. Louis. The concentration of the enzyme was measured spectrophotometrically using a molecular weight of 23400¹²⁾ and $E_{1\text{cm}}^{1\%}$ at 278 nm of 25.¹³⁾ Benzoyl-L-arginine *p*-nitroanilide (BAPA) was obtained from Peptide Institute, Inc., Osaka. Cupric acetate (Cu^{2+}) and L-ascorbic acid (AsA) were from Nacalai Tesque Co., Ltd., Kyoto. Sephadex G-25 (superfine) was from Pharmacia Fine Chemicals AB, Uppsala. All other chemicals were of the highest purity available and were used without further purification.

Assay The hydrolytic activity of papain was routinely assayed using BAPA as a substrate. To 0.3 ml of the activation solution, containing 62.5 mM cysteine-HCl, 12.5 mM disodium ethylenediaminetetraacetic acid (EDTA), 62.5 mM NaOH, and 50 mM acetate buffer (pH 5.6), 0.3 ml of an enzyme solution to be assayed was added, and the mixture was incubated at 30 °C over 30 min for activation.¹⁴⁾ The reaction was started by adding 0.5 ml of the activated enzyme solution to 2.0 ml of the substrate solution, which contained 0.5 mM BAPA and 50 mM acetate buffer (pH 5.6). After reacting at 30 °C for 10 min, the reaction was stopped by 0.5 ml of 0.3% iodoacetic acid. The enzyme activity was determined by the increase in absorption at 410 nm.¹⁵⁾

Inactivation Studies A typical reaction mixture contained enzyme (25 μM), AsA (250 μM), Cu^{2+} (25 μM), and acetate buffer (50 mM, pH 5.6). After incubation at 30 °C for various durations, aliquots (0.3 ml) were transferred into 0.3 ml of the activation solution containing EDTA to stop the reaction, because EDTA terminates markedly the oxidation of AsA by Cu^{2+} .¹⁶⁾ In order to examine the effect of iron ions, ferrous sulfate or ferric chloride were used in place of Cu^{2+} . A blank experiment was carried out in the same manner, except that the activation solution was added to

the enzyme solution, containing enzyme, metal ions, and acetate buffer (50 mM, pH 5.6), before the addition of AsA.

Oxidation of Ascorbic Acid In order to examine the rate of the oxidation of AsA by Cu^{2+} under the same conditions in which the Inactivation Studies were performed, aliquots (0.3 ml) of the reaction mixture were transferred into 0.3 ml of a solution containing 12.5 mM EDTA and 50 mM acetate buffer (pH 5.6), made up with water to a volume of 2.4 ml, at various times. The rate of the oxidation of AsA was followed by a decrease in absorption at 265 nm.¹⁷⁾

Binding of Cupric Ions The binding of Cu^{2+} to papain was analyzed according to the procedure of Hummel and Dreyer.¹⁸⁾ To a 1.5×12 cm column of Sephadex G-25 (superfine), equilibrated with a 0.05 mM solution of Cu^{2+} in 20 mM acetate buffer (pH 5.6), 2.4 mg papain (0.1 μmol), dissolved in 2 ml of the same Cu^{2+} solution, was applied. Elution was carried out with the same Cu^{2+} solution. The effluent solution was collected and the concentration of Cu^{2+} was measured spectrophotometrically using $E_{1\text{cm}}^{1\text{M}}$ at 230 nm to be 1400.

Results and Discussion

The inactivation of papain by AsA in the presence of Cu^{2+} was examined at 30°C and pH 5.6 (acetate buffer). AsA alone was completely innocuous to the enzyme. No significant inactivation occurred with Cu^{2+} alone, and the enzyme activity of the Cu^{2+} -added enzyme was in good agreement with that of the blank.

Inactivation at a Low Concentration of AsA While the concentration of AsA was kept constant at a low concentration (molar ratio of enzyme to AsA of 1 : 10), the rate of inactivation increased as the concentration of Cu^{2+} was increased. The inactivation was found to be an apparent first order reaction. At higher concentrations of Cu^{2+} (molar ratio of enzyme to Cu^{2+} of 1 : 5—10) the inactivation was characterized by a rapid initial phase followed by a much slower phase (Fig. 1). This is possibly explained by the notion that AsA is rapidly oxidized at higher concentrations of Cu^{2+} , thus resulting in a decrease in the concentration of AsA.

The rate of oxidation of AsA increased as the concentration of Cu^{2+} was increased, and at higher concentrations of Cu^{2+} , AsA was rapidly oxidized. The rate of oxidation of AsA in the presence of the enzyme was decreased in comparison with that in the absence of the enzyme (Fig. 2).

The rate of inactivation was pH dependent in the pH range investigated (acetate buffer; pH 3.6—5.6). Maximum inactivation was observed at pH 5.6 under the conditions tested. At pH's below 4, no significant inactivation occurred (data not shown). This finding corresponds fairly well to the fact that the rate of the Cu^{2+} - (and Fe^{3+} -) catalyzed oxidation of AsA depends on the concentration of monoionic species of AsA.¹⁹⁾ This result of pH dependence will be kinetically discussed later.

From the above findings, it appears that the redox reaction between Cu^{2+} and AsA (monoionic species) is related to the inactivation of the enzyme. For this inactivation there is a possibility that Cu^+ , formed as a result from the interaction between Cu^{2+} and AsA,²⁰⁾ reacts with the active site-SH group of the enzyme to form a Cu^+ -mercaptide enzyme.^{2,3)} If so, it is thought that reducing substances, such as cysteine, mercaptoethanol, etc., reactivate the enzyme by removing the bound Cu^+ and regenerating the SH group. However, the partially inactivated enzyme by pre-treatment with Cu^{2+} and AsA, was not reactivated by the activation solution containing cysteine and EDTA, even though the concentration of

Fig. 1. Inactivation of Papain by AsA in the Presence of Cu^{2+}

The reaction mixture contained 25 μM papain, 250 μM AsA, and 12.5—250 μM Cu^{2+} in 50 mM acetate buffer (pH 5.6) at 30°C. Aliquots were periodically added to the activation solution for assay. Activities were assayed using BAPA as a substrate. The concentration of Cu^{2+} was as follows: $\circ$, 12.5 μM ; $\bullet$, 25 μM ; $\square$, 62.5 μM ; $\blacksquare$, 125 μM ; $\triangle$, 250 μM .

Fig. 2. Oxidation of AsA by Cu^{2+}

The reaction mixture contained 25 μM papain, 250 μM AsA, and 25 or 250 μM Cu^{2+} in 50 mM acetate buffer (pH 5.6) at 30°C. Aliquots were periodically added to the EDTA solution for AsA determination. The oxidation of AsA was followed by a decrease in absorption at 265 nm. The concentration of Cu^{2+} was as follows: $\circ$, $\bullet$, 25 μM ; $\square$, $\blacksquare$, 250 μM . Open and closed symbols represent the presence and the absence of papain, respectively.

cysteine was increased. In addition, the enzyme was not inactivated by Cu^+ alone. When solutions of Cu^{2+} and of AsA (molar ratio of 1 : 10) were mixed and allowed to stand at 30°C prior to the addition to the enzyme, inactivation was greatly decreased. After pre-incubation for 3 min, only a small amount of AsA remained and no significant inactivation occurred (Fig. 3). In addition, dehydroascorbic acid, an oxidative product of AsA, was completely innocuous to the enzyme. The aerobic combination of Cu^{2+} and AsA caused a rapid inactivation of the enzyme, whereas the anaerobic combination was not deleterious to the activity. From these results, it is suggested that the enzyme may be affected by oxygen free radicals, to be generated during the redox reaction between Cu^{2+} and AsA.^{19,21,22)}

Fig. 3. Effect of Prior Mixing of Cu^{2+} and AsA

Aliquots of the mixture of Cu^{2+} and AsA (molar ratio of 1:10) were periodically added to an enzyme solution. After incubation with the enzyme for 3 min, the enzyme solution was added to the activation solution. Similarly, aliquots of the mixture of Cu^{2+} and AsA were periodically added to the EDTA solution. The enzyme assay and the AsA determination were as in Fig. 1 and in Fig. 2, respectively. The reaction mixture contained $25 \mu\text{M}$ papain, $250 \mu\text{M}$ AsA, and $25 \mu\text{M}$ Cu^{2+} in 50 mM acetate buffer (pH 5.6) at 30°C .

Effect of Iron Ions Iron ions, one of the transition metals, also catalyze the autoxidation of AsA.^{1,19} The rate of oxidation of AsA increased as the concentration of iron ions, ferrous and ferric ions, was increased. However, the enzyme was not inactivated by AsA in the presence of various concentrations of iron ions, even though the reaction mixture was allowed to stand for a long time. Furthermore, the rate of oxidation of AsA by iron ions in the presence of the enzyme was similar to that in the absence of the enzyme, though the rate by Cu^{2+} in the presence of the enzyme was decreased in comparison with that in the absence of the enzyme. Then, in order to verify that Cu^{2+} is specific to the inactivation of the enzyme by AsA, the effect of iron ions on inactivation by the Cu^{2+} -AsA system was examined. The rate of oxidation of AsA increased as iron ions were added, but the inactivation in the co-presence of iron ions was markedly decreased in comparison with that in the absence of iron ions (data not shown). These findings support the notion that the oxidation of AsA catalyzed by iron ions is not responsible for the inactivation, thereby resulting in a decrease in concentration of AsA and preventing the inactivation of the enzyme. Therefore, it is possible to presume that Cu^{2+} may be able to form a Cu^{2+} -enzyme complex, to be responsible for the inactivation by AsA, and that iron ions, which are in a free state, participate in a redox reaction that is not involved in the inactivation.

Binding of Cu^{2+} The formation of a Cu^{2+} -enzyme complex was examined by means of gel filtration.¹⁸ At some point after the protein peak, the concentration of Cu^{2+} in the eluate was decreased below the base-line level to form a trough (Fig. 4). The appearance of a trough in the elution profile indicates the binding of Cu^{2+} to the enzyme. From the area of the trough, it was found that the ratio of Cu^{2+} to the enzyme is 1:1, even though the concentration of Cu^{2+} in the eluent solution increased. Furthermore, in the co-presence of iron ions the same profile

Fig. 4. Binding Cu^{2+} to Papain

To a column of Sephadex G-25, equilibrated with 20 mM acetate buffer (pH 5.6) containing 0.05 mM Cu^{2+} , papain in the same Cu^{2+} solution was applied and eluted with the same Cu^{2+} solution. Fractions (2 ml) were measured at 230 nm . Closed symbols represent the absence of Cu^{2+} .

was obtained.

Relation between the Inactivation and the Oxidation of AsA At lower concentrations of Cu^{2+} (molar ratio of enzyme to Cu^{2+} of 1:<1) the extent of inactivation showed the same dependence against the extent of oxidation of AsA (Fig. 5a). However, at higher concentrations of Cu^{2+} , the extent of inactivation against the extent of oxidation of AsA was reduced as compared with that at lower concentrations of Cu^{2+} . From this finding, it is suggested that, at higher concentrations of Cu^{2+} , the surplus of Cu^{2+} is in a free state, or, not bound to the enzyme, and that this fraction of free Cu^{2+} competes with the enzyme-bound Cu^{2+} for AsA and participates in a redox reaction that is not involved in the inactivation, similarly to the effect of iron ions observed above. Therefore, we can conclude that Cu^{2+} binds to a certain locus on the enzyme and the oxidation of AsA by Cu^{2+} at the locus is responsible for the inactivation.

Inactivation at Higher Concentration of AsA While the concentration of AsA was kept constant at a higher concentration (molar ratio of enzyme to AsA of 1:100), the rate of inactivation increased as the concentration of Cu^{2+} was increased. However, maximum inactivation was observed at a Cu^{2+} concentration of $62.5 \mu\text{M}$ (molar ratio of enzyme to Cu^{2+} of 1:2.5), while at higher concentrations of Cu^{2+} the rate of inactivation declined (Fig. 6). At lower concentrations of Cu^{2+} (molar ratio of enzyme to Cu^{2+} of 1:<1) the relation between the inactivation and the oxidation of AsA showed the same dependence in comparison with that at a low concentration of AsA (Fig. 5b). However, at higher concentrations of Cu^{2+} (molar ratio of enzyme to Cu^{2+} of 1:5<), the extent of oxidation of AsA against the extent of inactivation was significantly increased as compared with that at a low concentration of AsA. Then, the inactivation was examined as a function of the AsA concentration.

Dependence of Inactivation on AsA Concentration At lower concentrations of Cu^{2+} (molar ratio of enzyme to Cu^{2+} of 1:<2.5) the rate of inactivation depended on the

Fig. 5. Relation between Inactivation of Papain and Oxidation of AsA by Cu^{2+}

The reaction mixture contained 25 μM papain, 250 μM or 1.25 mM AsA, and 12.5–250 μM Cu^{2+} in 50 mM acetate buffer (pH 5.6) at 30°C. The extent of oxidation of AsA was plotted against the extent of inactivation. The concentration of Cu^{2+} was as follows: $\circ$, $\bullet$, 12.5 μM ; $\triangle$, $\blacktriangle$, 25 μM ; $\square$, $\blacksquare$, 62.5 μM ; ∇ , $\blacktriangledown$, 125 μM ; $\diamond$, $\blacklozenge$, 250 μM . Open and closed symbols represent the presence of 250 μM or 1.25 mM AsA, respectively.

Fig. 6. Inactivation of Papain by a Higher Concentration of AsA in the Presence of Cu^{2+}

The reaction mixture contained 25 μM papain, 2.5 mM AsA, and 12.5–250 μM Cu^{2+} in 50 mM acetate buffer (pH 5.6) at 30°C. The assay conditions were as in Fig. 1. The concentration of Cu^{2+} was as follows: $\circ$, 12.5 μM ; $\bullet$, 25 μM ; $\square$, 62.5 μM ; $\blacksquare$, 125 μM ; $\triangle$, 250 μM .

concentration of AsA. However, at higher concentrations of Cu^{2+} (molar ratio of enzyme to Cu^{2+} of 1:5<), increasing the AsA concentrations led to a higher rate of inactivation, and the rate fell beyond a certain concentration of AsA (Fig. 7). Maximum inactivation was observed at lower concentration of AsA as the concentration of Cu^{2+} was increased. These findings suggest that the inactivation of the enzyme by AsA in the presence of Cu^{2+} cannot be sufficiently explained by only the effect of the formation of free radicals on the basis of the redox reaction between AsA and the enzyme-bound Cu^{2+} .⁷⁻⁹⁾ The phenomena under the higher concentrations of Cu^{2+} will be discussed later.

Fig. 7. Dependence of Inactivation of Papain on AsA Concentration

The reaction mixture contained 25 μM papain, 0.25–2.5 mM AsA, and 12.5–250 μM Cu^{2+} in 50 mM acetate buffer (pH 5.6) at 30°C. The k_{app} values were obtained from the initial velocity of inactivation. The concentration of Cu^{2+} was as follows: $\circ$, 12.5 μM ; $\bullet$, 18.75 μM ; $\square$, 25 μM ; $\blacksquare$, 37.5 μM ; $\triangle$, 62.5 μM ; $\blacktriangle$, 125 μM ; $\times$, 250 μM .

Kinetic Studies The hyperbolic dependence against the concentration of AsA at lower concentrations of Cu^{2+} (Fig. 7) indicates the existence of an enzyme–AsA complex. To establish whether or not, in the inactivation of the enzyme by AsA in the presence of Cu^{2+} , an intermediate complex is formed between the enzyme and AsA, the AsA concentration dependence of inactivation was kinetically studied. On the assumption that the formation of the Cu^{2+} –enzyme complex occurs initially, the following scheme was assumed:

where E, I, EI, and E' are the Cu^{2+} -enzyme complex, AsA, E-AsA complex, and an irreversible inactive enzyme, respectively. The equations for the rate of inactivation are

$$[E^0] = [E] + [EI] + [E'] = [E^*] + [E'] \quad (2)$$

$$[E][I]/[EI] = K_1 \quad (3)$$

$$-d[E^*]/dt = k_2[EI] \quad (4)$$

where $[E^0]$ and $[E^*]$ are the total enzyme concentration and $\{[E] + [EI]\}$, respectively. The solution of Eqs. 2–4 is

$$\ln([E^0]/[E^*]) = k_2 t / \{1 + (K_1/[I])\} \quad (5)$$

Then,

$$k_2 / \{1 + (K_1/[I])\} = (1/t) \ln([E^0]/[E^*]) = k_{app} \quad (6)$$

$$1/k_{app} = (K_1/k_2)(1/[I]) + 1/k_2 \quad (6')$$

where k_{app} is an apparent first order inactivation rate constant.

As the inactivation of an enzyme by AsA at various concentrations obeyed first-order kinetics through a major portion of the inactivation process, the k_{app} values obtained (Fig. 6) were applicable to Eq. 6'. $[I]$ was replaced $[I^0]$, the total concentration, because the k_{app} value is obtained from the initial velocity, and $[EI]$ is negligible in comparison with $[I^0]$ under the conditions employed. A reciprocal plot of k_{app} values versus AsA concentration gave a positive intercept on the ordinate (Fig. 8), indicating saturation of the enzyme by AsA with a K_1 value of 0.42 mM. This indicates the formation of an enzyme-AsA complex on the process of inactivation. A k_2 value of 0.52 min^{-1} was obtained in the presence of $25 \mu\text{M}$ Cu^{2+} (molar ratio of enzyme to Cu^{2+} of 1:1).

From the above results, it is suggested that the Cu^{2+} -enzyme complex is formed first, and then monoionic AsA is attracted to the Cu^{2+} -enzyme complex, to be responsible for the inactivation of the enzyme. Hence, for the formation of an enzyme-AsA complex there are two possible explanations as to a binding site. One is an enzyme-bound Cu^{2+} ,^{7,8)} and the other is a locus near an enzyme-bound Cu^{2+} . In the former case, the negatively charged AsA would be attracted to a positively charged copper, and consequently, no change of k_2 but with a change in K_1 would be observed with respect to the concentration of Cu^{2+} . In the latter case, a negatively charged AsA may combine directly with the enzyme at a locus of the enzyme near an enzyme-complexed Cu^{2+} to form a ternary complex, and the rate-limiting formation of the inactive enzyme may involve the action of Cu^{2+} . Therefore, no change of K_1 but with a change in k_2 would be observed with respect to the concentration of Cu^{2+} . Then, the dependence of K_1 and k_2 values against the concentration of Cu^{2+} was investigated. At various concentrations of Cu^{2+} , no change of K_1 was found (Fig. 8). The k_2 value showed a linear relation with the Cu^{2+} with concentrations up to $25 \mu\text{M}$ (molar ratio of enzyme to Cu^{2+} of 1:1) (Fig. 9). These results suggest that AsA is attracted to the enzyme at a locus near an enzyme-bound Cu^{2+} to form a ternary complex, whereupon the oxidation of AsA by Cu^{2+} in the locus takes place, causing the site-specific inactivation.

Dependence of Inactivation on Cu^{2+} Concentrations At higher concentrations of Cu^{2+} (125 and $250 \mu\text{M}$) the rate of

Fig. 8. Saturation Kinetics in Inactivation of Papain by AsA

The k_{app} values of inactivation at various AsA concentrations giving first-order inactivation kinetics (Fig. 6) were replotted in double-reciprocal fashion in accordance with Eq. 6' to determine the kinetic properties of the enzyme-AsA complex. The concentration of Cu^{2+} was as follows: $\circ$, $12.5 \mu\text{M}$; $\bullet$, $18.75 \mu\text{M}$; $\square$, $25 \mu\text{M}$; $\blacksquare$, $37.5 \mu\text{M}$; $\triangle$, $62.5 \mu\text{M}$.

Fig. 9. Relation between k_2 Value and Cu^{2+} Concentration

The k_2 values obtained in Fig. 7 were plotted against Cu^{2+} concentrations. The solid line represents a linear relation on the assumption that the k_2 value depends on the Cu^{2+} concentration.

inactivation fell beyond a certain concentration of AsA (Fig. 7). The above phenomena are possibly explained on the basis of the formation of a ternary complex, as follows: on the reaction pathways for the oxidation of monoionic AsA (AH^-) by Cu^{2+} , an AsA radical in an ionized form ($\cdot\text{AH}^-$) is produced as an intermediate, and then, $\cdot\text{AH}^-$, which is

Fig. 10. Saturation Kinetics in Inactivation of Papain by AsA as a Function of pH

The reaction mixture contained $25\ \mu\text{M}$ papain, $250\text{--}750\ \mu\text{M}$ AsA, and $25\ \mu\text{M}$ Cu^{2+} , and $50\ \text{mM}$ acetate buffer at various pH's at 30°C . The k_{app} values of inactivation at various AsA concentrations were plotted in double-reciprocal fashion in accordance with Eq. 6'. The pH value was as follows: ■, 4.4; □, 4.8; ●, 5.2; ○, 5.6.

produced by the surplus of Cu^{2+} , may compete with AH^- at a binding site for AsA, thereby preventing the formation of a ternary complex that would be involved in the inactivation. No observation of this phenomena in the presence of lower concentrations of Cu^{2+} confirms that the above explanation is reasonable, because of the absence of Cu^{2+} in a free state.

Effect of pH In the pH range investigated (3.6–5.6), the rate of inactivation was pH dependent. The extent of inactivation by various concentrations of AsA in the presence of Cu^{2+} (molar ratio of enzyme to Cu^{2+} of 1:1) as a function of pH is shown in Fig. 10 (plotted in accordance with Eq. 6'). While the concentration of Cu^{2+} was kept constant, no change of k_2 was found at various pH values tested. The value of K_1 increased when pH became lower. Therefore, pH dependence of the inactivation of an enzyme by AsA in the presence of Cu^{2+} must be due to the extent of the formation of an enzyme–AsA complex, and the formation of an enzyme– Cu^{2+} complex is not affected by

pH under the conditions tested.

The Mechanism of Inactivation From the experimental evidence presented above, a possible mechanism of the inactivation of papain by AsA in the presence of Cu^{2+} is characterized by the following features. AsA, as well as Cu^{2+} ,^{7–9} also binds to the enzyme at a locus near an enzyme-bound Cu^{2+} to form a ternary complex, and, consequently, the redox reaction between Cu^{2+} and AsA in the locus generates the highly reactive free radicals that will be involved in the inactivation. These reactive free radicals will probably react with the available target on the enzyme, resulting in site-specific damage, that is, “site-specific inactivation”, rather than random injury. More detailed experiments to clarify the inactivation of the enzyme are now under investigation.

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