On the Mechanism of Inactivation of Active Papain by Ascorbic Acid in the Presence of Cupric Ions

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An inactivation mechanism of active papain (EC 3.4.22.2) by the Cu^{2+} -ascorbic acid (AsA) system was examined. Incubation of active papain, which contains an active sulfhydryl (SH) group, with the Cu^{2+} -AsA system under aerobic conditions resulted in an irreversible loss of enzyme activity. The enzyme was not inactivated at a molar ratio of enzyme to Cu^{2+} of 1: <1, whereas at a molar ratio of 1:1–2, the extent of inactivation showed the same dependence on the extent of oxidation of AsA. Saturation kinetics were observed with respect to the concentration of AsA. The degree of inactivation was dependent on the decrease in SH content of the enzyme. Catalase at a low concentration partially protected the enzyme from inactivation, but did not affect the oxidation of AsA. In addition, catalase at a high concentration completely protected both the enzyme from inactivation and AsA from oxidation. The present results suggest that an additional function of H_2O_2 , besides producing hydroxyl radicals ($\cdot\text{OH}$), is to promote the conversion of Cu^+ into Cu^{2+} , and that an active SH group of papain is site-specifically modified by the $\cdot\text{OH}$, resulting in inactivation of the enzyme.

Keywords site-specific inactivation; cupric ion-ascorbic acid system; active papain; hydroxyl radical; active sulfhydryl group

Many studies on the oxidative modification of enzymes^{1–4)} have established that the oxidation is dependent upon an electron donor (NADPH, ascorbic acid, etc.), oxygen and metal ions (Fe^{3+} , Cu^{2+}), and is inhibited by chelating agents. It is therefore assumed that the inactivation of enzymes is caused by a metal-catalyzed oxidation (MCO) system.⁵⁾ L-Ascorbic acid (AsA) has been extensively used in order to understand the mechanism of enzyme inactivation by free radicals, particularly in the presence of Cu^{2+} .^{2–4)} In general, random and global modification of many different amino acid residues occurs when enzymes are exposed to free radicals produced by high energy radiation.⁶⁾ By contrast, only one or a few amino acid residues are modified when enzymes are exposed to MCO systems.⁵⁾ There is substantial evidence that the metal-catalyzed oxidative modification of enzymes is a site-specific process.^{5,7)} For example, in the case of the Cu^{2+} -AsA model system, Cu^{2+} binds to a metal-binding site on an enzyme molecule, and then hydroxyl radicals ($\cdot\text{OH}$) repeatedly formed *via* a redox reaction between the bound Cu^{2+} and AsA preferentially attack the specific site near the metal-binding site on the enzyme in a “multi-hit” manner.⁷⁾ We have examined in detail the mechanism of the site-specific inactivation of an enzyme by the Cu^{2+} -AsA system using papain (EC 3.4.22.2), and have proposed the following mechanism.⁸⁾ Namely, both Cu^{2+} and AsA bind to the enzyme to form a ternary complex, and $\cdot\text{OH}$ is site-specifically formed and reacts preferentially with the enzyme at the site of its formation. However, there remains some uncertainty about the exact mechanism of enzyme inactivation. This paper describes the mechanism of site-specific inactivation of an enzyme by the Cu^{2+} -AsA system using active papain, which contains an active sulfhydryl (SH) group.⁹⁾

MATERIALS AND METHODS

Chemicals Benzoyl-L-arginine *p*-nitroanilide (BAPA) and glycyl-glycyl-tyrosyl-arginine were obtained from Peptide Institute Inc., Osaka; Sepharose 4B and Sephadex G-25 (superfine) from Pharmacia, Uppsala, Sweden; 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) from Wako Pure Chemical Industries, Ltd., Osaka; cupric acetate (Cu^{2+}) and AsA from Nacalai Tesque, Inc. Kyoto; and catalase and superoxide dismutase (SOD) from Sigma Chemical Co., St. Louis, U.S.A. All other chemicals were of the highest purity available and were used without further purification.

Preparation of Active Papain Papaya latex was obtained from Kawari Co., Ltd., Kyoto. Active papain was prepared by affinity chromatography according to the method of Funk *et al.*⁹⁾ with slight modification, using Sepharose 4B coupled with glycyl-glycyl-tyrosyl-arginine.¹⁰⁾ Crude papain (ammonium sulfate precipitate) was prepared from papaya latex by the method of Kimmel and Smith.¹¹⁾ The crude papain was incubated with a 50 mM acetate buffer (pH 4.3) containing 62.5 mM cysteine and 12.5 mM EDTA. After 30 min of incubation at 30 °C, the enzyme solution was applied to the above affinity column, equilibrated with 20 mM acetate buffer (pH 4.3) containing 5 mM EDTA. Elution was carried out with the equilibration buffer, then with 20 mM acetate buffer (pH 4.3), and finally with water. Active papain was eluted with water from the affinity column. Disc gel electrophoresis at pH 4.0¹²⁾ of the active fraction gave a single protein band (Fig. 1). The papain preparation thus obtained was found to be the same as active papain, reported previously,¹³⁾ in amino acid composition and SH content.

Determination of Enzyme Activity Papain activity was determined using BAPA as described previously.⁸⁾ In inactivation studies, active papain was preincubated in 50 mM acetate buffer (pH 5.6) containing AsA, Cu^{2+} , and

Fig. 1. Polyacrylamide Gel Electrophoresis at pH 4.0 of Papain Preparations

Left), crude papain, precipitated by ammonium sulfate; right), active papain, prepared by affinity chromatography from crude papain.

other additives at 30 °C. Aliquots were periodically transferred to the cysteine-EDTA solution (activation solution⁸⁾) to stop the reaction, and then the mixture was assayed for enzyme activity.

Oxidation of Ascorbic Acid The rate of oxidation of AsA was followed by a decrease in absorption at 265 nm,¹⁴⁾ as described previously.⁸⁾

Amino Acid Analyses Amino acid analyses were performed with a Hitachi 835 Amino Acid Analyzer, following hydrolysis with 6N HCl *in vacuo* at 110 °C for 22 h. SH contents were determined by the method of Ellman using DTNB.¹⁵⁾

Binding of Cupric Ions The binding of Cu^{2+} to active papain was analyzed according to the method of Hummel and Dreyer¹⁶⁾ with slight modification.⁸⁾

RESULTS

Inactivation of Active Papain by the Cu^{2+} -AsA System Active papain was rapidly inactivated by the Cu^{2+} -AsA system under aerobic conditions, and the activity of this inactivated enzyme was not restored by treatment with an activation solution (data not shown). The inactivation by the Cu^{2+} -AsA system was observed at a molar ratio of enzyme to Cu^{2+} of 1:1 <, but at lower concentrations of Cu^{2+} (molar ratio of 1:<1), this inactivation was not observed.

The extent of inactivation showed the same dependence on the extent of oxidation of AsA when the molar ratio of enzyme to cupric ions was 1:1—2 (Fig. 2). However, at a higher concentration of Cu^{2+} (molar ratio of enzyme to Cu^{2+} of 1:10), the extent of inactivation compared

Fig. 2. Relationship between the Inactivation of Active Papain and the Oxidation of AsA by the Cu^{2+} -AsA System

The reaction mixture contained 25 μM active papain, 1.0 mM AsA, and 25–250 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: $\circ$, 25 μM ; $\bullet$, 37.5 μM ; $\triangle$, 50 μM ; $\blacktriangle$, 250 μM .

Fig. 3. Saturation Kinetics in Inactivation of Active Papain by AsA

The k_{app} values of inactivation at various AsA concentrations giving first-order inactivation kinetics were plotted in double-reciprocal fashion. The concentration of Cu^{2+} was as follows: $\circ$, 25 μM ; $\bullet$, 37.5 μM ; $\triangle$, 50 μM .

with the extent of oxidation of AsA was reduced in comparison that observed at lower concentrations of Cu^{2+} .

The formation of a Cu^{2+} -enzyme complex was examined by means of gel filtration.¹⁶⁾ It was found that active papain formed a complex with Cu^{2+} in a 1:2 molar ratio (data not shown), though papain formed the complex in a 1:1 molar ratio.⁸⁾ The complex of active papain and Cu^{2+} had no free SH group and exhibited no enzyme activity. After treatment of the complex with an activation solution, the enzyme's activity level was restored, as was a single SH group.

The inactivation of active papain by the Cu^{2+} -AsA system was found to be an apparent first order reaction (data not shown). A reciprocal plot of k_{app} value, an apparent first order inactivation rate constant, versus AsA concentration⁸⁾ gave a positive intercept on the ordinate (Fig. 3), indicating saturation of the enzyme by AsA. The k_2 value, an inactivation constant, was dependent on the

Fig. 4. Effect of Catalase on the Relationship between the Inactivation of Active Papain and the Oxidation of AsA by the Cu^{2+} -AsA System

The reaction mixture contained $25 \mu\text{M}$ active papain, 1.0 mM AsA, $25 \mu\text{M}$ Cu^{2+} , and catalase 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 catalase was as follows: $\circ$, none; $\blacktriangle$, $0.094 \mu\text{M}$; $\triangle$, $0.94 \mu\text{M}$.

Fig. 5. Effect of Radical Scavengers on the Relationship between the Inactivation of Active Papain and the Oxidation of AsA by the Cu^{2+} -AsA System

The reaction mixture contained $25 \mu\text{M}$ active papain, 1.0 mM AsA, $25 \mu\text{M}$ Cu^{2+} , and 2.5 mM radical scavenger 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 radical scavenger was as follows: $\circ$, none; $\bullet$, mannitol; $\triangle$, histidine.

concentration of Cu^{2+} , but no change in K_i , a dissociation constant of the enzyme-AsA complex, was observed.

To obtain information about the inactivated enzyme by the Cu^{2+} -AsA system, amino acid analysis of the enzyme was performed. The amino acid composition of the inactivated papain was indistinguishable from that of the active papain, except for the loss of a SH group (data not shown). The degree of inactivation was dependent on the decrease in SH content.

Effect of Radical Scavengers Oxidation of AsA produces hydrogen peroxide (H_2O_2) and dehydroascorbic acid (DHA).¹⁷⁾ Neither of these products, alone or together, inactivated the enzyme (data not shown). However, catalase at a low concentration partially protected the enzyme from inactivation by the Cu^{2+} -AsA system, though it did not affect the oxidation of AsA (Fig.

4). Heat-inactivated catalase had no effect on the inactivation by the Cu^{2+} -AsA system. In addition, catalase at a high concentration completely inhibited either the inactivation of the enzyme or the oxidation of AsA. On the other hand, SOD did not affect either the inactivation of the enzyme by the Cu^{2+} -AsA system or the oxidation of AsA (data not shown).

Histidine, which has been reported to scavenge $\cdot\text{OH}$ ¹⁸⁾ and singlet oxygen,¹⁹⁾ completely prevented the inactivation of the enzyme and partially prevented the oxidation of AsA (Fig. 5). The addition of thiourea or of sodium thiocyanate, a $\cdot\text{OH}$ scavenger,²⁰⁾ completely protected the enzyme from inactivation, and the oxidation of AsA was characterized by a rapid initial phase followed by a stable phase with no oxidation (data not shown). D-Mannitol, a $\cdot\text{OH}$ scavenger,²¹⁾ at concentrations that are usually effective in scavenging $\cdot\text{OH}$, failed to protect the enzyme from inactivation and the oxidation of AsA (Fig. 5). The effect of other $\cdot\text{OH}$ scavengers,²¹⁾ including sodium formate and sodium benzoate, was similar to that of mannitol.

DISCUSSION

An irreversible inactivation of active papain was observed only when both Cu^{2+} and AsA were added to the enzyme solution under aerobic conditions. The inactivation was due to the oxidation of an active SH group of the enzyme. No inactivation was observed at a molar ratio of enzyme to Cu^{2+} of $1 : <1$. At a molar ratio of $1 : 1-2$, the extent of inactivation showed the same dependence on the extent of oxidation of AsA (Fig. 2). These findings suggest that a Cu^{2+} -mercaptide enzyme is initially formed between an active SH group of the enzyme and Cu^{2+} , which is not concerned with the inactivation of the enzyme by the Cu^{2+} -AsA system, and another Cu^{2+} binds to a certain locus on the enzyme, and the oxidation of AsA by the Cu^{2+} at the locus is responsible for the inactivation.

Saturation kinetics were observed with respect to the concentration of AsA, indicating saturation of the enzyme by AsA. At various concentrations of Cu^{2+} , no change of K_i but a change in k_2 was found (Fig. 3). This finding is in accord with the previously proposed site-specific mechanism,⁸⁾ postulating that AsA combines directly with the enzyme at a locus near enzyme-bound Cu^{2+} to form a ternary complex responsible for the inactivation.

Based on the above findings, it seems likely that the redox reaction between Cu^{2+} and AsA at the specific site generates the highly reactive free radicals responsible for the inactivation. The function of monoionic AsA (AH^-) in the Cu^{2+} -AsA system is to reduce Cu^{2+} to Cu^+ with the accompanying production of a superoxide radical ($\cdot\text{O}_2^-$) and an AsA radical in its ionized form ($\cdot\text{A}^-$)²²⁾ (Eq. 1) which subsequently served as a source of H_2O_2 , formed via

dismutation²³⁾ (Eq. 2), and dehydroascorbic acid (DHA)²⁴⁾ (Eq. 3).

Neither of these products, H_2O_2 or DHA, inactivated the enzyme. Furthermore, SOD, which catalyzes the dismutation of $\cdot\text{O}_2^-$,²⁵⁾ did not affect either the inactivation of the enzyme or the oxidation of AsA by the Cu^{2+} -AsA system. These suggest that H_2O_2 and $\cdot\text{O}_2^-$ are not responsible for the inactivation of an enzyme by the Cu^{2+} -AsA system, but rather, they serve as a source for a secondary, highly reactive species, probably $\cdot\text{OH}$.^{18,21)} When the reaction between H_2O_2 and $\cdot\text{O}_2^-$ is catalyzed by Cu^{2+} , it seems likely that $\cdot\text{O}_2^-$ acts to reduce Cu^{2+} to Cu^+ (Eq. 4-1), followed by a reaction of this Cu^+ with H_2O_2 (Eq. 4-2), giving an overall reaction (Eq. 4).¹⁸⁾ If so, then it is likely that the addition of catalase prevents the conversion of Cu^+ into Cu^{2+} (Eq. 4-2) and, consequentially, the oxidation of AsA (Eq. 1). At a low concentration of catalase, the enzyme was partially protected from inactivation by the Cu^{2+} -AsA system, but the oxidation of AsA was not affected (Fig. 4). This suggests that $\cdot\text{OH}$ is not formed *via* a copper-catalyzed Haber-Weiss reaction (Eq. 4). It can therefore be presumed that H_2O_2 reacts directly with $\cdot\text{O}_2^-$ to form $\cdot\text{OH}$ (Haber-Weiss reaction²⁶⁾) (Eq. 5). On the other hand, at a high concentration of catalase, either the inactivation of the enzyme or the oxidation of AsA was completely inhibited. This suggests that H_2O_2 may also promote the conversion of Cu^+ into Cu^{2+} (Eq. 6) and thus the redox reaction between Cu^{2+} and AsA must proceed continuously.

Mannitol at concentrations that are usually effective in scavenging $\cdot\text{OH}$ failed to provide any protection against the inactivation of the enzyme or the oxidation of AsA (Fig. 5), even though the concentration was increased (up to 1 M). This suggests that $\cdot\text{OH}$ is not responsible for inactivation of the enzyme. However, the explanation for similar negative results given in previous papers^{2,3,27,28)} is that $\cdot\text{OH}$ formed near or on an enzyme itself is not scavenged, since the specific site is not readily accessible to the trapping agents. It can therefore be assumed that $\cdot\text{OH}$ is site-specifically formed. Thus, mannitol was inactive (Fig. 5).

Histidine completely prevented the inactivation of the enzyme, but did not completely prevent the oxidation of AsA (Fig. 5). This suggests that histidine may be able to form a Cu^{2+} -histidine complex responsible for the oxidation of AsA, and this redox reaction is not involved in inactivation. Furthermore, on the addition of either thiourea or sodium thiocyanate, the enzyme was completely protected from inactivation, and the oxidation

of AsA was characterized by a rapid initial phase followed a stable phase with no oxidation. This finding is possibly explained by the notion that the Cu^{2+} trapped by these agents is reduced by AsA to give a cuprous complex. This results in the oxidation of AsA, only initially, because these reactions are irreversible under normal conditions. These suggest that the above agents can trap metal ions (Cu^{2+} or Cu^+) and thereby prevent the formation of a ternary complex that is involved in the inactivation. That is, the above protection is due to the removal of the metal ions at the specific site for inactivation, as discussed in our previous paper.²⁷⁾

In conclusion, a possible mechanism of the inactivation of active papain by the Cu^{2+} -AsA system is characterized by the following features. Both Cu^{2+} and AsA (AH^-) bind to the enzyme to form a ternary complex (Eq. 7),⁸⁾ and then the redox reaction between Cu^{2+} and AsA in the specific site generates $\cdot\text{OH}$.²⁷⁾ This proposed mechanism involves another role of H_2O_2 , besides producing $\cdot\text{OH}$ *via* a Haber-Weiss reaction (Eq. 5). That is, H_2O_2 also promotes the conversion of Cu^+ into Cu^{2+} (Eq. 6), and thus the continuous oxidation of AsA by Cu^{2+} occurs. A "multi-hit" effect in the MCO system proposed by Chevion⁷⁾ can be explained in terms of the repeated redox reaction between Cu^{2+} and AsA in the specific site owing to the above mechanism. The $\cdot\text{OH}$ reacts preferentially with an active SH group of the enzyme, resulting in site-specific damage rather than random injury.

It must be verified that the mechanism described above is universal on the inactivation of an enzyme by the Cu^{2+} -AsA system. This is now under investigation.

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