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 Cu²⁺–ascorbic acid (AsA) system was examined. Incubation of active papain, which contains an active sulphydryl (SH) group, with the Cu²⁺–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 Cu²⁺ 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 H₂O₂, besides producing hydroxyl radicals (•OH), is to promote the conversion of Cu⁺ into Cu²⁺, and that an active SH group of papain is site-specifically modified by the •OH, resulting in inactivation of the enzyme.

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

Many studies on the oxidative modification of enzymes¹⁻⁴ have established that the oxidation is dependent upon an electron donor (NADPH, ascorbic acid, etc.), oxygen and metal ions (Fe³⁺, Cu²⁺), 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 Cu²⁺.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.⁷ There is substantial evidence that the metal-catalyzed oxidative modification of enzymes is a site-specific process.⁸,⁷ For example, in the case of the Cu²⁺–AsA model system, Cu²⁺ binds to a metal-binding site on an enzyme molecule, and then hydroxyl radicals (•OH) repeatedly formed via a redox reaction between the bound Cu²⁺ and AsA preferentially attack the specific site near the metal-binding site on the enzyme in a "multi-hit" manner.⁹ We have examined in detail the mechanism of the site-specific inactivation of an enzyme by the Cu²⁺–AsA system using papain (EC 3.4.22.2), and have proposed the following mechanism.⁸ Namely, both Cu²⁺ and AsA bind to the enzyme to form a ternary complex, and •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 Cu²⁺–AsA system using active papain, which contains an active sulphydryl (SH) group.⁹

MATERIALS AND METHODS

Chemicals: Benzoyl-L-arginine p-nitroanilide (BAPA) and glycyglycyl-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 (Cu²⁺) 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 glycyglycyl-tyrosyl-arginine.⁰ 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 acetaldehyde (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 acetic acid buffer (pH 4.3) containing 5 mM EDTA. Elution was carried out with the equilibration buffer, then with 20 mM acetic acid buffer (pH 4.3), and finally with water. Active papain was eluted with water from the affinity column. Disc gel electrophoresis at pH 4.0²³ 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,¹⁻³ 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 acetic acid buffer (pH 5.6) containing AsA, Cu²⁺, and
other additives at 30°C. Aliquots were periodically transferred to the cysteine-EDTA solution (activation solution) 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, as described previously.

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

**Binding of Cupric Ions**  The binding of Cu²⁺ to active papain was analyzed according to the method of Hummel and Dreger with slight modification.

**RESULTS**

**Inactivation of Active Papain by the Cu²⁺-AsA System**  Active papain was rapidly inactivated by the Cu²⁺-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 Cu²⁺-AsA system was observed at a molar ratio of enzyme to Cu²⁺ of 1:1 < , but at lower concentrations of Cu²⁺ (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 Cu²⁺ (molar ratio of enzyme to Cu²⁺ of 1:10), the extent of inactivation compared with the extent of oxidation of AsA was reduced in comparison that observed at lower concentrations of Cu²⁺.

The formation of a Cu²⁺-enzyme complex was examined by means of gel filtration. It was found that active papain formed a complex with Cu²⁺ in a 1:2 molar ratio (data not shown), though papain formed the complex in a 1:1 molar ratio. The complex of active papain and Cu²⁺ 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 Cu²⁺-AsA system was found to be an apparent first order reaction (data not shown). A reciprocal plot of kₐp value, an apparent first order inactivation rate constant, versus AsA concentration gave a positive intercept on the ordinate (Fig. 3), indicating saturation of the enzyme by AsA. The k₂ 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 Cu²⁺–AsA System

The reaction mixture contained 25 μM active papain, 1.0 mM AsA, 25 μM Cu²⁺, 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: ○, none; ▲, 0.094 μM; △, 0.94 μM.

Fig. 5. Effect of Radical Scavengers on the Relationship between the Inactivation of Active Papain and the Oxidation of AsA by the Cu²⁺–AsA System

The reaction mixture contained 25 μM active papain, 1.0 mM AsA, 25 μM Cu²⁺, 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: ○, none; ●, mannitol; △, histidine.

concentration of Cu²⁺, but no change in Kᵣ, a dissociation constant of the enzyme–AsA complex, was observed.

To obtain information about the inactivated enzyme by the Cu²⁺–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 (H₂O₂) and dehydroascorbic acid (DHA). 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 Cu²⁺–AsA system, though it did not affect the oxidation of AsA (Fig. 4). Heat-inactivated catalase had no effect on the inactivation by the Cu²⁺–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 Cu²⁺–AsA system or the oxidation of AsA (data not shown).

Histidine, which has been reported to scavenge ·OH and singlet oxygen, 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 ·OH scavenger, 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 ·OH scavenger, at concentrations that are usually effective in scavenging ·OH, failed to protect the enzyme from inactivation and the oxidation of AsA (Fig. 5). The effect of other ·OH scavengers, including sodium formate and sodium benzoate, was similar to that of mannitol.

DISCUSSION

An irreversible inactivation of active papain was observed only when both Cu²⁺ 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 Cu²⁺ 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 Cu²⁺—mercaptide enzyme is initially formed between an active SH group of the enzyme and Cu²⁺, which is not concerned with the inactivation of the enzyme by the Cu²⁺–AsA system, and another Cu²⁺ binds to a certain locus on the enzyme, and the oxidation of AsA by the Cu²⁺ 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 Cu²⁺, no change in Kᵣ but a change in k₂ was found (Fig. 3). This finding is in accord with the previously proposed site-specific mechanism, postulating that AsA combines directly with the enzyme at a locus near enzyme-bound Cu²⁺ to form a ternary complex responsible for the inactivation.

Based on the above findings, it seems likely that the redox reaction between Cu²⁺ and AsA at the specific site generates the highly reactive free radicals responsible for the inactivation. The function of monoionic AsA (AH⁻) in the Cu²⁺–AsA system is to reduce Cu²⁺ to Cu⁺ with the accompanying production of a superoxide radical (·O₂⁻) and an AsA radical in its ionized form (·A⁻) (25) (Eq. 1) which subsequently served as a source of H₂O₂, formed via

\[ \text{AH}^- + \text{Cu}^{2+} + \text{O}_2 -> \text{Cu}^+ + 2\text{H}^+ + \cdot\text{O}_2^- + \cdot\text{A}^- \]  

(1)

dismutation \(2^{-3}\) (Eq. 2), and dehydroascorbic acid (DHA) \(2^{-4}\) (Eq. 3).
\[
\text{O}_2^- + \text{H}^+ + \text{O}_2\text{H} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\] (2)

\[
\text{A}^- + \text{O}_2\text{H} \rightarrow \text{DHA} + \cdot \text{O}_2^-
\] (3)

Neither of these products, \(\text{H}_2\text{O}_2\) or DHA, inactivated the enzyme. Furthermore, SOD, which catalyzes the dismutation of \(\cdot \text{O}_2^-\), did not affect either the inactivation of the enzyme or the oxidation of AsA by the \(\text{Cu}^{2+}\)-AsA system. These suggest that \(\text{H}_2\text{O}_2\) and \(\cdot \text{O}_2^-\) are not responsible for the inactivation of an enzyme by the \(\text{Cu}^{2+}\)-AsA system, but rather, they serve as a source for a secondary, highly reactive species, probably \(\cdot \text{OH}\). When the reaction between \(\text{H}_2\text{O}_2\) and \(\cdot \text{O}_2^-\) is catalyzed by \(\text{Cu}^{2+}\), it seems likely that \(\cdot \text{O}_2^-\) acts to reduce \(\text{Cu}^{2+}\) to \(\text{Cu}^+\) (Eq. 4-1), followed by a reaction of this \(\text{Cu}^+\) with \(\text{H}_2\text{O}_2\) (Eq. 4-2), giving an overall reaction (Eq. 4). If so, then it is likely that the addition of catalase prevents the conversion of \(\text{Cu}^+\) into \(\text{Cu}^{2+}\) (Eq. 4-2) and, consequently, the oxidation of AsA (Eq. 1). At a low concentration of catalase, the enzyme was partially protected from inactivation by the \(\text{Cu}^{2+}\)-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 \(\text{H}_2\text{O}_2\) reacts directly with \(\cdot \text{O}_2^-\) to form \(\cdot \text{OH}\) (Haber–Weiss reaction) (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 \(\text{H}_2\text{O}_2\) may also promote the conversion of \(\text{Cu}^+\) into \(\text{Cu}^{2+}\) (Eq. 6) and thus the redox reaction between \(\text{Cu}^{2+}\) and AsA must proceed continuously.

\[
\text{H}_2\text{O}_2 + \cdot \text{O}_2^- \rightarrow \cdot \text{O}_2 + \text{O}_2 + \text{H}_2\text{O}
\] (4)

\[
\text{Cu}^{2+} + \cdot \text{O}_2^- \rightarrow \text{Cu}^+ + \text{O}_2
\] (4-1)

\[
\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \cdot \text{OH} + \cdot \text{OH}
\] (4-2)

\[
\text{H}_2\text{O}_2 + \cdot \text{O}_2^- + \cdot \text{OH} + \cdot \text{OH}
\] (5)

\[
\text{H}_2\text{O}_2 + 2\text{H}^+ + 2\text{Cu}^{2+} \rightarrow 2\text{Cu}^{2+} + 2\text{H}_2\text{O}
\] (6)

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 \(1 \text{m}\)). 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 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 \(\text{Cu}^{2+}\)-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 by a stable phase with no oxidation. This finding is possibly explained by the notion that \(\text{Cu}^{2+}\) 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 (\(\text{Cu}^{2+}\) or \(\text{Cu}^+\)) 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.

In conclusion, a possible mechanism of the inactivation of active papain by the \(\text{Cu}^{2+}\)-AsA system is characterized by the following features. Both \(\text{Cu}^{2+}\) and AsA (AH) bind to the enzyme to form a ternary complex (Eq. 7), and then the redox reaction between \(\text{Cu}^{2+}\) and AsA in the specific site generates \(\cdot \text{OH}\). This proposed mechanism involves another role of \(\text{H}_2\text{O}_2\), besides producing \(\cdot \text{OH}\) via a Haber–Weiss reaction (Eq. 5). That is, \(\text{H}_2\text{O}_2\) also promotes the conversion of \(\text{Cu}^+\) into \(\text{Cu}^{2+}\) (Eq. 6), and thus the continuous oxidation of AsA by \(\text{Cu}^{2+}\) occurs. A "multi-hit" effect in the MCO system proposed by Cheyvin can be explained in terms of the repeated redox reaction between \(\text{Cu}^{2+}\) 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.

\[
E + \text{Cu}^{2+} + \text{AH}^- \rightarrow E \cdot \text{Cu}^{2+} + \text{AH}^-
\] (7)

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

REFERENCES