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Oxidative Deamination by Hydrogen Peroxide in the Presence of Metals

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Various amines, including lysine residue of bovine serum albumin, were oxidatively deaminated to form the corresponding aldehydes by a H_2O_2/Cu^{2+} oxidation system at physiological pH and temperature. The resulting aldehydes were measured by high-performance liquid chromatography. We investigated the effects of metal ions, pH, inhibitors, and O_2 on the oxidative deamination of benzylamine by H_2O_2. The formation of benzaldehyde was the greatest with Cu^{2+}, and catalysis occurred with Co^{2+}, VO^{2+}, and Fe^{3+}. The reaction was greatly accelerated as the pH value rose and was markedly inhibited by EDTA and catalase. Dimethyl sulfoxide and thiourea, which are hydroxyl radical scavengers, were also effective in inhibiting the generation of benzaldehyde, indicating that the reaction is a hydroxyl radical-mediated reaction. Superoxide dismutase greatly stimulated the reaction, probably due to the formation of hydroxyl radicals. O_2 was not required in the oxidation, and instead slightly inhibited the reaction. We also examined several oxidation systems. Ascorbic acid$/O_2/Cu^{2+}$ and hemoglobin$/H_2O_2$ systems also converted benzylamine to benzaldehyde. The proposed mechanism of the oxidative deamination by H_2O_2/Cu^{2+} system is discussed.

Keywords: Aldehyde; Amine; Free radical; Fenton reaction; Hydroxyl radical; Oxidative deamination

Abbreviations: ACPP, 1-amino-1-carboxy-5,5-bis-p-hydroxyphenylpentane; ASA, ascorbic acid; BSA, bovine serum albumin; Cu–Zn SOD, copper–zinc superoxide dismutase; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Hb, hemoglobin; HPLC, high-performance liquid chromatography; poly-Lys, poly-L-lysine

INTRODUCTION

Reactive oxygen species, which are formed by several physiological processes and by induced oxidative stress, react with various biological molecules and cause oxidative damage to the body.[1] Oxidative damage is implicated in the pathogenesis of a number of diseases, as well as certain physiological processes such as aging, ischemia-reperfusion injury, and protein turn over.[2–4] Oxidative damage to protein, which induces protein–protein cross-links,[5–8] cleavage of some peptide bonds,[9,10] and oxidative modification of amino acid residues,[11–17] is also believed to be closely related to these processes. There exist a number of different sources *in vivo* that generate oxygen free radicals that could potentially lead to the oxidative modification of proteins. Among these sources, the metal-catalyzed oxidation systems are well established and reviewed by Stadtman *et al.*[13–17] The ability of the system to catalyze the oxidative modification of proteins is attributable to interaction with hydrogen peroxide (H_2O_2) and iron or copper ion, i.e. a Fenton reaction. The main pathway for the formation of the hydroxyl radical in biological systems may be through the following sequence of reactions.

$$H_2O_2 + M^{n+} \rightarrow {}^{\bullet}OH + OH^- + M^{(n+1)+}$$

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In this mechanism, it is assumed that the copper- or iron-binding site on proteins serve as a center for the production of hydroxyl radicals via the Fenton reaction.[18] Then hydroxyl radicals react in a site-specific manner to modify amino acid residues at the metal binding sites on the protein.[11,14,16]. Various iron complexes present in vivo promote the generation of the hydroxyl radical from H_2O_2 via the Fenton reaction, and decompose lipid peroxides with formation of alkoxy and peroxy radicals[1] Because the oxidative modification of proteins by the metal-catalyzed oxidation system leads to the conversion of some amino acid residues to carbonyl derivatives, which are detected by the formation of 2,4-dinitrophenylhydrazone,[19–24] the level of protein carbonyl groups has often been used as a measure of oxygen radical-mediated protein damage under various physiological conditions. An increase in protein carbonyl content of tissues is observed in aging and in a number of pathological disorders, including rheumatoid arthritis, Alzheimer's disease, respiratory distress syndrome, Parkinson's disease, and atherosclerosis.[3,16,17,25] Previous researches have demonstrated that histidine, arginine, and proline residues were damaged by the metal-catalyzed oxidation to form carbonyl groups.[26–28] More recently, a major oxidation product, namely, α-aminoadipic-δ-semialdehyde (allysine) originating from lysine, has been identified in serum albumin.[29] When oxidative stress is induced in rats by treatment with tert-butyl hydroperoxide (t-BuOOH) or acrolein, this aldehyde is found to be significantly higher compared with control rats. Furthermore, the content of allysine in plasma proteins shows a positive correlation with the rat's age. The allysine content in serum albumin or in plasma proteins collected from eight different mammalian species was found to be inversely proportional to their maximum lifespan potential.[29]

Since the highly reactive and hydrophobic allysine residue is quite different from the lysine residue and known to be a precursor of crosslinks of elastin and collagen, its oxidative deamination is considered to induce serious damage to a living body. Hence, it is important to understand the cause and the physiological mechanism of the oxidation. In this paper, we report direct evidence that various kinds of amines, including lysine residues of bovine serum albumin (BSA), were oxidatively deaminated by hydroxyl-radical attack, which was generated by H_2O_2/Cu^{2+} system, at physiological pH and temperature (at pH 7.4 and 37°C). This system produces hydroxyl radical according to the following equations

$$Cu^{2+} + H_2O_2 \rightarrow Cu^{+} + H^{+} + HO_2^{\bullet}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + {}^{\bullet}OH.$$

The present work was also undertaken to determine clearly the effects of pH, metal ions, inhibitors, and molecular oxygen (O_2) on the reaction. Such information is fundamental for the assessment of the role of the Fenton reaction in the oxidative modification of biomolecules. From these results, we discuss the mechanism of oxidative deamination by H_2O_2/Cu^{2+} system.

MATERIALS AND METHODS

Chemicals

H_2O_2 (30% aqueous solution) was purchased from Santoku Chemical Industries, Tokyo, Japan. Acetonitrile was of high-performance liquid chromatography (HPLC) grade from Kanto Chemicals, Tokyo, Japan. Catalase from bovine liver, N-methyl-n-butylamine, and N-n-butyldimethylamine were from Tokyo Kasei, Tokyo, Japan. Poly-L-lysine hydrobromide (molecular weight: approx. 1000) was from Sigma Chemical, St. Louis, MO, USA. All other chemicals were of analytical grade from Nacalai Tesque, Kyoto, Japan.

Oxidative Deamination by the H_2O_2/Cu^{2+} System

Benzylamine

Reaction mixtures (500 μl) in a micro test tube contained 10 mM benzylamine, 0.5 mM $CuSO_4$, and 0.1 M sodium phosphate buffer, pH 7.4. The reaction was started by the addition of 0.5 mM H_2O_2 (500 μl). The reaction mixtures were incubated at 37°C with shaking in the dark. After incubation, the reaction was terminated by the addition of acetic acid (500 μl). We confirmed that this procedure completely stopped the reaction. Then the resulting benzaldehyde was analyzed by HPLC on a C-18 reversed phase column (Cosmosil $5C_{18}$-AR-II, Nacalai Tesque, Kyoto, Japan), using acetonitrile/0.2% aqueous phosphoric acid (4:6, v/v) as eluent, and monitoring the eluate at 245 nm. Benzaldehyde was eluted at 5.2 min at a flow rate of 1.0 ml/min.

n-Butylamine, N-Methyl-n-Butylamine, and N-n-Butyldimethylamine

Reaction mixtures (1.0 ml) in a Pyrex test tube contained 10 mM n-butylamine, N-methyl-n-butylamine, or N-n-butyldimethylamine, 1.0 mM $CuSO_4$, and 0.1 M sodium phosphate buffer, pH 7.4. The reaction was started by the addition of 2.0 mM H_2O_2 aqueous solution (1.0 ml). The reaction mixtures were incubated at 37°C for 24 h with shaking in the dark. After incubation, the reaction mixture was extracted with 2.0 ml of ethyl ether. Then 100 μl of 20 mM 2, 4-dinitrophenylhydrazine-0.1 N HCl-methanol was added to an ethyl ether layer (1.0 ml), and then the mixture was allowed to react

for 5 h at room temperature. We confirmed that this operation completely prevented the progress of the oxidative deamination. Then n-butyraldehyde-2,4-dinitrophenylhydrazone was analyzed by HPLC on a C-18 reversed phase column (COSMOSIL $5C_{18}$-AR-II), using acetonitrile / water (8 : 2, v / v) as eluent, and the eluate was monitored at 358 nm. n-butyraldehyde-2,4-dinitrophenyl hydrazone was eluted at 3.6 min at a flow rate of 1.0 ml / min.

Lysine Residue

Reaction mixtures (1.0 ml) in a Pyrex test tube with a Teflon-lined screw cap contained 20.0 mg / ml BSA, 1.0 mM $CuSO_4$, and 0.1 M sodium phosphate buffer (pH 7.4). The reaction was started by the addition of 2.0 mM of H_2O_2 solution (1.0 ml). The reaction mixtures were incubated at 37°C for 24 h with shaking in the dark. After incubation, the protein was precipitated by the addition of 70% trichloroacetic acid (1.0 ml), and centrifuged at 2000 g for 20 min at room temperature followed by washing with 2.0 ml of distilled water and 1.0 ml of 70% trichloroacetic acid. Then, 1-amino-1-carboxy-5,5-bis-p-hydroxyphenylpentane (ACPP), a bisphenol derivative of allysine, was determined by a modification of the previous method.[30,31] The protein was hydrolyzed in a conventional manner for 48 h at 110°C with 2.0 ml of 6 N HCl containing 3% (v / v) phenol. The hydrolysate was extracted twice with 2.0 ml of ethyl ether, and the water layer was dried by rotary evaporation followed by reconstitution in 500 μl of distilled water. A Sep-Pak plus C_{18} environmental cartridge (Waters, Milford, MA, USA) was used for pre-purification as follows. The Sep-Pak cartridge was flushed with methanol (10 ml) and then distilled water (20 ml), and the sample (500 μl) was put onto the cartridge. After the cartridge was washed with 10.0 ml of distilled water, ACPP was eluted with 5.0 ml of distilled water / methanol (1:1, v / v). The eluate was evaporated to dryness, and then reconstituted in 500 μl of distilled water. A 20 μl portion of it was injected into an HPLC apparatus with a C-18 reversed phase column (COSMOSIL $5C_{18}$-AR-II) eluted by a linear gradient of 0–20% methanol in 20 min. The HPLC analysis was performed on a Perkin Elmer Liquid Chromatograph Integral 4000 system (Norwalk, CT, USA) and the eluate was monitored at 278 nm with the diode array detector. The flow rate was 1.0 ml / min and the column oven was maintained at 40°C. ACPP was eluted in a t_R of 18.9 min.

Oxidative Deamination Under O_2 and N_2

Reaction mixtures (500 μl) in a Pyrex test tube contained 10 mM benzylamine, 0.5 mM $CuSO_4$, and 0.1 M sodium phosphate buffer (pH 7.4). The test tube was tightly fitted with a silicone rubber cap. The tube was evacuated and then filled with O_2 and N_2 gas through a hypodermic needle for the reaction under O_2 and N_2, respectively. After another hypodermic needle was inserted in the tube to serve as outlet port, gas was passed through the incubation mixture for 10 min and charged until the pressure of 0.049 MPa inside the tube was reached. Then the reaction was started by the addition of H_2O_2 solution ((500 μl) using a hypodermic syringe.

Effect of Catalase and Copper–zinc Superoxide Dismutase (Cu–Zn SOD) on Oxidative Deamination of Benzylamine

Reaction mixtures (500 μl) in a micro test tube contained 300 U / ml catalase or Cu–Zn SOD from bovine erythrocytes, 10 mM benzylamine, 0.50 mM $CuSO_4$ and 0.1 M sodium phosphate buffer, pH 7.4. Heat-inactive catalase and Cu–Zn SOD[32] were prepared by heating at 100°C for 5 and 30 min in distilled water, respectively. The reaction was started by the addition of 0.5 mM H_2O_2 (500 μl). The reaction mixtures were incubated at 37°C with shaking in the dark. After incubation for 1 h, the reaction was terminated by the addition of acetic acid (500 μl). Then the production of benzaldehyde was measured by HPLC as described above.

Statistical Analysis

The results are shown as means ± SEM for single measurements of individual aliquots from three separate incubations.

RESULTS AND DISCUSSION

Oxidative Deamination by H_2O_2/Cu^{2+} System

To determine the oxidative deamination of various amines by H_2O_2/Cu^{2+} system, 5.0 mM of each amine, 1.0 mg / ml poly-Lys, or 10.0 mg / ml BSA was incubated with H_2O_2 and 0.5 mM $CuSO_4$ in 50 mM sodium phosphate buffer (pH 7.4) for 24 h at 37°C. Then the resulting aldehydes were measured using HPLC. Allysine residue was determined as ACPP, which is a condensation product of one allysine residue and two phenol molecules.[30,31] ACPP, the bisphenol derivative of allysine, was observed in hydrolysate of BSA treated with 10.0 mM H_2O_2, but not in hydrolysates of BSA treated with 1.0 mM H_2O_2 and native BSA. This oxidation system resulted in the formation of 0.075 mM allysine derived from the lysine residues of BSA (Table I). The ACPP peak was also observed in hydrolysate of poly-Lys oxidized with 1.0 mM H_2O_2. As shown in Table I, benzylamine and n-butylamine were oxidatively deaminated to

TABLE I Oxidative deamination of various kinds of amines by H_2O_2/Cu^{2+} system

Amine	H_2O_2 (mM)	Aldehyde production mean ± SEM (mM)
BSA (10.0 mg / ml)	1.0	Not detected
BSA (10.0 mg / ml)	10.0	0.075 ± 0.001
Poly-Lys (1.0 mg / ml)	1.0	0.058 ± 0.004
Benzylamine (5.0 mM)	1.0	0.21 ± 0.00
n-Butylamine (5.0 mM)	1.0	0.41 ± 0.06
N-methyl-n-butylamine (5.0 mM)	1.0	0.11 ± 0.00
N,N-dimethyl-n-butylamine (5.0 mM)	1.0	Not detected

Each amine was incubated with H_2O_2 and 0.5 mM $CuSO_4$ in 50 mM sodium phosphate buffer, pH 7.4, at 37°C for 24 h. Then each aldehyde was measured by HPLC as described under section "Materials and Methods".

form the corresponding aldehydes, i.e. benzaldehyde and n-butyraldehyde, respectively. Interestingly, N-methyl-n-butylamine was also converted to n-butyraldehyde but not N-n-butyldimethylamine, implying that secondary amines also undergo deamination by the H_2O_2/Cu^{2+} system, whereas, incubation of n-butylamine with H_2O_2 and Cu^{2+} gave a significant amount of n-butyraldehyde (0.41 mM), showing that 41% of the added H_2O_2 (1.0 mM) reacted with n-butylamine. These results suggest that various amines, including the ε-amino group of lysine residue, can be oxidized to carbonyls by the H_2O_2/Cu^{2+} system at physiological pH and temperature. Generally, the hydroxyl radical is characterized by very high kinetic constants for its reaction with a variety of biological molecules. This radical can cause breaks in the polymeric backbone of proteins[6,11,13,33] and oxidative modifications of amino and residues[11–17] by abstracting a hydrogen atom or by adding to a double bond. Therefore, the lower production of allysine within poly-Lys and BSA is thought to be due to the attack of the hydroxyl radical against the polypeptide backbone and other amino acid residues.

Effect of Metal Ions

The effects of physiologically important metal ions on the oxidation of benzylamine by H_2O_2 are summarized in Fig. 1. Benzaldehyde production was not observed in the reaction of benzylamine with H_2O_2 alone. The greatest extent of reaction was observed with Cu^{2+} in the presence of H_2O_2. The Fe^{3+}, Co^{2+}, and VO^{2+} also effectively catalyzed the reaction. However, other metal ions showed very little effect on the generation of benzaldehyde. In the absence of H_2O_2, incubation of benzylamine with any metal ions did not generate benzaldehyde, suggesting that metal ions are reduced by H_2O_2 and then induce the formation of hydroxyl radicals. This result is consistent with a previous report[34] that Cu^{2+} is more effective than Fe^{3+} in the generation of carbonyl groups in BSA by H_2O_2. These observations can be explained by the fact that the Cu^{2+}/H_2O_2 system generates hydroxyl radicals at much greater rate than the Fe^{3+}/H_2O_2 system.[35] Co^{2+}/H_2O_2 system is also known to form the hydroxyl radical. In addition, an intake of excessive amounts of cobalt is known to be toxic to the living body, probably because of increased generation of hydroxyl radicals.[36]

Dependence of H_2O_2 and Cu^{2+}

The time dependence of oxidative deamination of benzylamine by the H_2O_2/Cu^{2+} system was determined by measuring the production of benzaldehyde. We used benzylamine and Cu^{2+} for the oxidative deamination reaction because of the ease of analysis. Figure 2A shows the results obtained when a fixed amount of Cu^{2+} (0.25 mM) and benzylamine (5.0 mM) were incubated with various concentration of H_2O_2 (0–0.5 mM) for 0–24 h at physiological pH and temperature. Benzaldehyde production continued to increase up to 24 h in the presence of H_2O_2, but benzaldehyde slightly decreased thereafter. Nevertheless, benzoic acid and perbenzoic acid, the oxidation products of benzaldehyde, were not detected by HPLC. As shown in Fig. 2B, the initial rate of benzaldehyde formation is linear with respect to the concentration of H_2O_2 between 0 and 0.5 mM, indicating that the reaction is first order with respect to H_2O_2.

Figure 3A shows the results obtained when a fixed amount of H_2O_2 (0.25 mM) and Cu^{2+} (5.0 mM) were incubated with various concentrations of copper ion (0–0.5 mM) for 0–24 h at 37°C. In the presence of Cu^{2+}, the maximum production of benzaldehyde was almost equivalent regardless of Cu^{2+} concentration. However, incubation of benzylamine with H_2O_2 alone instead of Cu^{2+} did not give rise to benzaldehyde. The initial rate of the oxidative deamination increased as the concentration of Cu^{2+} increased in a non-linear fashion (Fig. 3B). The data suggests that copper ions catalyze hydroxyl radical formation, and that Cu^{2+} acts as an electron acceptor in the oxidation of the intermediate radical to the aldehyde. Thus, metal ions should undergo cyclic reduction and reoxidation during the oxidative-deamination reaction as will be described later (see Fig. 9). From the dose-dependent effect of Cu^{2+} and H_2O_2, it is evident that the hydroxyl radical

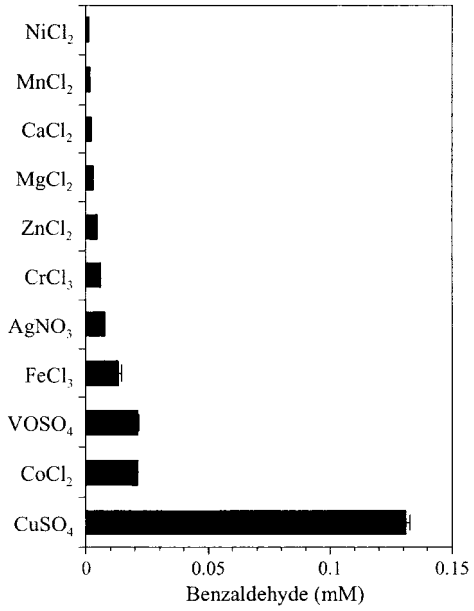

FIGURE 1 Effect of various kinds of metal ion on oxidative deamination of benzylamine by hydrogen peroxide. Benzylamine (5.0 mM) was incubated with 0.25 mM H_2O_2 and 0.25 mM metal ion in 50 mM sodium phosphate buffer, pH 7.4, at 37°C for 24 h. After the reaction was terminated, the production of benzaldehyde was measured by HPLC.

generated through the Fenton reaction between copper ion and H_2O_2 is responsible for the oxidative deamination.

Effect of PH

Figure 4 shows the effect of pH on the oxidative deamination by the H_2O_2/Cu^{2+} system. Reaction mixtures at pH values varying from 6.0 to 10.0, containing 5.0 mM benzylamine, 0.25 mM $CuSO_4$, and 0.25 mM H_2O_2 were incubated at 37°C for 1 h, and then the initial rate of the reaction was determined. The reaction was faster at a higher pH, but very slow at pH 6. At pH 10, benzaldehyde production was nearly equal to the amount of the added H_2O_2 (0.25 mM) after 1 h. Presumably benzylamine is not oxidized at pH 6 because the hydroxyl radical is not produced by the H_2O_2/Cu^{2+} system in acidic media.[37] In addition, the pH-dependent increase in the production of benzaldehyde may reflect the deprotonation of the amino group of benzylamine. Thus the deprotonated amines may be targeted by the hydroxyl radical.

Inhibition by EDTA and Hydroxyl Radical Scavengers

To investigate the reactivity of the hydroxyl radical formed by the H_2O_2/Cu^{2+} system and responsible for the oxidation of benzylamine, we used the chelating agent, ethylenediaminetetraacetic acid (EDTA), and hydroxyl radical scavengers, thiourea and dimethyl sulfoxide (DMSO). Benzylamine (5.0 mM) was incubated with 0.25 mM H_2O_2 and 0.25 mM Cu^{2+} in the presence of each scavenger (50 mM) or EDTA (0.25 mM). As shown in Fig. 5, DMSO and thiourea significantly inhibited the production of benzaldehyde (72 and 71% inhibition, respectively). This result suggests that the hydroxyl radicals released into solution by H_2O_2/Cu^{2+} system attack benzylamine and can be scavenged by DMSO and thiourea. The addition of EDTA (0.25 mM) exhibited a marked inhibition (92% inhibition). EDTA–Cu^{2+} complexes have a stabilizing effect on the decomposition of H_2O_2.[38] Additionally, EDTA–transition metal complexes are attacked by hydroxyl radical.[39,40] Therefore, the inhibition of the oxidative deamination appears to be due to both the depression of the hydroxyl radical formation and the competition between benzylamine and EDTA for reaction of hydroxyl radical.

Effect of O_2

In order to investigate the effect of O_2 on the oxidation, we incubated benzylamine with H_2O_2 and Cu^{2+} under air, O_2, or N_2. As shown in Fig. 6, the reaction under O_2 significantly decreased the production of benzaldehyde to approx. 65% of that under air. On the contrary, a slight increase in the production was observed under N_2 compared with air. The large differences in the production of benzaldehyde was observed between the air and O_2 conditions, possibly because the reaction under O_2 was carried out at much higher atmospheric pressure than that under air. These results show that the oxidative deamination by H_2O_2/Cu^{2+} system requires no molecular oxygen. The decreased rate of the benzaldehyde production under O_2 may be due to the competition of O_2 with H_2O_2 for the reaction with Cu^+. Oxygen may convert Cu^+ to Cu^{2+}, thereby preventing the hydroxyl radical formation by the Fenton reaction. Alternatively, oxygen may add to the carbon-centered radical generated by the hydroxyl radical attack as described later (see Fig. 9), thus reducing the yield of benzaldehyde.

Effects of Catalase and Superoxide Dismutase

Figure 7 shows the effects of catalase and Cu–Zn SOD on the initial rate of oxidative deamination of benzylamine by the H_2O_2/Cu^{2+} system. The formation of benzaldehyde in the presence of heat-inactivated enzymes was significantly less than that in the absence of enzymes, probably because heat-inactivated enzymes were attacked by hydroxyl radicals. As expected, active catalase (150 U/ml) almost completely inhibited the production of benzaldehyde, but heat-inactivated

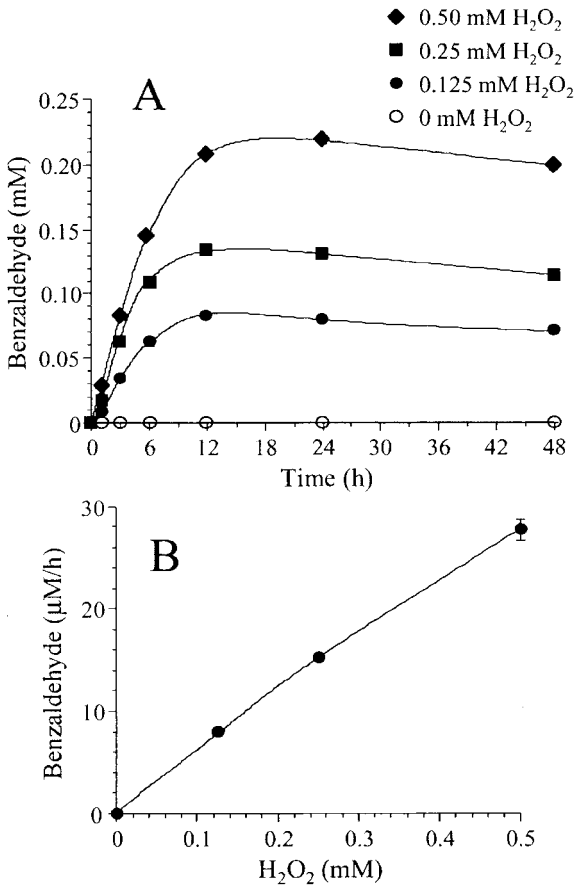

FIGURE 2 (A) Time course of oxidative deamination of benzylamine by H_2O_2/Cu^{2+} system as a function of H_2O_2 concentration. Benzylamine (5.0 mM) was incubated with 0.25 mM $CuSO_4$ and 0–0.50 mM H_2O_2 in 50 mM sodium phosphate buffer, pH 7.4, at 37°C. After the reaction was terminated, the production of benzaldehyde was measured by HPLC. (B) The initial rate of oxidative deamination of benzylamine (5.0 mM) as a function of H_2O_2 concentration at a fixed concentration of $CuSO_4$ (0.25 mM).

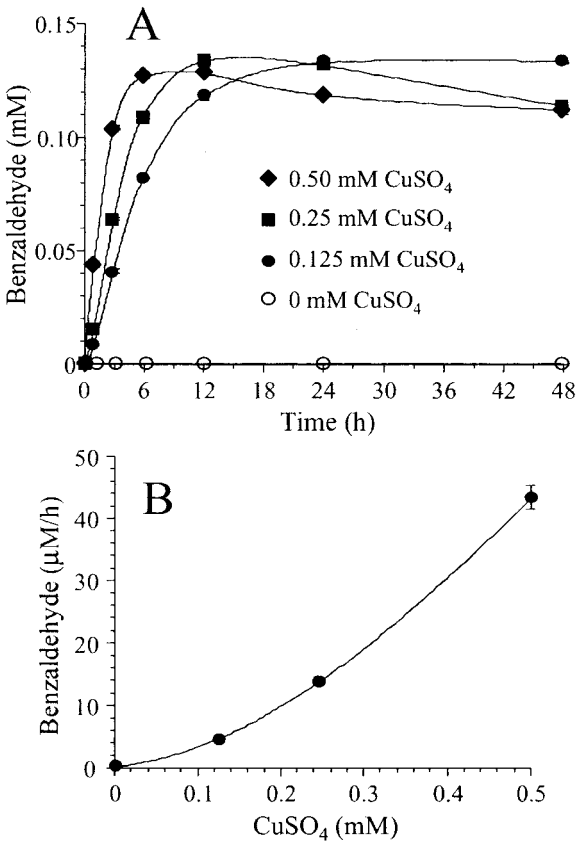

FIGURE 3 (A) Time course of oxidative deamination of benzylamine by H_2O_2/Cu^{2+} system as a function of $CuSO_4$ concentration. Benzylamine (5.0 mM) was incubated with 0.25 mM H_2O_2 and 0–0.50 mM $CuSO_4$ in 50 mM sodium phosphate buffer, pH 7.4, at 37°C. After the reaction was terminated, the production of benzaldehyde was measured by HPLC. (B) The initial rate of oxidative deamination of benzylamine (5.0 mM) as a function of $CuSO_4$ concentration at a fixed concentration of H_2O_2 (0.25 mM).

catalase did not. In contrast, an accelerating effect of Cu–Zn SOD was seen on the rate of the reaction. Interestingly, active Cu–Zn SOD (150 U/ml) generated a much higher amount of benzaldehyde than heat-inactivated Cu–Zn SOD, whereas incubation of benzylamine with active Cu–Zn SOD in the absence of H_2O_2 did not generate benzaldehyde absolutely. This observation can be rationalized by the previous report[41,42] that Cu–Zn SOD catalyzes the formation of hydroxyl radical using H_2O_2 as substrate. Thus, hydroxyl radicals formed by Cu–Zn SOD may accelerate the reaction. This reaction is one possible source of hydroxyl radical formation in vivo and may be responsible for the inactivation of Cu–Zn SOD.

Oxidative Deamination of Various Oxidation Systems

Various metal-catalyzed oxidation systems have been used hitherto to catalyze the oxidative modification of proteins. Among these systems, the ascorbic acid (ASA)/O_2- or H_2O_2-mediated systems are well established to cause the oxidative alteration and inactivation of protein. ASA can facilitate the generation of potentially damaging oxygen species, implying a possible source of the

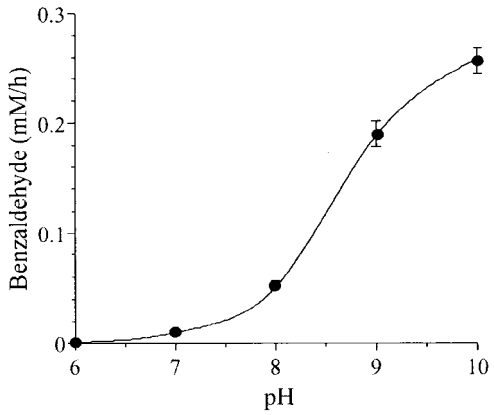

FIGURE 4 Effect of pH on oxidative deamination of benzylamine by H_2O_2/Cu^{2+} system. Benzylamine (5.0 mM) was incubated with 0.25 mM H_2O_2 and 0.25 mM $CuSO_4$ in 50 mM sodium phosphate buffer, pH 6–10, at 37°C for 1 h. After the reaction was terminated, the production of benzaldehyde was measured by HPLC.

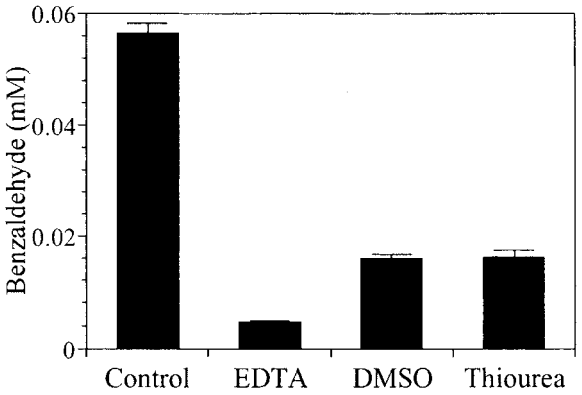

FIGURE 5 Inhibition of the oxidative deamination of benzylamine by chelating agent and hydroxy radical scavengers. Reaction mixtures containing 5.0 mM benzylamine, 0.25 mM H_2O_2, 0.25 mM $CuSO_4$, and 50 mM sodium phosphate buffer, pH 7.4, was incubated with 0.25 mM EDTA, 50 mM DMSO, or 50 mM thiourea at 37°C for 3 h. After the reaction was terminated, the production of benzaldehyde was measured by HPLC.

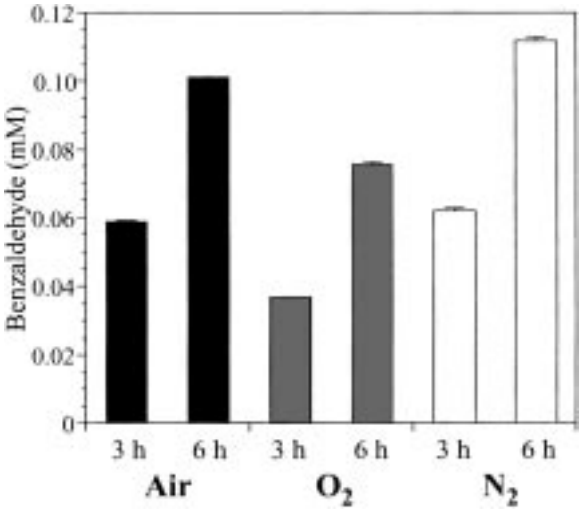

FIGURE 6 Effect of O_2 on the oxidative deamination by H_2O_2/Cu^{2+} system. Benzylamine (5.0 mM) was incubated with 0.25 mM $CuSO_4$ and 0.25 mM H_2O_2 in 50 mM sodium phosphate buffer, pH 7.4, at 37°C for 3 or 6 h under air, O_2, or N_2. After the reaction was terminated, the production of benzaldehyde was measured by HPLC.

generation of reactive oxygen species in biological systems.[43] The oxidation of ASA, catalyzed by Fe^{3+} or Cu^{2+}, yields the reduced metal ion, followed by one-electron transfer to O_2 to form the superoxide radical anion, which yields H_2O_2 via dismutation. Thus, the resulting H_2O_2 should react via the Fenton reaction generating the hydroxyl radical. Then the oxidized metal ion can be recycled by ASA. We examined the ability of several oxidation systems to convert benzylamine to benzaldehyde. Reaction mixtures containing 5.0 mM benzylamine and 50 mM sodium phosphate buffer, pH 7.4, were incubated with 0.25 mM H_2O_2, $CuSO_4$, and/or ASA at 37°C for 1 h. As shown in Fig. 8, incubation of benzylamine with ASA in the presence of Cu^{2+} generated benzaldehyde in amounts greater than that by

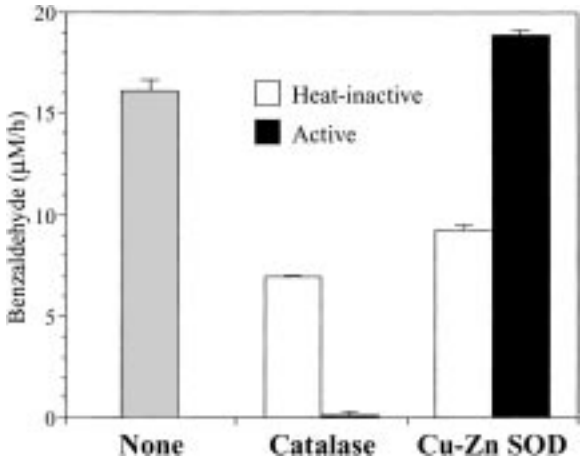

FIGURE 7 Effects of catalase and Cu–Zn SOD on oxidative deamination of benzylamine by H_2O_2/Cu^{2+} system. Benzylamine (5.0 mM) was incubated with 0.25 mM $CuSO_4$, 0.25 mM H_2O_2 in 50 mM sodium phosphate buffer (pH 7.4) in the presence or absence of 150 U/ml catalase and 150 U/ml Cu–Zn SOD at 37°C for 1 h. After the reaction was terminated, benzaldehyde was measured by HPLC. Heat-inactive catalase and Cu–Zn SOD were prepared by heating at 100°C for 5 and 30 min, respectively.

H_2O_2. However, the addition of catalase completely inhibited the formation of aldehyde, suggesting the participation of H_2O_2 in the oxidation (data are not shown). Moreover, incubation of benzylamine with ASA in the absence of Cu^{2+} also generated benzaldehyde to a small extent, probably due to trace amounts of metal ions in buffer. In the presence of H_2O_2 and Cu^{2+}, ASA effectively enhanced the generation of benzaldehyde. Interestingly, bovine hemoglobin (Hb)/H_2O_2 system also produced a significant amount of benzaldehyde.

Biological Significance and Mechanism of the Oxidative Deamination by H_2O_2/Cu^{2+} System

A proposed mechanism for the oxidative deamination by H_2O_2/Cu^{2+} system is illustrated in Fig. 9. It is consistent with the previous mechanism proposed by Stadtman *et al.*[11,14–16] Reaction of H_2O_2 with Cu^{+}, the so-called Fenton reaction, leads to the generation of a hydroxyl radical. Then the hydroxyl radical would abstract a hydrogen atom from the carbon adjacent to the amino groups to form a carbon-centered radical. Immediately Cu^{2+} would accept the lone electron of the carbon radical to generate Cu^{+} followed by the formation of the imine. Finally, spontaneous hydrolysis of the imino group can lead to the release of NH_3 and the formation of aldehyde. In this mechanism, it is assumed that the amino group serves as one of several ligands to which the metal ion is bound to form a coordination complex.

Today, many normal physiological processes, such as phagocytosis and mitochondrial metabolism, are known to involve the formation of

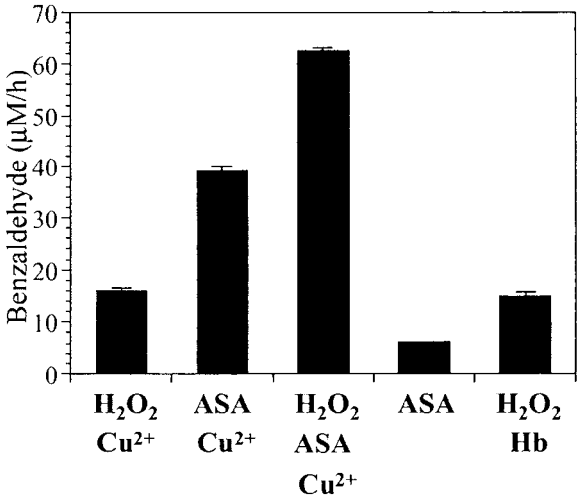

FIGURE 8 Effect of various oxidation systems on oxidative deamination of benzylamine. Benzylamine (5.0 mM) was incubated with 50 mM sodium phosphate buffer, pH 7.4. H_2O_2, with 0.25 mM H_2O_2; Cu^{2+}, with 0.25 mM $CuSO_4$; ASA, with 0.25 mM ascorbic acid; Hb, with 10 mg / ml Hb; Plasma, in human plasma. After 1 h of incubation at 37°C, the reaction was terminated by addition of acetic acid, and the production of benzaldehyde was measured.

hydroxyl radicals.[44] In addition, solar ultraviolet light and many toxic agents seem to generate free radicals, and their toxicity is apparently due to the subsequent reactions of the hydroxyl radical.[18,45] From these facts, various amines may be attacked by hydroxyl radicals and undergo oxidative deamination *in vivo*. Actually, allysine residue has been identified in serum albumin.[29] The conversion of lysine residues to aldehydes might reflect changes in protein conformation, and then the protein will be inactivated. Furthermore, the lysine residue has been suggested to participate in cross-linking reactions induced by some oxidation systems.[17] On the other hand, lysyl oxidase (EC 1.4.3.13), one kind of copper-containing amine oxidases, catalyzes the oxidative deamination of certain lysine residues in elastin and collagen, which are connective tissue proteins, to form allysine. Thus allysine is well known to form various inter- and intramolecular cross-links via aldol condensation or Schiff's base formation spontaneously.[46–48] Therefore, allysine residues are likely candidates of a precursor for the formation of cross-links in oxidized protein. These processes might be responsible for aging and many diseases.

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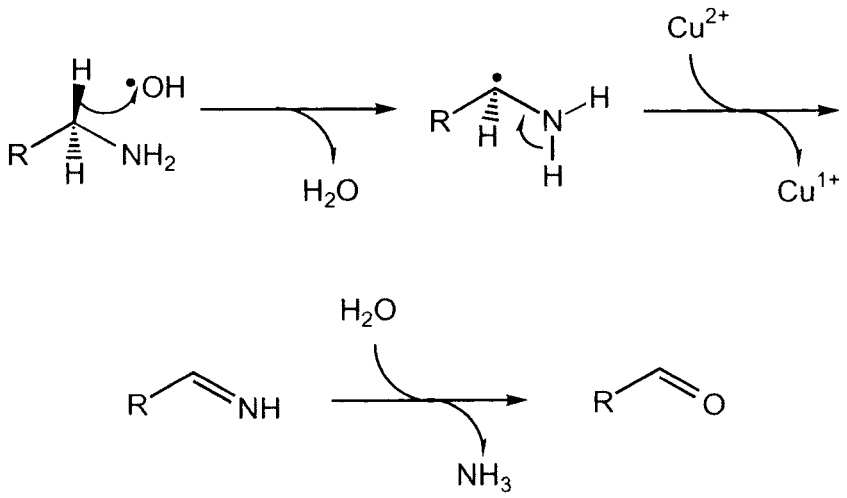

FIGURE 9 Proposed mechanism for the oxidative deamination induced by the H_2O_2 / Cu^{2+} system.

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