Reaction of a Histidyl Residue Analogue with Hydrogen Peroxide in the Presence of Copper(II) Ion

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Site-specific damage to a specific amino acid residue of a protein by Cu^{2+}/H_2O_2 was investigated. Observation of specific damage to the histidine residue of the protein was fully supported by a model experiment using *N*-benzoylhistidine. The reaction of H_2O_2 toward the substrate was characteristic of a Cu^{2+}-catalyzed system and was significantly influenced by pH. Other peroxides and peracids were slightly reactive to the substrate even in the presence of Cu^{2+}. Under simulated physiological conditions (pH 7.2, room temperature), incubation with Cu^{2+}/H_2O_2 (0.5 mM $CuSO_4$, 50 mM H_2O_2) resulted in complete destruction of the substrate within 1 h. Four products including *N*-benzoyl derivatives of asparagine, aspartic acid, aspartylurea, and formylasparagine were detected as the ring-ruptured products, and three products including 5-(2-benzamidovinyl)imidazole, benzamide, and benzoate were detected as the oxidation products at the α- and β-positions of the substrate. Plausible mechanisms for oxidation of histidine are proposed.

Partially reduced oxygen species have been implicated by many authors to be important causative agents of oxygen toxicity in cancer, aging, and other human diseases. Consecutive univalent reduction of molecular oxygen to water produces three active intermediates, superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$). These intermediates may be responsible for the action of various biological and synthetic materials as enzymes, antibiotics, carcinogen, and reducing materials under physiological conditions (Halliwell and Gutteridge, 1986).

The hydroxyl radical, a highly reactive oxidant, has been implicated in peroxide-mediated oxidation of a variety of substrates (Walling, 1975). The univalent reduction of H_2O_2 has been postulated to explain the metal (M^+) dependent decomposition of H_2O_2 (eq 1).

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

Much of the attention has been given to the iron-dependent decomposition of H_2O_2; however, it has been suggested that the rate constant for the reaction of Cu^+ with H_2O_2 is several orders of magnitude greater than that for Fe^{2+} (Halliwell and Gutteridge, 1985). Simpson et al. have detected $^{\bullet}OH$, generated by a Cu^{2+}/H_2O_2 system, by lucigenin-amplified chemiluminescence, deoxyribose degradation, and benzoate hydroxylation (Simpson et al., 1988). We have previously shown that Cu^{2+}/H_2O_2 exhibited marked reactivity toward various saccharides and the oxidation mechanism mediated by this reagent was similar to the other $^{\bullet}OH$-generating system (Uchida and Kawakishi, 1987, 1988a). The reaction of H_2O_2 with Cu^{2+} is represented as follows (Gutteridge and Wilkins, 1983):

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

$$HO_2^{\bullet} \rightarrow H^+ + O_2^- \quad (3)$$

$$Cu^{2+} + O_2^- \rightarrow Cu^+ + O_2 \quad (4)$$

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + {}^{\bullet}OH + OH^- \quad (5)$$

Although the reactivity of $^{\bullet}OH$ is rapid and nonspecific toward various compounds, a site-specific mechanism for $^{\bullet}OH$ as to protein damage has been suggested, in which metal ions are bound to proteins (Gutteridge and Wilkins, 1983; Marx and Chevion, 1985). The chemical nature of the oxidized protein is poorly understood; however, the specific loss of histidine residues, the most characteristic change in the primary structure of protein, has been demonstrated (Uchida and Kawakishi, 1988b, 1989a). As for the oxidation product of histidine, it has been characterized in detail by sensitized photooxidation (Tomita et al., 1969), UV irradiation (Johns and Jaskewycz, 1965), and ascorbate autoxidation (Uchida and Kawakishi, 1986, 1989b) studies, while the mechanism on the Fenton's reagent has not yet been established.

Hence, in order to establish the oxidaion mechanism of histidine with the Cu^{2+}/H_2O_2 system, we have undertaken a model study using *N*-benzoylhistidine, an analogue of histidine residue in protein, as the substrate. In this report, we discuss some factors affecting the oxidation of histidine and also propose the mechanism for destruction of histidine mediated by a copper-dependent Fenton's reaction.

MATERIALS AND METHODS

Materials. Bovine serum albumin was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo). Hydrogen peroxide (31%, w/v) was obtained from Mitsubishi Gas Co. (Tokyo), and *tert*-butyl hydroperoxide (70%, w/v) and cumene hydroperoxide (80% w/v) were from Aldrich. Catalase (from bovine liver), superoxide dismutase (from bovine erythrocytes), and *N*-benzoylhistidine were obtained from Sigma Chemical Co. *N*-Benzoylaspartic acid and *N*-benzoylasparagine were synthesized by the authentic method (Fischer, 1899). Benzoate was prepared from the acid hydrolysis of benzoyl chloride. Trifluoroacetic acid (>99%) was purchased from Tokyo Kasei Kogyo Co., Ltd. All other reagents were of the highest grade commercially available.

Reaction of Bovine Serum Albumin with Cu^{2+}/H_2O_2. The reactions were carried out at room temperature. The solutions (25 mL) contained 0.04% (w/v) bovine serum albumin, 5

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mM H_2O_2, and 50 μM Cu^{2+} in phosphate buffer (pH 7.2). The reactions were stopped by the addition of EDTA (1 mM) and catalase (10 μM) solutions.

Amino Acid Analysis. Amino acid analysis was performed with a Jeol JLC-6AH amino acid analyzer, for which the samples were prepared as follows: The reaction mixtures contained 0.04% bovine serum albumin, 5 mM H_2O_2, and 50 μM Cu^{2+} in phosphate buffer (25 mL, pH 7.2). After the mixtures were freeze-dried, they were desalted on Sephadex G-25 and then hydrolyzed with 6 N HCl at a concentration of 2 mg of protein/mL at 120 °C for 24 h. The hydrolysates were concentrated, dissolved in aqueous HCl (pH 2.2), and then submitted to the analysis.

Reaction of *N*-Benzoylhistidine with Cu^{2+}/H_2O_2. The usual reaction was carried out at room temperature. The solutions (25 mL) were composed of 1 mM *N*-benzoylhistidine, 50 mM H_2O_2, and 0.5 mM $CuSO_4$ in phosphate buffer (pH 7.2).

The quantitative determination of *N*-benzoylhistidine was undertaken by HPLC on a reversed-phase column. The chromatographic conditions were as follows: column, Develosil ODS-5 (4.6 × 250 mm); eluent, 0.1% trifluoroacetic acid–methanol (3:1); flow rate, 0.8 mL/min; detection, UV absorbance at 230 nm.

Areas of the chromatographic peaks of each materials were calculated by Shimadzu Chromatopac integrator C-R3A.

Isolation of the Products. The reaction was carried out in phosphate buffer (200 mL, pH 7.2) containing 1 mM *N*-benzoylhistidine, 50 mM H_2O_2, and 0.5 mM $CuSO_4$. The reaction mixture was kept at room temperature for 48 h. The residual substrate and oxidation products in the reaction mixture were determined by HPLC at various time intervals. After the reaction was terminated with EDTA (1 mM) and catalase (10 μM), the mixture was freeze-dried, extracted with methanol (3 × 100 mL) to remove a large quantity of the inorganic salts, and then evaporated in vacuo. The extract was dissolved in a small amount of dimethyl sulfoxide and then subjected to preparative HPLC on a Lop ODS column (24 × 300 mm) (Nomura Chemical Co., Ltd.). The products were eluted with a solution of 0.1% trifluoroacetic acid–methanol (3:2) and monitored by UV absorbance at 230 nm. The eluate was fractionated and further purified on a Develosil ODS-5 column.

Instrumental Analyses. Nuclear magnetic resonance (NMR) spectra were taken on a Jeol JNM-FX200 spectrometer in dimethyl-d_6 sulfoxide with tetramethylsilane as the internal standard. Fast atom bombardment mass spectrometry (FAB-MS) was performed with a Jeol JMS-DX303 mass spectrometer. For this, the samples were dissolved in dimethyl sulfoxide and added to a drop of glycerol on the target.

Spectral data of each products are as follows.

1: ^1H NMR δ 2.72 (2 H, d), 4.80 (1 H, m), 7.00 (2 H, s), 7.52 (3 H, m), 7.82 (2 H, m), 8.68 (1 H, d), 12.72 (1 H, b); ^{13}C NMR δ 36.0, 48.9, 127.7, 128.6, 131.5, 133.9, 166.3, 171.8, 173.4; FAB-MS *m/z* 237 (M + 1).

2: ^1H NMR δ 7.48 (3 H, m), 7.85 (2 H, m), 12.80 (1 H, b); ^{13}C NMR δ 127.1, 127.8, 130.8, 134.4, 168.0; FAB-MS *m/z* 122 (M + 1).

3: ^1H NMR δ 2.95 (2 H, dd), 4.82 (1 H, m), 7.12 (1 H, m), 7.51 (3 H, m), 7.60 (1 H, s), 7.84 (2 H, m), 8.75 (1 H, d), 10.24 (1 H, s), 12.68 (1 H, b); ^{13}C NMR δ 38.5, 48.6, 126.9, 127.9, 131.1, 133.4, 153.3, 165.6, 171.4, 172.1; FAB-MS *m/z* 280 (M + 1), 302 (M + Na).

4: ^1H NMR δ 2.83 (2 H, dd), 4.77 (1 H, m), 7.51 (3 H, m), 8.75 (1 H, d), 12.76–13.20 (2 H, b); ^{13}C NMR δ 35.5, 49.1, 126.9, 127.9, 131.0, 133.4, 165.6, 171.3, 172.0; FAB-MS *m/z* 238 (M + 1), 252 (M + Na).

5: ^1H NMR δ 3.00 (2 H, dd), 4.80 (1 H, m), 7.51 (3 H, m), 7.86 (2 H, m), 8.97 (1 H, d), 9.02 (1 H, d), 11.28 (1 H, d), 12.80 (1 H, b); ^{13}C NMR δ 37.1, 48.3, 126.9, 127.9, 131.1, 133.4, 162.6, 165.6, 171.3, 171.9; FAB-MS *m/z* 265 (M + 1), 287 (M + Na), 303 (M + K).

6: ^1H NMR δ 6.16 (1 H, d), 7.40 (3 H, m), 7.52 (1 H, m), 7.68 (1 H, dd), 7.80 (2 H, m), 8.88 (1 H, s), 10.64 (1 H, d), 14.24 (1 H, b); ^{13}C NMR δ 97.9, 114.2, 127.1, 127.3, 128.2, 130.2, 132.5, 131.8, 133.7, 163.9; FAB-MS *m/z* 214 (M + 1).

7: Product 7 was identical by HPLC analysis to benzoate. HPLC conditions: column, Develosil ODS-5 (4.6 × 250 mm); eluent, 0.1% trifluoroacetic acid–methanol (2:1); flow rate, 0.8 mL/min; detection, UV absorbance at 230 nm.

Table I. Changes in Amino Acid Compositions of Bovine Serum Albumin during Incubation with the Copper(II)/H_2O_2 System

amino acid	molar ratio,[a] %				
	0 h	2 h	4 h	8 h	24 h
Asp	10.5	10.6	10.6	10.9	10.6
Thr	5.8	6.5	6.0	6.1	5.8
Ser	4.8	4.4	5.0	4.5	5.1
Glu	14.5	15.2	14.6	14.8	14.4
Pro	4.8	4.4	5.0	4.5	5.1
Gly	3.2	3.4	3.6	3.9	3.5
Ala	7.8	8.3	7.9	8.4	8.1
Cys	2.9	2.3	2.4	2.5	2.4
Val	6.5	6.7	6.5	7.0	6.7
Met	0.4	0.4	0.4	0.4	0.4
Ile	2.4	2.6	2.5	2.5	2.6
Leu	10.9	10.5	11.1	10.4	11.1
Tyr	3.3	3.1	2.8	3.0	3.2
Phe	4.8	4.7	4.8	4.6	4.9
His	3.3	2.9	3.0	2.7	2.4
Lys	10.0	9.5	9.7	9.1	9.5
Arg	4.1	4.3	4.1	4.7	4.1

[a] Molar ratio was represented by the mole concentration of each amino acid per total amino acid.

RESULTS

Selective Damage to Histidine Residues of the Protein. It has been well established that exposure of protein to Cu^{2+}/hydroperoxide results in protein fragmentation (Wolff et al., 1986; Wolff and Dean, 1986; Hunt et al., 1988). We have also confirmed significant fragmentation of protein during incubation with Cu^{2+}/H_2O_2 by SDS–polyacrylamide gel electrophoresis (unpublished data). On the basis of these situations, we subsequently examined the site of damage in protein, since $^{\bullet}OH$ generation at a site-specific location on the protein molecule has been suggested in a copper-dependent Fenton system.

Changes in the primary structure of protein during incubation with Cu^{2+}/H_2O_2 were determined by amino acid analysis. As shown in Table I, loss of histidine residues was the most prominent. Losses of lysine and cysteine residues were also regarded as being significant. Of the aromatic amino acids, neither tyrosine nor phenylalanine residues were damaged. As for the tryptophan residue, a change in the tryptophan content of the protein was determined by the measurement of native fluorescence with excitation at 280 nm and emission at 340 nm, and a slight decrease of its content was observed (Uchida and Kawakishi, unpublished data).

Reaction of *N*-Benzoylhistidine with Cu^{2+}/H_2O_2. In order to establish the oxidation mechanism of histidine, we have undertaken a model reaction of histidine-containing materials with a Cu^{2+}/H_2O_2 system using *N*-benzoylhistidine as described below.

The system composed of 50 mM H_2O_2 and 0.5 mM Cu^{2+} exhibited marked reactivity toward *N*-benzoylhistidine (Figure 1). The substrate *N*-benzoylhistidine was completely diminished within 1 h of incubation and, instead, a number of newly formed products (1–6) were detected (Figure 2). By contrast, *N*-benzoylglycine was less reactive to Cu^{2+}/H_2O_2, suggesting the requirement of the imidazole group to react with the copper-dependent Fenton's reagent (Figure 1). In addition, reaction of Cu^{2+}/H_2O_2 with the substrate was significantly influenced by pH. As shown in Figure 3, loss of *N*-benzoylhistidine by Cu^{2+}/H_2O_2 increases with increasing pH. His-

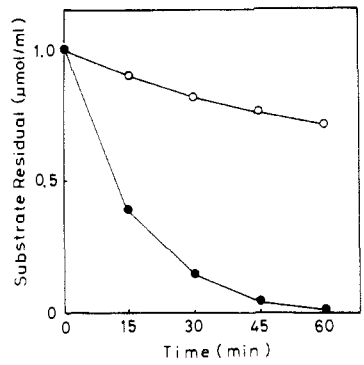

Figure 1. Time-dependent loss of *N*-benzoylhistidine and *N*-benzoylglycine during incubation with the Cu^{2+}/H_2O_2 system. The substrate (1 mM) was exposed to the Cu^{2+}/H_2O_2 system composed of 50 mM H_2O_2 and 0.5 mM Cu^{2+}. Symbols: ●, *N*-benzoylhistidine; ○, *N*-benzoylglycine.

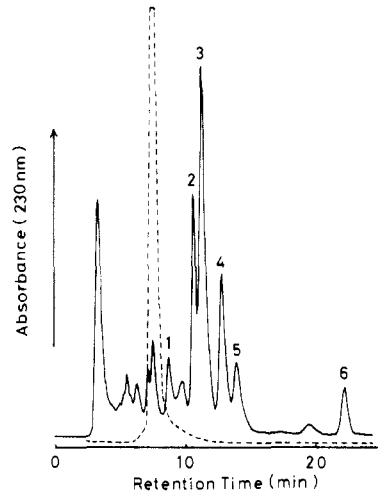

Figure 2. HPLC profile of the oxidation products of *N*-benzoylhistidine. The reaction mixture in phosphate buffer (25-mL total volume), pH 7.2, containing 1 mM *N*-benzoylhistidine, 50 mM H_2O_2, and 0.5 mM Cu^{2+} was incubated at room temperature for 60 min. The broken line represents *N*-benzoylhistidine (1 mM) without exposure to the Cu^{2+}/H_2O_2 system. Peak numbers (1–6) represent the products numbers in the remainder of the figures and in the text.

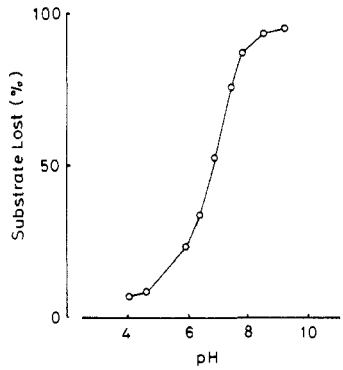

Figure 3. pH-dependent reaction efficiency of Cu^{2+}/H_2O_2 toward *N*-benzoylhistidine. The reaction mixtures in phosphate buffer (25-mL total volume) containing 1 mM *N*-benzoylhistidine, 50 mM H_2O_2, and 0.5 mM Cu^{2+} were incubated at room temperature for 60 min.

tidine is the only amino acid whose side chain can serve either as an acid or as a base in the physiological pH range, suggesting that, under neutral pH, the reactivity of histidine with Cu^{2+}/H_2O_2 depends upon the degree of dissociation of imidazole nitrogen.

Table II. Effect of Metal Ions on the Hydrogen Peroxide Induced Oxidation of *N*-Benzoylhistidine[a]

metal	% oxidn	metal	% oxidn
none	0	Co^{2+}	12
Cu^{2+}	100	Ti^{3+}	2
Fe^{2+}	35	Mn^{2+}	0
Fe^{3+}	22		

[a] The mixtures were kept for 60 min at room temperature. As for the iron-catalyzed systems, iron was solubilized to the distilled water with the same concentration of EDTA prior to addition into the reaction mixture.

Table III. Oxidation of *N*-Benzoylhistidine through Reaction with Peroxide or Peracid in the Presence and Absence of Copper(II) Ion[a]

system	% oxidn
H_2O_2	8
Cu^{2+}/H_2O_2	100
tert-butyl hydroperoxide	0
Cu^{2+}/*tert*-butyl hydroperoxide	0
cumene hydroperoxide	0
Cu^{2+}/cumene hydroperoxide	0
m-chloroperbenzoic acid	10
Cu^{2+}/*m*-chloroperbenzoic acid	20

[a] The mixtures were kept for 2 h at room temperature.

Then, we determined the effect of various metal ions on the reaction of H_2O_2 toward the substrate (Table II). Without the addition of metal ion, only a slight decrease of the substrate was observed. The iron-catalyzed systems stimulated the reaction less efficiently than the Cu^{2+}-catalyzed system. Other metal ions, Mn^{2+}, Co^{2+}, and Ti^{3+}, were all inactive. Thus, the reaction of H_2O_2 toward the histidine derivative was highly characteristic in the Cu^{2+}-catalyzed system.

On the other hand, it has been reported that exposure of protein to hydroperoxide, such as *tert*-butyl hydroperoxide (TBHP), cumene hydroperoxide (CHP), and peroxidized lipid, resulted in protein fragmentation (Wolff et al., 1986; Wolff and Dean, 1986; Hunt et al., 1988). Hence, we examined the activity of organic peroxide (TBHP and CHP) and peracid (*m*-chloroperbenzoic acid) on the destruction of *N*-benzoylhistidine in the presence and absence of Cu^{2+} (Table III). Whether Cu^{2+} was absent or present, all oxidants other than H_2O_2 could not give rise to substantial loss of the substrate. It was therefore proven that specificity to histidine was highly characteristic of the Cu^{2+}/H_2O_2 system.

Products 1–6 in Figure 2 were found to be identical to FAB-MS, ^1H NMR, and ^{13}C NMR spectrometry (see Materials and Methods) to *N*-benzoylasparagine (1), benzamide (2), *N*-benzoylaspartylurea (3), *N*-benzoylaspartic acid (4), *N*-benzoyl-*N'*-formylasparagine (5), and 5-(2-benzamidovinyl)imidazole (6) obtained from the reaction of *N*-benzoylhistidine with Cu^{2+}/ascorbate (Uchida and Kawakishi, 1986, 1989b). Other than products 1–6, benzoate (7) was also detected in HPLC chromatogram (see Materials and Methods). To our surprise, however, the imidazolone derivative (compound 8 in Figure 4), which has been isolated as the major product in the Cu^{2+}/ascorbate system, could not be detected in the Cu^{2+}/H_2O_2 system. This is a serious difference between the Cu^{2+}/H_2O_2 and the Cu^{2+}/ascorbate systems. The data led to the assumption that the mechanism of Cu^{2+}/H_2O_2, including active species, is basically distinct from that of Cu^{2+}/ascorbate; the details remain to be investigated further.

Mechanism. We propose a plausible mechanism for the ring-opening reaction of imidazole ring with Fenton's reagent (Figure 4). The oxidant (•OH) generated

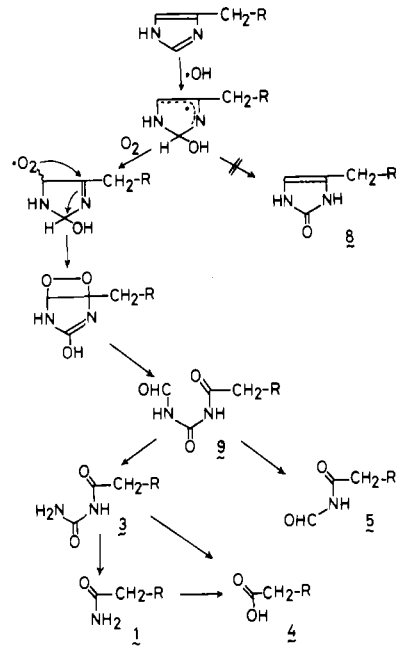

Figure 4. Proposed mechanism for oxidation of *N*-benzoylhistidine with Cu^{2+}/H_2O_2.

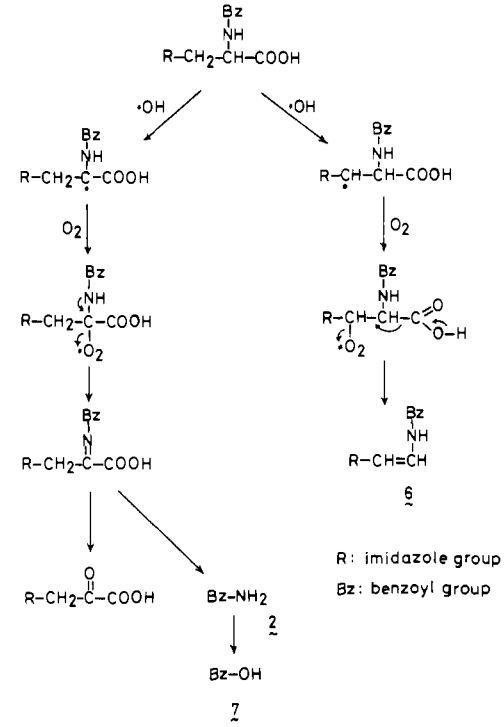

Figure 5. Proposed mechanism for oxidation of the α- or β-position of *N*-benzoylhistidine with Cu^{2+}/H_2O_2.

by the Fenton reaction attacks a histidyl imidazole (ImH) to generate an imidazole radical (Im$^{\bullet}$) (Hodgson and Fridovich, 1975a), followed by reaction with O_2 to give ring-ruptured product **9** (Figure 4). Product **9** seems to be a precursor for a subsequent oxidation product; however, an attempt to detect **9** has been unsuccessful probably because of its unstability. Decomposition of **3** is assumed to be followed by the formation of further oxidation products via a deformylation or deamidation reaction. When compound **3** was further exposed to Cu^{2+}/H_2O_2, approximately 55% of **3** was utilized to yield small amounts of **1**, **2**, and **4** (data not shown). In addition, it was also confirmed that the deamidation reaction of *N*-benzoylasparagine mediated by Cu^{2+}/H_2O_2 resulted in its loss (54%) to yield *N*-benzoylaspartic acid (11.5%). There is little doubt that oxidation products **1** and **3**–**5** are formed through the reaction onto the imidazole moiety, whereas formation of the other products (**2**, **6**, and **7**) might be induced by the oxidation at the α- or β-position of *N*-benzoylhistidine (Figure 5).

DISCUSSION

The results obtained in the present study suggest the toxic action of Cu^{2+}-dependent Fenton's reagent toward histidine-related compounds in vivo. However, we are aware of the limitation of our model system since the situation of a histidine derivative used in this study is quite distinct from that of protein. However, of particular importance is the fact that, in our model system, $^{\bullet}OH$ generated by Cu^{2+}/H_2O_2 is extremely reactive and specific to the low molecular weight histidine derivative, similar to the histidine residue in the protein.

As shown in Table I, copper-catalyzed Fenton's reagent gave rise to selective damage to the histidine residue in the protein. Although the mechanism underlying the oxidation has not been elucidated in detail yet, it has been presumed that the selective reaction is due to the ligand formation of histidine with Cu^{2+}. The imidazole ring of histidine plays a role in ligand formation with Cu^{2+}, which might induce the oxidative reaction due to oxygen radicals generated at a site-specific location on the protein molecule. Rowley and Halliwell (1983) have observed that free histidine, or serum albumin, lowered the bulk concentration of $^{\bullet}OH$ in a system in which the radical was generated by a Cu^{+}/Cu^{2+} couple. The Cu^{2+}–protein complexes might bring about site-specific damage to protein in the presence of H_2O_2, in spite of the tight binding of Cu^{2+} to the protein molecules. Hodgson and Fridovich (1975a,b) have previously reported that H_2O_2 caused a rapid and reversible reduction of the enzyme (superoxide dismutase) bound Cu^{2+}, which was associated with a bleaching of its absorption in the visible and near-UV regions. The data were interpreted in terms of reduction of the enzyme-bound Cu^{2+} to Cu^{+} by H_2O_2. This might be followed by a Fenton-type reaction of Cu^{+} with additional H_2O_2 to form Cu^{2+}–$^{\bullet}OH$ or Cu^{2+}–O^{-}, which could attack an adjacent histidine (Hodgson and Fridovich, 1975b). Recently, Borders and his co-workers reported that HO_2^{-}, rather than H_2O_2, is the reactive species in the inactivation of Cu,Zn-superoxide dismutase (Blech and Borders, 1983; Fuchs and Borders, 1983). This is based on a study of the effect of pH on the kinetic parameters of inactivation. They also suggest that one histidine residue in the subunit of the protein is lost with decreasing enzyme activity.

From our study, the occurrence of similar specific reactions is suggested in copper-containing fluids such as plasma and synovial fluid when the free-radical reactions are initiated by H_2O_2 at the metal-binding site of proteins. The formation of the free radicals in situ may give rise to the site-specific oxidation of histidine residues in protein. Such reactions may therefore be physiologically important in connection with oxygen toxicity or protein turnover on the mixed-function oxidation systems. Our results raise the possibility that serum albumins or histidine-containing materials play a role of antioxidant against the $^{\bullet}OH$-induced biological damage. The present study may also give some information about the inactivation reaction mechanism of metal-binding protein by H_2O_2. Studies concerning the more detailed reaction mechanism of Fenton's reagent toward histidine and other materials will be extended.

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