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Ascorbate-Mediated Specific Oxidation of the Imidazole Ring in a Histidine Derivative

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In the presence of a catalytic amount of metal ion, ascorbate gave rise to site-specific damage to the protein and peptides accompanied with the selective loss of histidine residues. Hence, we undertook a model reaction of *N*-benzoylhistidine with the metal/ascorbate system to chemically characterize the site-specific mechanism. Under the physiological conditions, the substrate (*N*-benzoylhistidine) was almost completely degraded in this system (5 mM ascorbate and 0.05 mM Cu^{2+}) within 24 h of incubation. The reaction of Cu^{2+}/ascorbate toward the substrate was considerably O_2-dependent. We have confirmed eight products **(II–IX)** including a 2-oxo compound, *N*-benzoyl-β-(2-oxo-imidazolonyl)alanine **(III),** as the main product and various ring-ruptured products such as *N*-benzoylasparagine **(II),** *N*-benzoylaspartate **(VI),** *N*-benzoylaspartylurea **(V),** and *N*-benzoyl-*N'*-formylasparagine **(VII).** Oxidation of the imidazole group was assumed to be initiated at the C-2 position of the imidazole ring to yield **III,** which was then followed by the formation of the ring-ruptured products. In addition, other products, 5-(2-benzamidovinyl)imidazole **(VIII),** benzamide **(IV),** and benzoate **(IX),** were also detected, which were tentatively formed by the hydrogen abstraction of the α- or β-position of the substrate.

INTRODUCTION

Metal ions in biological systems may be concentrated at specific metal binding sites, such as those which often occur in proteins. The reducing agent, such as ascorbate *in situ*, can trigger a chain reaction and generate highly reactive oxygen radicals (*1–3*). The function of ascorbate is to reduce the metal ion [M(*n* + 1)] (Eq. [1]) and to serve as a source for superoxide (O_2^-) (Eq. [2]) and H_2O_2 (Eq. [3]). The reduced metal ion [M(*n*)] is conducted via a Fenton's reaction to generate the most potent oxidant, the hydroxyl radical (·OH) (Eq. [4]).

$$\text{M}(n+1)\text{-protein} \xrightarrow{\text{ascorbate}} \text{M}(n)\text{-protein} \quad [1]$$

$$\text{M}(n)\text{-protein} + O_2 \rightarrow \text{M}(n+1)\text{-protein} + O_2^- \quad [2]$$

$$O_2^- + 2H^+ \rightarrow H_2O_2 \quad [3]$$

$$\text{M}(n)\text{-protein} + H_2O_2 \rightarrow \text{M}(n+1)\text{-protein} + \cdot OH + OH^- \quad [4]$$

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It has been suggested that these radicals tend to react with the first available target in the ligand (protein) resulting in specific damage rather than random injury to the protein (*4–13*). This mechanism has been recently proposed for the nonenzymatic free radical (*4–12*) or mixed-function oxidation (*9, 10, 12, 13*) systems as "the site-specific mechanism" for protein damage (*4–13*). Examples of the lowering of the function of proteins through the incubation with the metal-mediated oxidation system are albumins (*5, 6, 11*), glutamine-dependent carbamyl phosphate synthetase (*14*), superoxide dismutase (*15*), penicillinase (*16*), glutamine synthetase (*8–10*), ribonucleotide reductase (*17*), acetylcholinesterase (*4*), phosphoglucomutase (*18*), etc.

The chemical nature of the oxidized protein is poorly understood; however, specific loss of histidine residues, the most characteristic change in the primary structure of protein, has been demonstrated (*8–12*). In connection with the specific damage to the histidine residue in protein, Levine *et al.* have demonstrated that glutamine synthetase underwent a modification mediated by a mixed-function oxidation as well as the ascorbate model systems (*8–10, 12, 19–22*).

As for the oxidation product of histidine, it has been characterized in detail by sensitized photooxidation (*23*) and uv[2] irradiation (*24*) studies, while the mechanism of the ascorbate or other free radical oxidation systems has not yet been established. Hence, in order to characterize the oxidation product of histidine and to establish the oxidation mechanism, we have undertaken a model study using *N*-benzoylhistidine, an analog of histidine residues in protein, as the substrate. And, in the course of this study, we have detected a monooxygenated form of the substrate as the major product (*25*).

In this report, we discuss some factors affecting the oxidation of the histidine derivative and describe the identification and characterization of the products resulting from both oxidative cleavage of the imidazole ring and hydrogen abstraction of the α- or β-position of the histidine derivative. We also show some oxidation mechanisms of *N*-benzoylhistidine through a series of free radical reactions.

MATERIALS AND METHODS

Chemicals

L-Ascorbic acid and L-histidine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka), and bovine serum albumin was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo). Catalase (from bovine liver), superoxide dismutase (from bovine erythrocytes), *N*-benzoylhistidine, glycylglycyl-L-histidine, L-histidyl-L-phenylalanine, L-histidyl-L-tyrosine, and poly-L-histidine (m_r 1.1×10^4) were obtained from the Sigma Chemical Co. *N*-Benzoylaspartic acid and *N*-benzoylasparagine were synthesized by the authentic method (*26*). Benzoate was prepared from the acid hydrolysis of benzoylchloride. Trifluoroacetic acid (>99%)

[2] Abbreviations used: uv, ultraviolet; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; FAB–MS, fast atom bombardment–mass spectrometry; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetate.

was purchased from Tokyo Kasei Kogyo Co., Ltd. All other reagents were of the highest grade commercially available.

Reaction of the Histidine-Containing Material with Ascorbate in the Presence of Copper Ion

The reaction was carried out at room temperature. The solutions (25 ml) contained the histidine-containing material (0.04% bovine serum albumin, 0.08% poly-histidine, 1 mM L-histidine, or 1 mM histidine-containing peptide), 5 mM ascorbate, and 0.05 mM $CuSO_4$ in phosphate buffer (pH 7.2).

Loss of bovine serum albumin was chromatographically determined by gel filtration on a TSK-GEL G3000 SW column (7.5 × 600 mm). Samples were eluted at a rate of 1.0 ml/min with 0.1 M phosphate buffer (pH 7.0) containing 0.1 M NaCl, the elution being monitored at 210 nm.

Chromatographic determination of poly-histidine was performed by gel filtration on a TSK-GEL G3000 PW column (7.5 × 600 mm). Since the reaction mixture was emulsified under the conditions (pH 7.2, room temperature), 0.5 ml of HCl (0.2 N) was added to a portion of the mixture (1.0 ml) each time to dissolve poly-histidine and then subjected to HPLC analysis. Samples were eluted at a rate of 1.0 ml/min with 0.1 M phosphate buffer (pH 4.4) containing 0.1 M KCl, the elution being monitored at 210 nm.

Chromatographic determination of other smaller peptides, glycylglycyl-histidine, histidyl-phenylalanine, and histidyl-tyrosine, also was performed by HPLC on a Develosil ODS-5 column (4.6 × 250 mm). Chromatographic conditions were as follows: samples were eluted at a rate of 0.8 ml/min with a H_2O containing 0.1% trifluoroacetic acid (TFA) for glycylglycyl-histidine, H_2O (0.1% TFA)–methanol (3 : 1) for histidyl-phenylalanine, and H_2O (0.1% TFA)–methanol (5 : 1) for histidyl-tyrosine, the elutions being monitored at 210 nm.

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

Loss of histidine residue in each material was determined by amino acid analysis on a JEOL JLC-6AH amino acid analyzer, for which the samples were prepared as follows: after the mixtures were freeze-dried, they were hydrolyzed with 6 N HCl at 120°C for 24 h. The hydrolyzates were then concentrated, dissolved in aqueous HCl (pH 2.2), and submitted for analysis.

Reaction of N-Benzoylhistidine with Ascorbate in the Presence of Copper Ion

The usual reaction was carried out at room temperature. The solutions (25 ml) were composed of 1 mM *N*-benzoylhistidine, 5 mM ascorbate, and 0.05 mM $CuSO_4$ in phosphate buffer (pH 7.2).

Changes in the visible absorption of the mixture composed of 10 mM *N*-benzoylhistidine, 50 mM ascorbate, and 0.5 mM $CuSO_4$ in phosphate buffer (pH 7.2) were measured with a Hitachi Model 200-10 spectrophotometer.

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); eluant, TFA (0.1%)–methanol (3 : 1); flow rate, 0.8 ml/min; detection, uv absorbance at 230 nm.

Isolation of the Oxidation Products of N-Benzoylhistidine

The reaction was carried out in phosphate buffer (200 ml, pH 7.2) containing 1 mM *N*-benzoylhistidine, 50 mM ascorbate, and 0.5 mM $CuSO_4$. The reaction mixture was kept for 48 h at room temperature. 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), 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 TFA (0.1%)–methanol (3 : 2) being monitored by uv absorbance at 230 nm. The eluate was fractionated and further purified using a Develosil ODS-5 column.

Instrumental Analyses

Nuclear magnetic resonance (NMR) spectrum on a JEOL JNM-FX200 (50.2 Hz) spectrometer was taken in dimethyl sulfoxide-d_6 with tetramethylsilane as the internal standard.

Fast atom bombardment–mass spectrometry (FAB–MS) was performed with a JEOL JMS-DX303 mass spectrometer, for which the samples were dissolved in dimethyl sulfoxide and added to a drop of glycerol on the target.

RESULTS AND DISCUSSION

Selective Damage to Histidine Residue in Protein and Peptides

Reactivity of the histidine-containing peptides and proteins with the Cu^{2+}/ascorbate system was summarized in Table 1. The result demonstrates the clear relationship between the substrate consumption and the loss of the histidine residues. We have previously shown that other residues remained almost intact except for the tryptophan residue in bovine serum albumin (*11*). Therefore, there seems little doubt that the decrease of each substrate was predominantly due to the loss of the histidine residues.

Hence, in order to characterize the oxidation product of histidine, we have undertaken a model reaction of histidine-containing materials with a Cu^{2+}/ascorbate system using *N*-benzoylhistidine as described below.

Reaction of N-Benzoylhistidine with the Cu^{2+}/Ascorbate System

Similarly to the results in Table 1, the Cu^{2+}/ascorbate system exhibited a marked reactivity with *N*-benzoylhistidine. Approximately 90% of the substrate was lossed after 24 h and, instead, a number of newly formed products were

TABLE 1

Reaction of the Histidine-Containing Materials with the Copper/Ascorbate System

Substrate	Loss of substrate[a] (%)	Loss of histidine[b] residue (%)
L-Histidine	82.9	82.9
Glycylglycyl-L-histidine	72.5	73.5
Histidyl-L-phenylalanine	77.0	77.5
Histidyl-L-tyrosine	75.6	75.4
Poly-L-histidine[c]	84.7[d]	23.1
Bovine serum albumin	84.6[d]	54.2

[a] Loss of each substrates other than L-histidine was determined by HPLC. The reaction was carried out at room temperature for 24 h. The solutions were composed of the substrate (1 mM or 0.04%), 5 mM ascorbate, and 0.05 mM $CuSO_4$ in phosphate buffer (pH 7.2). Chromatographic conditions of each substrates are noted under Materials and Methods.

[b] Loss of histidine residues in each materials was determined by amino acid analysis.

[c] The reaction was performed in the suspension of polyhistidine.

[d] Expressed as the percentage of decrease of the peak height in the chromatogram.

detected (Fig. 1). Among them, **III** was a main product and its yield, based on the substrate, was approximately 33% after 24 h, while other products were obtained in yields of less than 5%.

Then, we determined the effect of various metal ions on the reaction of ascorbate toward the substrate (Table 2). Without an 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 ascorbate toward the histidine derivative was highly characteristic in the Cu^{2+}-catalyzed system.

On the other hand, loss of *N*-benzoylhistidine in this system was greatly accelerated in a O_2 atmosphere while entirely retarded in a N_2 atmosphere (Fig. 2). The reaction was therefore confirmed to be O_2-dependent, which strongly suggested the participation of an oxygen-derived free radical reaction (Eqs. [1]–[4]).

Isolation and Chemical Characterization of the Products

In order to characterize the chemical structure of the products, **II–IX,** isolation by preparative HPLC on the reversed-phase columns was undertaken. Finally, the products were crystallized from cold methanol and their structural elucidation was mainly performed by FAB–MS, ^1H NMR (Table 3), and ^{13}C NMR (Table 4) spectrometry.

TABLE 2

Effects of Various Metal Cations on the Oxidation of *N*-Benzoylhistidine[a]

Metal	% oxidation[b]
None	16
Cu^{2+}	100
Fe^{2+}	60
Fe^{2+}	63
Co^{2+}	36
Ti^{2+}	29
Mn^{2+}	21

[a] The reactions were carried out in 25 ml of phosphate buffer containing 1 mM *N*-benzoylhistidine, 5 mM ascorbic acid, and 0.05 mM metal ions. The mixtures were kept for 24 h at room temperature. As for the iron-catalyzed systems, iron was solubilized to the distilled water with the same concentration of EDTA prior to the addition into the reaction mixture.

[b] Expressed as a percentage of the substrate (*N*-benzoylhistidine) lost in the Cu^{2+}-catalyzed system.

As described in a previous report, the main product **III** is *N*-benzoyl-β-(2-oxoimidazolonyl)alanine (*25*).

By comparison of the spectral data with those of the authentic samples, **II** and **VI** were confirmed to be *N*-benzoylasparagine and *N*-benzoylaspartic acid, respectively.

The product, **IV**, provided the quasimolecular ion, *m/z* 121 (M + 1), on the FAB–MS spectrum, and together with the data from the ^{13}C and ^{1}H NMR, this product was identified as benzamide.

The ^{13}C NMR spectrum of **V** revealed that the signals of the imidazole carbons disappeared entirely and, instead, the newly formed carbonyl carbons at 171.4 and 153.3 ppm appeared in the spectrum. On the other hand, the ^{1}H NMR spectrum suggested the presence of a NH_2 group which revealed remote signals at 7.1 and 7.6 ppm. On the basis of these data, the structure of *N*-benzoylaspartylurea was proposed for **V**. The FAB–MS spectrum of **V** gave the peak of a quasimolecular ion, *m/z* 280 (M + 1), which coincided with the predicted structure.

In a similar manner to **V**, the ^{13}C NMR spectrum of **VII** revealed the disappear-

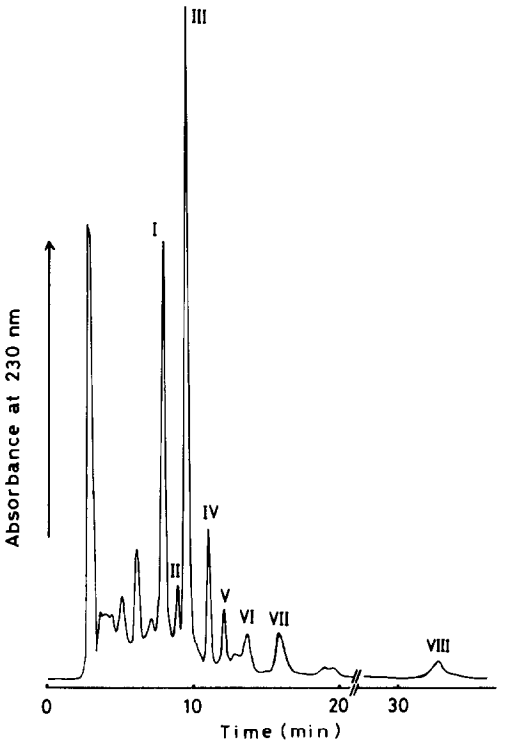

FIG. 1. HPLC profile of the oxidation products of *N*-benzoylhistidine. The reaction mixture composed of 1 mM *N*-benzoylhistidine, 5 mM ascorbate, and 0.05 mM Cu^{2+} in phosphate buffer (pH 7.2) was incubated at room temperature for 24 h. The peak **I** represents *N*-benzoylhistidine. Chromatographic conditions were as follows: column, Develosil ODS-5 (4.6 × 250 mm); eluate, H_2O (0.1% TFA)–methanol (3 : 1); flow rate, 0.8 ml/min; detection, absorbance at 230 nm.

TABLE 3

Chemical Shifts[a] of the Products **I–VIII** in ^1H NMR

I	II	III	IV	V	VI	VII	VIII
3.10 (2H, *d*) (CH_2)	2.72 (2H, *d*) (CH_2)	2.79 (2H, *d*) (CH_2)	7.48 (3H, *m*) (PhCO)	2.95 (2H, *dd*) (CH_2)	2.83 (2H, *dd*) (CH_2)	3.00 (2H, *dd*) (CH_2)	6.16 (1H, *d*) (Im—CH=)
4.60 (1H, *m*) (CH<)	4.80 (1H, *m*) (CH<)	4.58 (1H, *m*) (CH<)	7.85 (2H, *m*) (PhCO)	4.82 (1H, *m*) (CH)	4.77 (1H, *m*) (CH)	4.80 (1H, *m*) (CH)	7.40 (3H, *m*) (PhCO)
6.88 (1H, *s*) (Im-4)	7.00 (2H, *s*) (NH_2)	5.98 (1H, *s*) (CH=C<)		7.12 (1H, *s*) ($CONH_2$)	7.51 (3H, *m*) (PhCO)	7.51 (3H, *m*) (PhCO)	7.52 (1H, *m*) (Im-4)
7.04 (1H, *b*) (Im NH)	7.52 (3H, *m*) (PhCO)	7.49 (3H, *m*) (PhCO)		7.51 (3H, *m*) (PhCO)	7.84 (2H, *m*) (PhCO)	7.86 (2H, *m*) (PhCO)	7.68 (1H, *dd*) (=CH—N)
7.48 (3H, *m*) (PhCO)	7.82 (2H, *m*) (PhCO)	7.81 (2H, *m*) (PhCO)		7.60 (1H, *s*) ($CONH_2$)	8.75 (1H, *d*) (CONH)	8.97 (1H, *d*) (CONH)	7.80 (2H, *m*) (PhCO)
7.64 (1H, *s*) (Im-2)	8.68 (1H, *d*) (CONH)	8.54 (1H, *d*) (CONH)		7.84 (2H, *m*) (PhCO)	12.76–13.20 (2H, *b*) (COOH)	9.02 (1H, *d*) (CONHCO)	8.88 (1H, *s*) (Im-2)
7.84 (2H, *m*) (PhCO)	12.72 (1H, *b*) (COOH)	9.40 (1H, *s*) 9.72 (1H, *s*) (NHCONH)		8.75 (1H, *d*) (CONH)		11.28 (1H, *d*) (CHO)	10.64 (1H, *d*) (CONH)
8.72 (1H, *d*) (CONH)		12.72 (1H, *b*) (COOH)		10.24 (1H, *s*) (CONHCO)		12.80 (1H, *b*) (COOH)	14.24 (1H, *b*) (COOH)
12.72 (1H, *b*) (COOH)				12.68 (1H, *b*)			

[a] Expressed as *ppm* from tetramethylsilane. Signal patterns and results of signal integrations; *s*, *d*, *dd*, *m*, or *b* represents singlet, doublet, doublet–doublet, multiplet, or broad, respectively. **I** represents *N*-benzoylhistidine.

TABLE 4

Chemical Shifts[a] of the Products **I–VIII** in ^{13}C NMR

I	II	III	IV	V	VI	VII	VIII
28.3 (CH_2)	36.0 (CH_2)	26.9 (CH_2)	127.1 127.8	38.5 (CH_2)	35.5 (CH_2)	37.1 (CH_2)	97.9 (Im-CH)
52.8 (CH)	48.9 (CH)	51.4 (CH)	130.8 (Ph) 134.4	48.6 (CH)	49.1 (CH)	48.3 (CH)	114.2 (=CHNH)
116.2 (Im-4)	127.7 128.6	105.2 (NHCH=)	168.0 ($CONH_2$)	126.9 127.9	126.9 127.9	126.9 127.9	127.1 (Im-4)
126.9	131.5 (Ph)	117.7 (=C<)		131.1 (Ph)	131.0 (Ph)	131.1 (Ph)	127.3
128.0	133.9			133.4	133.4	133.4	128.2
131.0 (Ph)		127.0					130.2 (Ph)
133.7	166.3 (CONH)	127.9 131.0 (Ph)		153.3 (NH_2CONH)	165.6 (CONH)	162.6 (CHO)	132.5
133.5 (Im-5)	171.8 (COOH)	133.6		165.6 (CONH)	171.3 (CH_2COOH)	165.6 (CONH)	131.8 (Im-5)
134.4 (Im-2)	173.4 ($CONH_2$)	154.2 (NHCONH)		171.4 ($NHCOCH_3$)	172.0 (COOH)	171.3 ($NHCOCH_3$)	133.7 (Im-2)
165.6 (CONH)		165.9 (CONH)		172.1 (COOH)		171.9 (COOH)	163.9 (COOH)
172.6 (COOH)		172.4 (COOH)					

[a] Expressed as *ppm* from tetramethysilane. **I** represents *N*-benzoylhistidine.

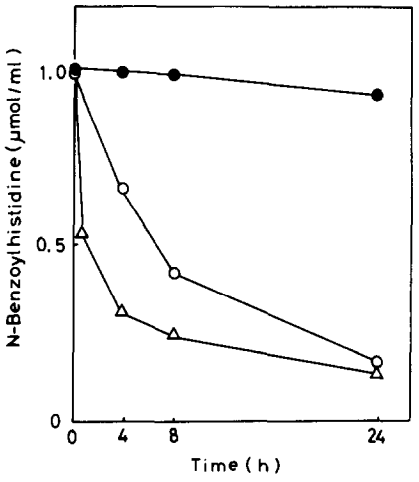

FIG. 2. Reaction of *N*-benzoylhistidine with the Cu^{2+}/ascorbate system under air, oxygen, and nitrogen atmospheres. *N*-Benzoylhistidine (1 mM) was exposed to the Cu^{2+}/ascorbate system composed of 5 mM ascorbate and 0.05 mM Cu^{2+} under air (○), O_2 (△), and N_2 (●) atmospheres. Residual *N*-benzoylhistidine at each times was determined by the reversed-phase HPLC.

ance of the imidazole carbons and, instead, the formation of new carbonyl carbons was observed at 171.3 and 162.6 ppm. The ^1H NMR spectrum suggested the presence of a formyl group (11.3 ppm). In addition, the FAB–MS spectrum gave a peak of quasimolecular ion, *m/z* 265 (M + 1). Consequently, it was confirmed that this compound had the structure of *N*-benzoyl-*N'*-formylasparagine.

As for the compound **VIII,** the NMR spectrum suggested the existence of an intact imidazole ring, while the disappearance of a carboxyl group and the presence of a new double bond were also revealed. Accompanied with the FAB–MS spectrum [*m/z* 214 (M + 1)], the structure of **VIII** was determined as 5-(2-benzamidovinyl)imidazole. It was proved that the vinyl protons of **VIII** were *trans* configured from their coupling constant (15 Hz).

Apart from the previously mentioned products, formation of a small amount of benzoate **(IX)** was confirmed by comparison with an authentic sample on the chromatogram. The product **IX** was eluted at 11 min in the retention time with 50% methanol in H_2O (0.1% TFA) as an eluant.

Ring Opening Reaction of III

The main product, **III,** seemed to be a precursor for other products **(II, V, VI,** and **VII).** Hence, the reaction of **III** (1 mM) with the Cu^{2+}/ascorbate system was undertaken.

As shown in Fig. 3, the HPLC profile of the reaction mixture after 24 h demonstrated the selective formation of **V** (yield 10.4%) accompanied with a significant loss of the substrate (approx 70%). It was therefore ascertained that **III** is an intermediate to yield **V** in the oxidation process of *N*-benzoylhistidine.

When compound **V** was further exposed to the Cu^{2+}/ascorbate system under

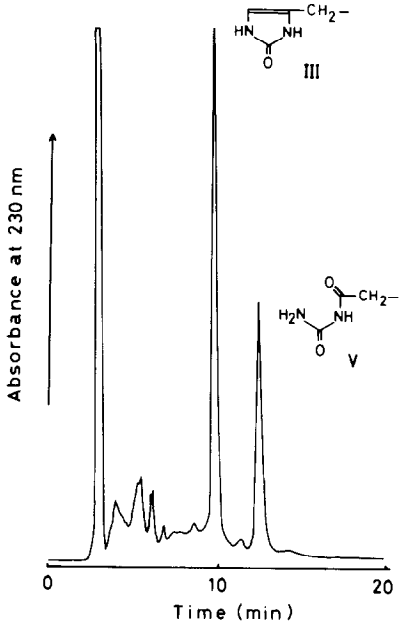

FIG. 3. HPLC profile of the oxidation products of **III.** The mixture composed of 1 mM **III,** 5 mM ascorbate, and 0.05 mM Cu^{2+} in phosphate buffer (pH 7.2) was kept at room temperature for 24 h. Chromatographic conditions were as follows: column, Develosil ODS-5 (5.0 × 250 mm); eluate, H_2O (0.1% TFA)–methanol (3 : 1); flow rate, 0.8 ml/min; detection, absorbance at 230 nm.

similar conditions, approximately 60% of **V** was utilized to yield small amounts of **II, IV,** and **VI** within 24 h of incubation (data not shown).

In addition, it was also confirmed that the deamidation reaction of *N*-benzoylasparagine **(II)** mediated by the Cu^{2+}/ascorbate system resulted in its loss (55%) to yield *N*-benzoylaspartic acid **(VI)** (7.9%) within 24 h of incubation.

Reaction Mechanism

The reaction of *N*-benzoylhistidine with Cu^{2+} produced a light blue complex which exhibited a broad absorption near 600–700 nm (Spectrum A in Fig. 4). This clearly indicated the characteristic interaction of *N*-benzoylhistidine with the copper ion. In this regard, it has been suggested that the reaction of histidine with Cu^{2+} is accompanied with a charge transfer from the histidine ring orbital to the Cu^{2+} (*27*), which might contribute to the occurrence of a chromophore. The characteristic absorption of this copper–substrate complex was completely diminished by the addition of ascorbate (Spectrum B in Fig. 4). This implies the likelihood of the one-electron reduction of the Cu^{2+} complex to the colorless Cu^{+} complex by ascorbate. The mixture assumed gradually a light yellow color, and the new absorption which might be due to the accumulation of the oxidized products of *N*-benzoylhistidine appeared near 400 nm (Spectra C and D in Fig. 4). The electron transfers from ascorbate to Cu^{2+} might be followed by a series of free

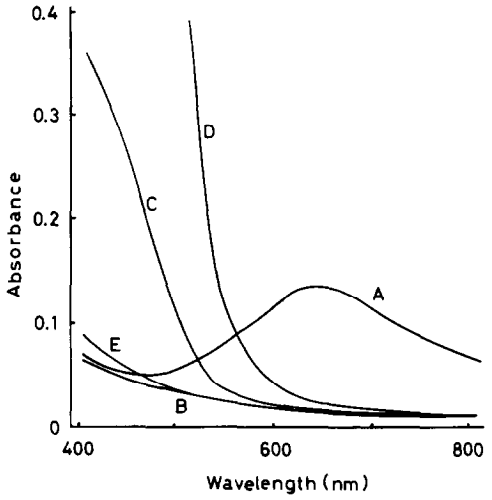

FIG. 4. Time-dependent changes in the visible absorption of the copper–*N*-benzoylhistidine complex by ascorbate. The reaction of *N*-benzoylhistidine (10 mM) with Cu^{2+} (0.5 mM) in the phosphate buffer (pH 7.2) was carried out at room temperature (Spectrum A). Ascorbate (final concentration was 50 mM) was then added into the mixture, and time-dependent changes in the visible absorption of the mixture were measured at 1 min (Spectrum B), 60 min (Spectrum C), and 180 min (Spectrum D). Spectrum E represents the mixture of ascorbate (50 mM) and Cu^{2+} (0.5 mM) incubated for 180 min. All spectrum were measured on the phosphate buffer (pH 7.2) as the reference.

radical reactions (Eqs. [1]–[4]) which result in the oxidative damage to the ligand (*N*-benzoylhistidine) itself.

A series of oxidation products of *N*-benzoylhistidine identified in this study can be arranged as shown in Figs. 5 and 6.

In Fig. 5, the reaction is initiated by the monooxygenation of the C-2 position of the imidazole group to give **III** which is further oxidized to **X.** The ring-ruptured product **X** seems to be a precursor for a subsequent oxidation product **(V);** however, an attempt to detect **X** has been unsuccessful. Degradation of **V** is assumed to be followed by the formation of further oxidation products via a deformylation or deamidation reaction.

There seems little doubt that the oxidation products, **II, III, V, VI,** and **VII,** are formed through the reaction onto the imidazole moiety, whereas the formation of the other products **(IV, VIII,** and **IX)** might be induced by the oxidation at the α- or β-position of *N*-benzoylhistidine. As for the formation of benzamide **(IV),** the reaction might be initiated by the hydrogen abstraction at the α-carbon of *N*-benzoylhistidine **(I)** (Scheme A in Fig. 6). The carbon-centered radical may react with molecular oxygen to give a peroxy intermediate which is then followed by the formation of a Schiff's base product. Under ambient conditions, the Schiff's base would be broken down to yield benzamide **(IV),** which is partly hydrolyzed to give benzoate **(IX).** The other product, **VIII,** includes an intact imidazole, and the reaction seems to be initiated by hydrogen abstraction at the β-carbon followed by the addition of molecular oxygen. This equivocal peroxy intermediate would be decomposed to **VIII** via the β-elimination reaction (Scheme B in Fig. 6).

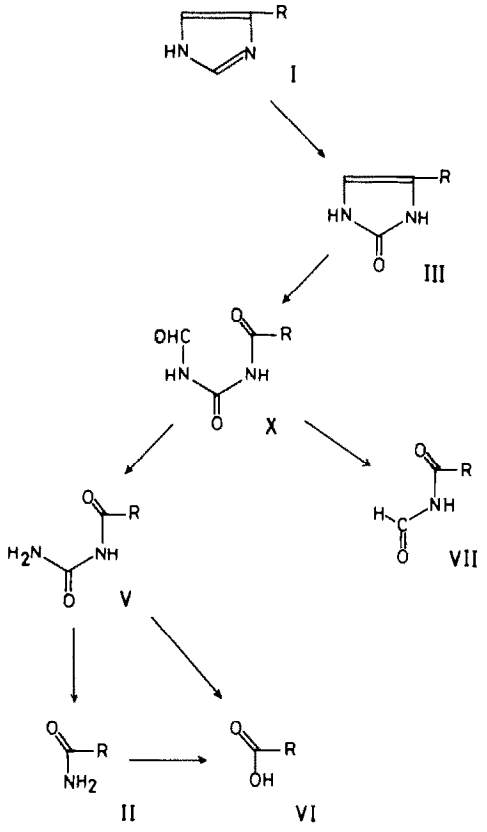

FIG. 5. A proposed mechanism for the ring opening reaction of the imidazole ring.

CONCLUSION

These results were partly consistent with the findings of other investigators who have studied the oxidative degradation of histidine and its derivatives under various oxidizing conditions. In the initial study, Imanaga suggested the formation of aspartic acid from L-histidine through the reaction with the ascorbate autoxidation system (*28*). Johns and Jaskewycz isolated many reaction products of the photolytic degradation of L-histidine in an aqueous solution (*24*). In the study of the sensitized photooxidation of the histidine derivative, Tomita *et al.* isolated a series of oxidation products during the photooxygenation of an imidazole compound by singlet oxygen ($^1O_2$) (*23*). They also proposed a total oxidation mechanism, in which the imidazole group was oxygenated via the 1,4-cycloaddition of $^1O_2$. Furthermore, formations of asparagine and aspartic acid have been demonstrated from the degradative reactions of the histidyl imidazole side chain with lipid hydroperoxide (*29–31*).

From our study, the occurrence of similar specific reactions is suggested *in vivo* when the free radical reactions are initiated by ascorbate at the metal-binding site

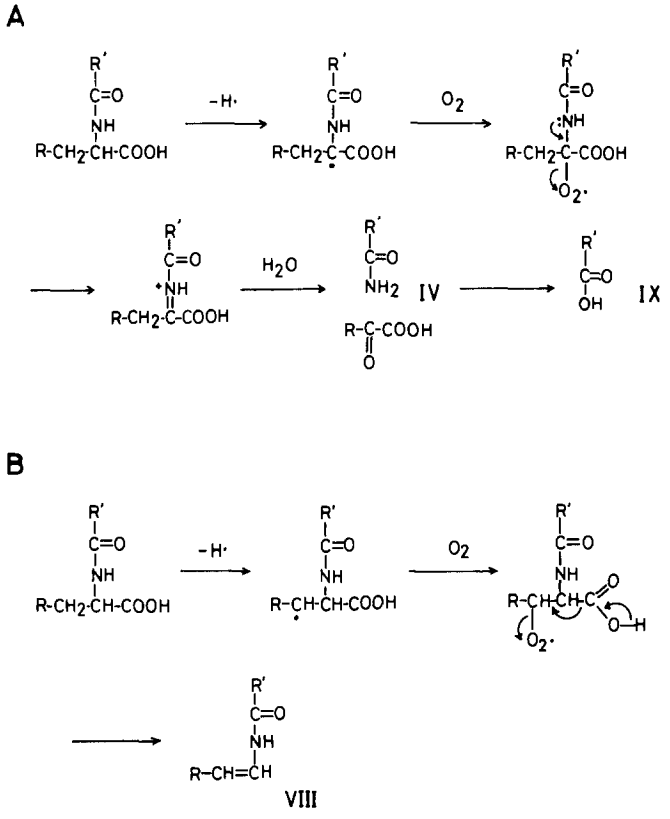

FIG. 6. Proposed mechanisms for formations of benzamide **(IV)**, benzoate **(IX)** (Scheme A), and 5-(2-benzamidovinyl)imidazole **(VIII)** (Scheme B). R and R' represent imidazole and phenyl groups, respectively.

of proteins. The formation of the free radicals *in situ* may give rise to the site-specific oxidation of the histidine residues in protein. Such reactions may therefore be physiologically important in connection with the oxygen toxicity or protein turnover on the mixed-function oxidation systems.

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