Formation of the 2-Imidazolone Structure within a Peptide Mediated by a Copper(II)/Ascorbate System

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The reaction of a histidine-containing tripeptide, hippurylhistidylleucine, with copper(II)/ascorbate under physiological conditions has been studied chemically. In the presence of a catalytic amount of copper(II) ion, ascorbate mediated the oxidative damage to the peptide via selective loss of the histidine residue. Furthermore, reaction of the copper(II)/ascorbate with the peptide gave a single product in good yield. The oxidized product was isolated and finally identified as the 2-imidazolone compound by ^1H NMR and tandem mass spectrometries. Especially in tandem mass spectrometry, we found a specific fragmentation characteristic of the 2-imidazolone structure. Therefore, it was confirmed that the reaction of Cu(II)/ascorbate occurs specifically at the C-2 position of the imidazole ring of the histidine residue in the peptide. The occurrence of an oxocopper(III) intermediate during the oxidation process of ascorbate is postulated.

INTRODUCTION

Ascorbate is relatively stable in pure water, while in the presence of a catalytic amount of metal ion, it is rapidly oxidized to dehydroascorbate through an electron transfer from ascorbate to metal (Khan et al., 1967a,b; Ogata et al., 1968). The rate of reaction is known to depend on pH, catalyst, oxygen pressure, temperature, etc. Especially, the acceleration of ascorbate autoxidation by copper(II) ion is well-known, which is accompanied by the one-electron reduction of molecular oxygen to yield some free-radical species such as superoxide (O_2^-) and the hydroxyl radical ($^\bullet OH$) (Khan et al., 1967a,b). Accordingly, the cytotoxicity of ascorbate in the presence of metal ions has been interpreted in terms of the generation of oxygen-derived free radicals (Samuni et al., 1983).

It has been reported that, in the presence of a micromolar concentration of copper(II) ion, ascorbate enhances the oxidation of various biological materials such as polysaccharides (Uchida and Kawakishi, 1986a), proteins (Samuni et al., 1983; Shinhar et al., 1983; Levine, 1984; Marx and Chevion, 1985; Uchida and Kawakishi, 1988), and DNA (Chiou, 1983, 1984; Wang and Ness, 1989). We have investigated to date the reaction of histidine residues in proteins and peptides with copper(II)/ascorbate (Uchida and Kawakishi, 1988, 1989a) and established a novel monooxygenation reaction of the imidazole ring of the histidine derivative (Uchida and Kawakishi, 1986b, 1989b; Uchida et al., 1989). Hence, we have attempted to detect this oxidized form of histidine residues using peptides and proteins as the substrates.

The present experiment has been designed so as to detect oxidized histidine residues in proteins through reaction with a Cu(II)/ascorbate-dependent redox system. In the course of this study, we found that Cu(II)/ascorbate caused selective oxidation of the imidazole moiety of the histidine residue in the peptide and gave a single product (2-imidazolone compound) in good yield. In the present paper, we demonstrate dramatic reactivity of a histidine-containing peptide, hippurylhistidylleucine (Hip-His-Leu), with copper(II)/ascorbate and present a structure of the oxidized peptide elucidated with NMR and tandem mass spectrometries.

MATERIALS AND METHODS

Materials. L-Ascorbate was purchased from Wako Pure Chemical Industries Ltd. (Osaka). Hippurylhistidylleucine (Hip-His-Leu) was obtained from the Peptide Institute, Inc. (Osaka). Trifluoroacetic acid (TFA >99%) was purchased from Tokyo Kasei Kogyo Co., Ltd. Other reagents were of the highest grades commercially available.

Reaction of Hip-His-Leu with Copper(II)/Ascorbate. The reaction was carried out at room temperature. The solution (2 mL) contained 1 mM peptide, 5 mM ascorbate, and 0.05 mM $CuSO_4$ in 0.1 M sodium phosphate buffer (pH 7.4). The reaction was initiated by the addition of ascorbate and stopped by the addition of EDTA solution (0.05 mM).

High-Performance Liquid Chromatography. The peptide was determined by reversed-phase HPLC on a Develosil ODS-5 column (4.6 × 250 nm). The reaction mixture was applied to a column equilibrated in a solution of 0.1% trifluoroacetic acid. The peptide was eluted with a linear gradient of methanol (0–100%) in 0.1% trifluoroacetic acid within 40 min at a flow rate of 0.8 mL/min, the elution being monitored by absorbance at 230 nm. Areas of the chromatographic peaks of each material were calculated by using a Shimadzu Chromatopac integrator, Model C-R3A.

Amino Acid Composition. Amino acid analysis was performed with a JEOL JLC-300 amino acid analyzer for which the sample was prepared as follows: the mixtures at a specific time were freeze-dried and then hydrolyzed with 6 N HCl at a concentration of 1 mg of peptide/mL at 110 °C for 20 h. The hydrolysates were concentrated, dissolved in aqueous HCl (pH 2.2), and then submitted for amino acid analysis.

Isolation and Identification of the Oxidized Peptide. The reaction mixtures (10 mL) in phosphate buffer (pH 7.2) containing 10 mM Hip-His-Leu, 0.5 mM $CuSO_4$, and 50 mM ascorbate were incubated for 6 h at room temperature. Both substrate and products in the reaction mixture were monitored by HPLC at various time intervals. After incubation, the mixture was freeze-dried, dissolved in a small amount of distilled water, and then subjected to preparative HPLC on a Develosil ODS-10 column (20 × 250 mm). The products were eluted with a solution of 50% methanol in 0.1% trifluoroacetic acid at a flow rate of 6.0 mL/min. Products were detected by

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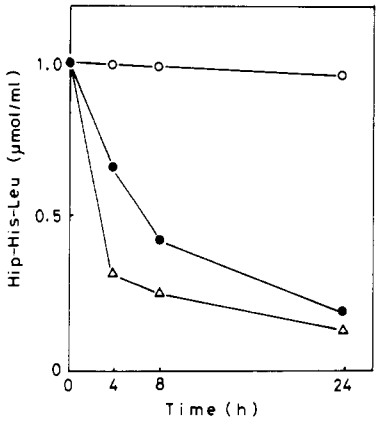

Figure 1. Time-dependent loss of Hip-His-Leu during incubation with the Cu(II)/ascorbate system. (●) Air; (△) O_2 atmosphere; (○) N_2 atmosphere.

absorbance at 230 nm. The peak of the oxidized peptide was fractionated and further purified by using a Develosil ODS-5 column.

Nuclear Magnetic Resonance Spectrometry. A nuclear magnetic resonance (NMR) spectrum on a JEOL JNM-FX200 spectrometer was taken in dimethyl sulfoxide-d_6 with tetramethylsilane as the internal standard.

Tandem Mass Spectrometry. FAB(+) collision-induced decomposition MS/MS (tandem mass spectrometry) was performed with a JEOL JMS-DX705 mass spectrometer. The sample was dissolved in glycerol, and 1 mmol in 0.5 μL of matrix was deposited on a stainless steel probe tip and placed in the ion source of MS-1 where it was bombarded with a beam of xenon atoms from a JEOL neutral atom gun (4 keV, 2-A cathode current, 10-mA emission). The precursor ion was then selected with sufficient resolution to transmit only the $(M + H)^+$ ion beam into the collision region. Helium was introduced into the collision region at a pressure that attentuated the precursor ion to 50–60% of its original abundance measured at detector 3 before and after the admission of helium. In this experiment, the resolution of MS-2 was 1:1500 and the secondary electron multiplier −10 kV postacceleration. The product ion spectrum was scanned from m/z 0 to beyond the precursor ion in 15 s with 1000-Hz filtering.

RESULTS AND DISCUSSION

Selective Damage to the Histidine Residue in Hip-His-Leu. We have previously shown that Cu(II)/ascorbate mediated the oxidative modification of histidine-containing peptides (Gly-Gly-His, His-Tyr, and His-Phe), under physiological conditions and approximately 70% of each peptide was diminished within 24 h of incubation (Uchida and Kawakishi, 1989a). Similar to these results, Hip-His-Leu exhibited a pronounced reactivity with Cu(II)/ascorbate (Figure 1). Loss of the peptide in the Cu(II)/ascorbate system was greatly accelerated in an O_2 atmosphere while entirely retarded in a N_2 atmosphere (Figure 1). In addition, it is of interest that the reaction of the peptide with Cu(II)/ascorbate gave a single product (peak 2) in good yield (Figure 2).

We then characterized the change in amino acid composition of the peptide. As shown in Table I, 42% of the histidine residue was selectively lost within 24 h of incubation. Therefore, there is little doubt that the loss of the peptide was predominantly due to the selective damage to the histidine residue. Accompanying the selective loss of the histidine residue, a considerable amount of ammonia was produced by the acid treatment of the modified peptide and, moreover, trace amounts of asparate, glutamate, and serine were also detected. Selective damage to the histidine residue in the peptide might have led to the liberation of ammonia from the oxidized imidazole moiety followed by the formation of acidic amino acids. These results were quite analogous to those of the other histidine-containing peptides (Uchida and Kawakishi, 1989a). Whereas without acid treatment of the oxidized peptide, trace amounts of histidine (0.17%) and leucine (1.59%) were detected on the amino acid chromatogram after 24 h of incubation. In addition, a trace amount of hippuric acid (2.6%) was also detected on the HPLC chromatogram in Figure 2. Therefore, it became apparent that Cu(II)/ascorbate only slightly reacted with the peptide bond but mainly reacted with the histidine residue in Hip-His-Leu.

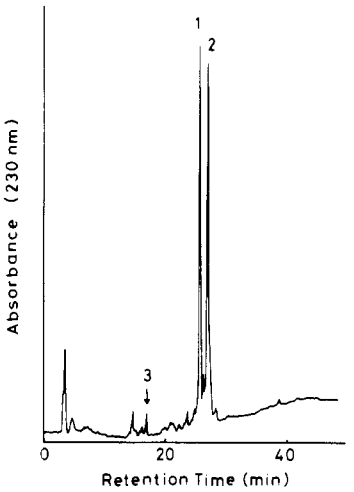

Figure 2. HPLC profile of the peptide treated with copper(II)/ascorbate. The reaction mixture (2 mL) in 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM peptide, 5 mM ascorbate, and 0.05 mM Cu(II) was incubated for 24 h at room temperature. Peaks 1, 2, and 3 represent Hip-His-Leu, the oxidized peptide, and hippuric acid, respectively.

Table I. Time-Dependent Changes in the Amino Acid Composition of Hip-His-Leu through the Reaction with Cu(II)/Ascorbate System

	molar ratio,[a] %			
	0 h	4 h	8 h	24 h
Asp	0	0.47	1.01	1.39
Ser	0	0.25	0.74	1.24
Glu	0	0.61	0.95	1.13
Gly	36.53	36.69	38.41	38.17
Leu	30.75	30.14	29.88	29.64
His	32.00	25.77	20.19	18.30
NH_4OH	0.74	3.93	5.13	5.72

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

Isolation and Identification of the Oxidized Hip-His-Leu. Structural assignments to the oxidized peptide were made on the basis of their spectral properties. To characterize the chemical structure of the product, isolation by preparative HPLC on reversed-phase columns was undertaken. Structural elucidation of the oxidized peptide was performed mainly by ^1H NMR and tandem mass spectrometries.

Figure 3 shows the ^1H NMR spectrum of Hip-His-Leu and the oxidized peptide. The data reveal the disappearance of the C-2 proton signal (c) and the shift of the C-4 proton signal (d) of the substrate to the higher field from 6.92 to 6.03 ppm (f), suggesting alteration of the imidazole moiety. A signal at 6.03 ppm is highly characteristic of the 2-imidazolone structure. In addition, the two proton atoms of the methylene group (2.92 ppm) attain equivalence in the native peptide; however, they were changed significantly in the oxidized peptide (data not shown),

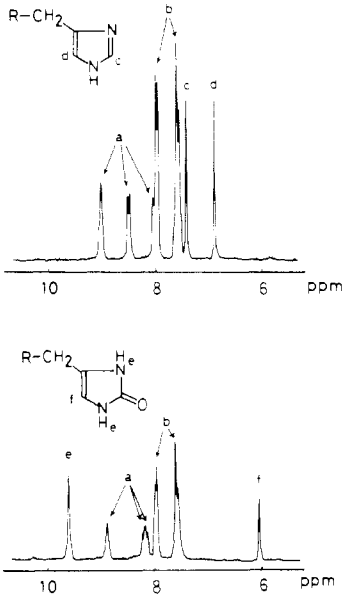

Figure 3. ^1H NMR spectra of Hip-His-Leu (upper) and the oxidized peptide (lower). Signals a and b correspond to the NH hydrogen of the peptide bond and the phenyl group, respectively.

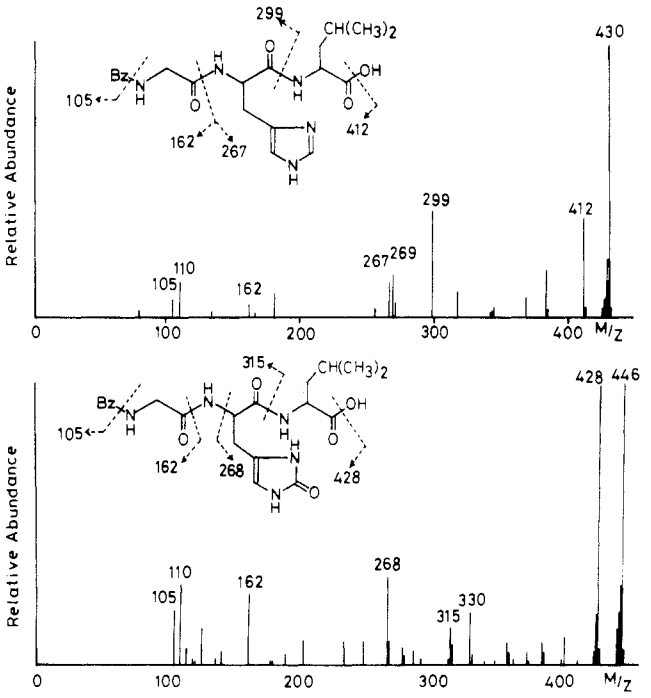

Figure 4. Tandem mass spectra of Hip-His-Leu (upper) and the oxidized peptide (lower). Bz represents the benzoyl group.

suggesting that free rotation of the imidazole residue was retarded by steric hindrance of the oxidized imidazole group.

The FAB-MS spectrum of the oxidized peptide gave a signal corresponding to the $(M + H)^+$ ion, m/z 446 (M + 1), which suggested the monooxygenation of the substrate.

The oxidized peptide was finally identified by comparison of its fragmentation in FAB(+) collision-induced decomposition MS/MS (tandem mass spectrometry) with the authentic 2-imidazolone compound prepared by the reaction of *N*-benzoylhistidine with Cu(II)/ascorbate (Figures 4 and 5). The specific fragmentation process was sufficient to characterize the structure of the oxidized peptide. In the series of fragmentations, the fragmentation between thee α-carbon and the nitrogen was the most characteristic process of the 2-imidazolone structure. The

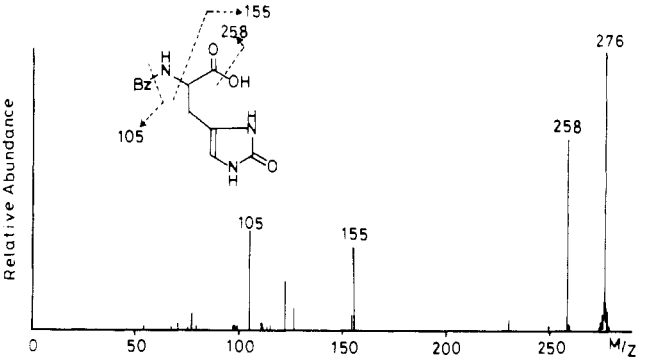

Figure 5. Tandem mass spectrum of the authentic 2-imidazolone compound. Bz represents the benzoyl group.

fragment ions corresponding to this fragmentation are m/z 268 of the oxidized peptide (Figure 4) and m/z 155 of the authentic sample (Figure 5). These results suggest that the oxidized peptide has undoubtedly the 2-imidazolone structure.

Proposed Mechanism. As to the active species in Cu(II)/ascorbate, the hydroxyl radical has long been regarded as the major cause of the biological damage. However, we speculate that a diffusible hydroxyl radical cannot occur in such highly site specific oxygenation of the imidazole moiety. To determine whether the active species of this specific oxygenation is the hydroxyl radical or not, the reaction of Hip-His-Leu with Fenton's reagent ($FeSO_4$/H_2O_2) or Udenfriend's [Fe(II)–EDTA/ascorbate] was examined; however, there was no 2-imidazolone product at all. In addition, the reaction of the substrate with Cu(II)/ascorbate was not prevented by the hydroxyl radical scavengers (data not shown). Moreover, we have recently found that Cu(II)/H_2O_2, a typical hydroxyl-radical-generating system, could not oxidize selectively at the C-2 position of the imidazole moiety of a histidine residue analogue (Uchida and Kawakishi, 1990). Therefore, the hydroxyl radical was excluded as an active species in the oxygenation of the C-2 position of the imidazole ring with Cu(II)/ascorbate.

On the other hand, we have previously demonstrated that the reaction of histidine residues in the peptides involves an electron transfer from ascorbate to copper(II) ion bound to the histidine residue (eq 1) (Uchida and

$$\text{His–Cu(II)} \xrightarrow{\text{ascorbate}} \text{His–Cu(I)} \quad (1)$$

$$\text{His–Cu(I)} + O_2 \rightarrow \text{His–Cu(I)–}O_2 \quad (2)$$

$$\text{His–Cu(I)–}O_2 \rightarrow \text{His–Cu(II)–}O_2^- \rightarrow \text{His–Cu(II)–}O_2\text{H} \quad (3)$$

$$\text{His–Cu(II)–}O_2\text{H} \rightarrow \text{His–Cu(III)=O} \quad (4)$$

Kawakishi, 1989a). The His–Cu(I) complex subsequently reacts with molecular oxygen to give an O_2 adduct (eq 2). The His–Cu(I)–O_2 complex is probably followed by generation of the His–Cu(II)–O_2^- complex (eq 3), and then the metal–peroxo complex (Cu(II)–O_2H degrades to the strongly oxidizing oxocopper(III) species (eq 4).

A subsequent reaction mechanism for oxygenation of the C-2 position of the imidazole ring with oxocopper(III) species has not yet been established in an ascorbate-oxidizing system. In relation to this, the oxocopper(III) species has been suggested in the orthohydroxylation of the pyridine nucleus via an azaoxometallacyclic intermediate in the copper(II)/iodosylbenzene system (Reg-

lier et al., 1989). Therefore, at present, we assume that an azaoxometallacyclic intermediate is the most probable oxidation route of the imidazole moiety of the histidine residue in the peptide. The oxocopper(III) species has been suggested as the hydroxylating species in monooxygenases such as tyrosinase or dopamine β-hydroxylase (Stewart and Klinman, 1987). We suppose that the high yield of the oxidized peptide in the present study can be reasonably interpreted by this mechanism. However, the oxocopper(III) species has not been established as the active species in the Cu(II)/ascorbate-dependent redox system. Further study is needed to detect the oxocopper(III) species in this system.

We have presented herein the proposed mechanism concerning a series of electron transfers from ascorbate to the copper–imidazole complex. The site-specific oxygenation of the peptide with Cu(II)/ascorbate could be of significance for the following reasons: (i) ascorbate, Cu(II), histidine, and its related materials are normal food and cellular constituents and (ii) site-specific oxygenation of the peptide happens at physiological pH, ionic strength, and temperature. Our present findings are also significant not only to interpret the site-specific oxygenation mechanism for oxidation of proteins with ascorbate but to exploit a new function of copper and ascorbate as a free-radical-generating system.

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Registry No. Hip-His-Leu, 31373-65-6; Cu(II), 7440-50-8; His, 71-00-1; ascorbic acid, 50-81-7; 2-imidazolone, 5918-93-4.