Selective Degradation of Histidine Residue Mediated by Copper(II)-Catalyzed Autoxidation of Glycated Peptide (Amadori Compound)

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The oxidative damage of protein induced by the Amadori compound/copper(II) system was recently clarified (Kawakishi, S.; Okawa, Y.; Uchida, K. *J. Agric. Food Chem.* **1990**, *38*, 13–17). In the present study, we characterized a selective degradation and a possible cleavage mechanism for histidine residue found in protein mediated by the glycated peptide (Amadori compound)/copper(II) system. The main oxidized intermediate of the histidine residue, a 2-imidazolone derivative, was also confirmed in this system, which was similar to that formed in the ascorbate/copper(II) system. Although glucosone, a secondary product generated from copper(II)-catalyzed autoxidation of the Amadori compound, has been identified as a strong oxidizing agent causing protein damage, the Amadori compound itself was also confirmed to be an oxidizing agent similar to glucosone in the present study. A proposed mechanism for oxidative damage of protein mediated by autoxidation of the glycated peptide (Amadori compound)/copper(II) system is discussed.

INTRODUCTION

Protein glycation, i.e., the nonenzymatic reaction between a reducing sugar and the primary amino group of proteins, is a specific example of the initial step of a multireaction pathway that has been generically known as the Maillard reaction. The early steps in this reaction assume that the Schiff base resulting from the initial sugar attachment to the protein primary amino group undergoes an Amadori rearrangement to yield a more stable but still chemically reversible Amadori product (Isbell and Frush, 1958; Gottschalk, 1972).

Glycation of protein has been found to occur both in vivo and in vitro. This reaction was suggested to be largely relevant to the complication of diabetes mellitus (Brownlee et al., 1984, 1988; Bunn, 1981; Cerami et al., 1979), aging (Kohn and Schnider, 1982; Monnier and Cerami, 1981; Chiou et al., 1981), cataracts (Ortwerth et al., 1988; Pirie, 1968), and so on. However, the chemical processes of this reaction are not clearly understood yet.

Amadori compounds are well-known as important intermediates in this browning reaction. Glycation produces Amadori derivatives, and thus it may cause the denaturation, polymerization, cross-linking, and insolubilization of tissue proteins in vivo. Whereas, in vitro, through the metal ion catalyzed autoxidation of the Amadori compound, protein damage (Kawakishi et al., 1990), lipid peroxidation (Sakurai et al., 1990, 1991), cleavage of DNA strands, and virus inactivation (Kashimura et al, 1986) have occurred. Recently, Ahmed et al. (1986, 1988) reported that N^{ϵ}-(carboxymethyl)lysine was generated from autoxidation of the Amadori compound (N^{α}-formyl-N^{ϵ}-fructoselysine) in phosphate buffer under aerated conditions. However, we found that glucosone, an active sugar moiety product, was produced directly from copper(II)-catalyzed autoxidation of the Amadori compound (N^{α}-*t*-Boc-N^{ϵ}-fructoselysine) (Kawakishi et al, 1991). The metal ion may be the main factor that caused the differences between our results and Ahmed's. Moreover, the oxidation of protein initiated by the Amadori compound/copper(II) system was completely inhibited by catalase or EDTA, indicating that some active oxygen species participate in the oxidative reaction. However, the concentration of the lenticular copper ion significantly increased with the progression of senile cataract to 4–10 times that of normal intact lenses (Shlopak, 1962, 1963, 1964; Nath et al., 1969; Racz and Ordogh, 1977; Cook and McGahan, 1986). This fact suggests that metal ion mediated oxidation of glycated protein in vivo may be an important process relating to aging, diabetes, cataracts, and other diseases which will be discussed further.

In the present study, we characterized the selective degradation of histidine residue in protein or peptide and its possible oxidation pathway induced by the glycated Gly-Leu-Tyr/copper(II) system. In addition, we clarified that either the Amadori compound or a secondary product (glucosone) could cause the same oxidative cleavage of the imidazole ring of the histidine derivative in the presence of copper ion.

EXPERIMENTAL PROCEDURES

Reagent. Polylysine (PL), glycyl-L-leucyl-L-tyrosine (Gly-Leu-Tyr), L-histidyl-L-tyrosine (His-Tyr), and N^{α}-benzoylhistidine (Bz-His) were purchased from Sigma Chemical Co. Bovine serum albumin (BSA) and β-alanyl-L-histidine (β-Ala-His) were obtained from Seikagaku Kogyo Co. Ltd. (Tokyo) and Peptide Institute Inc. (Osaka), respectively. D-Glucosone was prepared according to the procedure of Bayne (1963), and all other reagents were of the highest grades commercially available.

Preparation of Glycated Gly-Leu-Tyr and Glycated Polylysine. Gly-Leu-Tyr (10 mg) and glucose (100 mg) were dissolved in 2 mL of methanol and refluxed in a water bath (80–90 °C) with stirring for 45 min. After the addition of 0.2 mL of acetic acid, the mixture was refluxed further for 40 min and then concentrated by a rotary evaporator. Glycated Gly-Leu-Tyr was separated, purified by HPLC on a Develosil ODS-5 column, and eluted with 0.05 M ammonium acetate–methanol (100/35 v/v). The prepared glycated Gly-Leu-Tyr was identified by ^{1}H NMR and FAB mass (M + 1, 514). Glycated polylysine (G-PL) was prepared by incubation of polylysine (4 mg/mL) with glucose (1 M) in 67 mM phosphate buffer (pH 7.2) containing trace amounts of gentamicin and EDTA at 37 °C for 3 days. Excess glucose in the reaction mixture was dialyzed against distilled water at 4 °C for 48 h with several changes of distilled water. The dialysate was lyophilized and analyzed.

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Reaction Conditions. All reactions were carried out at 37 °C. The reaction mixtures contained 5 mM glycated Gly-Leu-Tyr, 1 mM histidine-containing peptide, and 50 μM $CuSO_4$ in 67 mM phosphate buffer (pH 7.2). Instead of Gly-Leu-Tyr, 0.1% glycated polylysine was used under the same conditions. The reactions were initiated by the addition of copper ion and stopped by the addition of EDTA solution (0.1 mM).

Determination of Sugar Moiety Products Generated in Copper(II)-Catalyzed Autoxidation of Glycated Gly-Leu-Tyr. The reaction mixture (5 mL) containing 5 mM glycated Gly-Leu-Tyr and 50 μM $CuSO_4$ was incubated at 37 °C for 6 h in 67m mM phosphate-buffer (pH 7.2). Thereafter, it was passed through a Dowex 50X8 (H form, 10 × 60 mm) column to separate the sugar moiety products (unadsorbed parts) from the amino acid derivatives (adsorbed parts) by eluting the column with 50 mL of distilled water. The eluate was lyophilized and dissolved in 3 mL of distilled water, followed by reduction with $NaBH_4$ (pH 9) for 2 h. After neutralization to pH 7 by the addition of Amberlite IR-120 resin, the solution was filtered and lyophilized. The residue was acetylated with acetic anhydride in anhydrous pyridine (1:1) and then submitted for analysis by HPLC. Chromatographic conditions were as follows: Samples were eluted at a rate of 1.5 mL/min with 0.1% TFA–acetonitrile (2/1 v/v) on a Develosil ODS-5 column (8 × 250 mm) monitored at 210 nm. The standard samples were directly acetylized from glycerol, erythritol, ribitol, arabinitol, xylitol, mannitol, and sorbitol by the same procedure described above. DL-Threitol tetraacetate was obtained from Sigma Chemical Co. directly.

Amino Acid Analysis. Amino acid analysis was performed on the JEOL JLC-300 amino acid analyzer. The sample was prepared as follows: The reaction mixtures containing 0.04% protein, 5 mM glycated Gly-Leu-Tyr, and 50 μM $CuSO_4$ in 67 mM phosphate buffer (pH 7.2) were removed at the indicated time and then treated with a 6% trichloroacetic acid (TCA) solution at 4 °C for 24 h. However, in the case of the mixture with the histidine-containing peptide (1 mM) instead of protein, the reaction mixtures were removed and lyophilized directly without reduction with $NaBH_4$. The precipitated protein or lyophilized peptide mixture was hydrolyzed by the gas-phase method with 6 N HCl at 110 °C for 24 h. The hydrolysates were concentrated to dryness, dissolved in a dilute HCl solution (pH 2.2), and then put into the amino acid analyzer.

High-Performance Liquid Chromatography (HPLC). The degradation of histidine-containing peptides induced by the glycated Gly-Leu-Tyr/copper(II) system was determined by reversed-phase 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 0.05 M ammonium acetate–methanol (AA–M) (97/3 v/v) for histidyltyrosine, and AA–M (75/25 v/v) for N^{α}-benzoylhistidine, and at a rate of 0.5 mL/min with AA–M (99/1 v/v) for β-alanylhistidine. The elutions were monitored at 210 nm.

However, the oxidized products of N^{α}-benzoylhistidine caused by the glycated Gly-Leu-Tyr/copper(II) system and the glycated polylysine/copper(II) system were determined on a Develosil ODS-5 column (8 × 250 mm) by eluting at a rate of 1.5 mL/min with 0.1% TFA–methanol (3/1 v/v) and monitoring at 230 nm. The chromatographic peak areas for each material were calculated using a Shimadzu Chromatopac C-R 3A integrator.

RESULTS

Selective Degradation of Histidine Residue in Protein with the Glycated Gly-Leu-Tyr/Copper(II) System. Our previous study (Kawakishi et al., 1990) confirmed that the Amadori compound/copper(II) system could cause the fragmentation of bovine serum albumin (BSA) and the degradation of its histidine residue. As shown in Figure 1, a marked oxidation of histidine residue in several proteins was also induced by glycated Gly-Leu-Tyr in the presence, but not in the absence, of copper ion. This suggests that the histidine residue was selectively degraded by copper(II) participating in the autoxidation of the glycated peptide. To clarify this degradation process of histidine residue, a model reaction of the histidine-containing peptides with the glycated Gly-Leu-Tyr/copper(II) system was undertaken here.

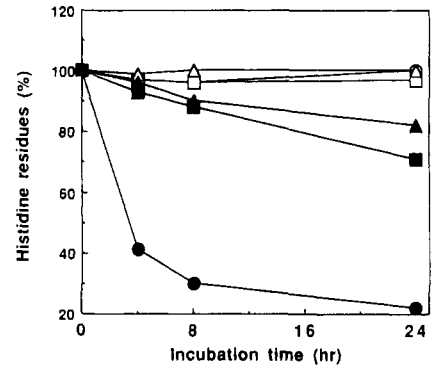


Figure 1. Oxidative damage of histidine residue in proteins by reaction with the glycated Gly-Leu-Tyr/copper(II) system. The reactions were carried out at 37 °C; the mixtures contained 5 mM glycated Gly-Leu-Tyr, 50 μM $CuSO_4$, and 0.04% proteins in 67 mM phosphate buffer (pH 7.2). Each portion (0.5 mL) of the reaction mixtures was sampled at the indicated time (0, 4, 8, 24 h) and treated with 0.5 mL of TCA solution (12%) at 4 °C for 24 h, and the precipitates were submitted for amino acid analysis. The data were calculated from amino acid analyses based on the molar ratio (percent) of histidine residue. (○) BSA/G-Gly-Leu-Tyr; (●) BSA/G-Gly-Leu-Tyr/Cu(II); (□) OVA/G-Gly-Leu-Tyr; (■) OVA/G-Gly-Leu-Tyr/Cu(II); (△) RNase/G-Gly-Leu-Tyr; (▲) RNase/G-Gly-Leu-Tyr/Cu(II).

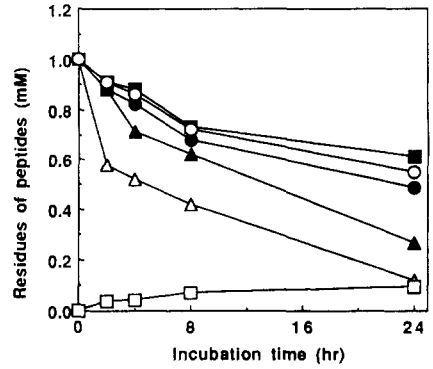


Figure 2. Oxidative degradation of histidine-containing peptides by reaction with the glycated Gly-Leu-Tyr (or polylysine)/copper(II) system. The reactions were carried out at 37 °C; the mixtures contained 5 mM glycated Gly-Leu-Tyr or 0.1% glycated polylysine, 50 μM $CuSO_4$, and 1 mM peptide in 67 mM phosphate buffer (pH 7.2). The peptide was determined by reversed-phase HPLC as described under Experimental Procedures. G-Gly-Leu-Tyr/Cu(II) system: (■) Bz-His; (●) β-Ala-His; (□) Bz-2-oxo-His; (▲) His-Tyr; (○) His-Ala. G-polylysine/Cu(II) system: (△) His-Tyr.

As shown in Figure 2, the decrease in histidine-containing peptides was determined by reversed-phase HPLC as described under Experimental Procedures. Each of them significantly degraded with incubation time. Along with the oxidation of N^{α}-benzoylhistidine, the N^{α}-benzoyl-2-oxohistidine intermediate was also detected. The glycated polylysine/copper(II) system can induce the same oxidation also. Their composition changes were determined by amino acid analysis and are shown in Tables I and II. The content of Gly-Leu-Tyr is also shown in each table as molar ratio (percent) because it was not removed from the reaction mixture before hydrolysis. Similar to the oxidation of protein (Figure 1), only the histidine residue was conspicuously degraded, while the other amino acids did not change appreciably.

Oxidative Cleavage of Imidazole Ring. To clarify this degradation process, the oxidation of N^{α}-benzoylhistidine was investigated. As shown in Figure 3, N^{α}-benzoylhistidine was degraded by oxidation to several new

Table I. Time-Dependent Changes in Amino Acid Compositions of L-Histidyltyrosine through Reaction with Glycated Gly-Leu-Tyr/Copper(II) System[a]

	molar ratio,[b] %				
amino acid	0	2 h	4 h	8 h	24 h
Gly	12.9	12.9	13.1	13.1	12.6
Leu	14.1	14.1	14.3	14.3	14.0
Tyr	13.9	13.9	13.6	13.6	13.1
His	2.6	2.4	2.2	1.9	1.5
NH_4OH	56.5	56.7	56.6	57.0	58.5

[a] The reactions were carried out at 37 °C on time course; the solutions contained 5 mM glycated Gly-Leu-Tyr, 50 μM $CuSO_4$, and 1 mM L-histidyltyrosine in 67 mM phosphate buffer (pH 7.2). The loss of histidine residue was determined by amino acid analysis as described under Experimental Procedures. [b] Molar ratio (%) is represented by the mole concentration of each amino acid per total amino acid.

Table II. Time-Dependent Changes in Amino Acid Compositions of L-Histidylalanine through Reaction with Glycated Gly-Leu-Tyr/Copper(II) System[a]

	molar ratio,[b] %				
amino acid	0	2 h	4 h	8 h	24 h
Gly	13.0	13.1	13.0	13.0	13.1
Leu	13.9	14.0	13.9	13.8	14.0
Tyr	12.2	12.7	12.2	12.1	12.1
Ala	2.3	2.3	2.4	2.2	2.1
His	2.0	1.8	1.7	1.6	1.3
NH_4OH	55.6	55.9	56.6	57.4	57.3

[a] The reactions were carried out at 37 °C on time course; the solutions contained 5 mM glycated Gly-Leu-Tyr, 50 μM $CuSO_4$, and 1 mM L-histidylalanine in 67 mM phosphate buffer (pH 7.2). The loss of histidine residue was determined by amino acid analysis as described under Experimental Procedures. [b] Molar ratio (%) is represented by the mole concentration of each amino acid per total amino acid.

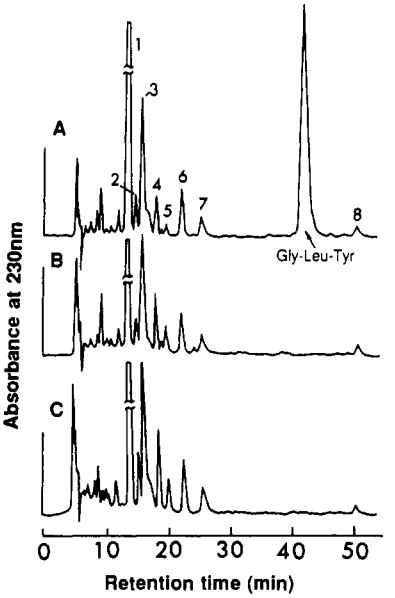


Figure 3. HPLC profiles of the oxidation of N^{α}-benzoylhistidine by reaction with glycated Gly-Leu-Tyr (A), glycated polylysine (B), and the ascorbate (C)/copper(II) system. The reaction mixtures containing 1 mM N^{α}-benzoylhistidine, 50 μM $CuSO_4$, and 5 mM glycated Gly-Leu-Tyr or 0.1% glycated polylysine or 5 mM ascorbate in 67 mM phosphate buffer (pH 7.2) were incubated at 37 °C for 24 h. Peaks 1 and 2 represent N^{α}-benzoylhistidine and N^{α}-benzoyl-2-oxohistidine, respectively. The HPLC analyses were done as described under Experimental Procedures.

products (peaks 2–8) induced by either glycated Gly-Leu-Tyr or glycated polylysine. Fortunately, these oxidations are the same as those induced by the ascorbate/copper(II) system. Uchida and Kawakishi (1989) reported the oxidation of N^{α}-benzoylhistidine mediated by the ascorbate/copper(II) system in detail. Compared to this previous study, the cleavage mechanism of the imidazole ring induced by the glycated peptide (Amadori compound)/copper(II) system was considered the same as that by the ascorbate/copper(II) system. As shown in Figure 4, the histidine residue was decomposed to aspartate and their derivative via the 2-imidazolone intermediate.

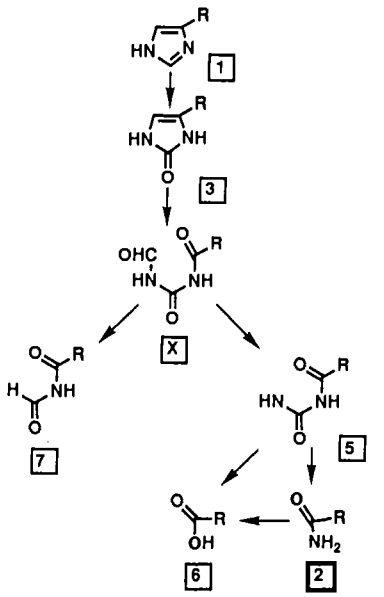
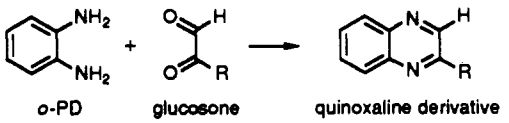


Figure 4. Possible mechanism for the oxidative cleavage of the imidazole ring by the glycated peptide (Amadori compound)/copper(II) system.

Determination of the Oxidation of N^{α}-Benzoylhistidine Caused by the Amadori Compound Itself. Recently, copper(II)-catalyzed autoxidation of the Amadori compound directly generated glucosone and amino acid (Kawakishi et al., 1991). Moreover, glucosone was confirmed to be a very active intermediate for the oxidation of protein catalyzed by the copper ion (Cheng et al., 1992). However, the Amadori compound itself being an oxidation promoter is not understood clearly; therefore, to clarify this question, some experiments were done in the present study.

The autoxidation of glycated Gly-Leu-Tyr mediated by copper ion was investigated as shown in Figure 5. Part A represents the decomposition of glycated Gly-Leu-Tyr (P) to free Gly-Leu-Tyr (P1), identified by ^1H NMR and FAB mass analyses. While the upper part of "B" represents the sugar moieties formed from the decomposition of glycated Gly-Leu-Tyr, the lower part is the standard acetates derived from glycerol (1), threitol (2), erythritol (3), ribitol (4), arabinitol (5), xylitol (6), mannitol (7), and sorbitol (8). Compared with these standards, hexitols derived from glucosone and glycerols were detectd in the glycated Gly-Leu-Tyr/copper(II) system. Since glucosone degrades rapidly in the presence of copper ion, the C3 moieties were considered as degradation products from glucosone.

However, in the presence of *o*-phenylenediamine (*o*-PD) in the reaction mixture, glucosone will be trapped to form a nonreactive quinoxaline derivative. Therefore, by



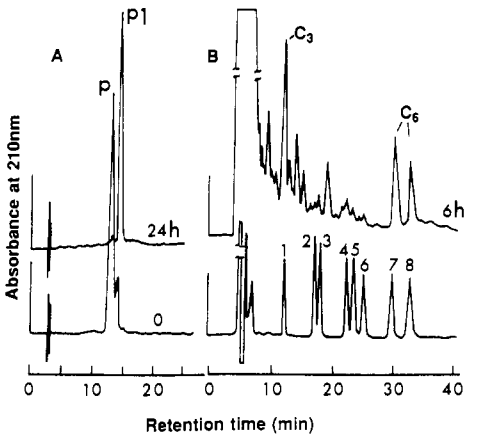


Figure 5. Oxidative degradation of glycated Gly-Leu-Tyr (A) and the determination of formed sugar moiety products (B) in the presence of copper ion. The reactions were carried out at 37 °C; the mixtures contained 5 mM glycated Gly-Leu-Tyr and 50 mM $CuSO_4$ in 67 mM phosphate buffer (pH 7.2). The HPLC analyses and the modification of sugar moiety products were done as described under Experimental Procedures. P represents the glycated Gly-Leu-Tyr.

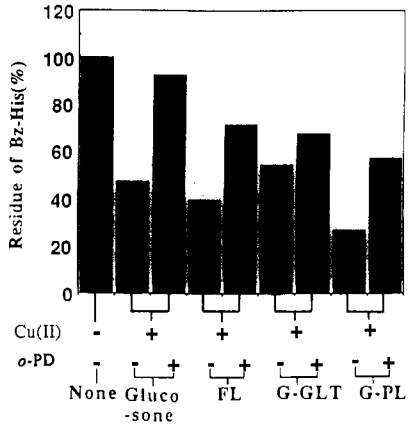


Figure 6. Oxidation of N^{α}-benzoylhistidine induced by the Amadori compound/copper(II) system in the presence or absence of *o*-phenylenediamine (*o*-PD). The reactions were carried out at 37 °C for 24 h; the mixtures contained 5 mM Amadori compound or glucosone or 0.1% glycated polylysine, 50 μM $CuSO_4$, 1 mM N^{α}-benzoylhistidine, and 25 mM *o*-PD in 67 mM phosphate buffer (pH 7.2). The determination of N^{α}-benzoylhistidine was done the same as in Figure 3.

the addition of *o*-PD to the Amadori compound/copper(II)/N^{α}-benzoylhistidine (1 mM) system, the oxidative degradation of N^{α}-benzoylhistidine caused from secondarily generated glucosone and copper(II) would be suppressed.

As shown in Figure 6, there was a large difference in the decrease in N^{α}-benzoylhistidine between the presence and absence of *o*-PD. As a reference, synthetic glucosone was also used here; the oxidation of N^{α}-benzoylhistidine induced by the glucosone/copper(II) system was completely inhibited by the addition of *o*-PD. These results suggest that not only the glucosone but the Amadori compound itself also has the ability to induce the oxidation of protein mediated by a metal ion. In addition, quinoxaline derivative derived from glucosone in the Amadori compound/copper(II) system increased with the reaction time, parallel with the decrease of the Amadori compound. Furthermore, it was also clarified that *o*-PD has almost no chelation effect on copper ion, by comparing the oxidative degradation of BSA induced by a H_2O_2/copper(II) system in the presence and absence of *o*-PD (data not shown).

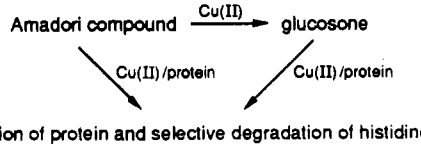
DISCUSSION

Nonenzymatic glycation of protein in vivo is deeply related to diabetes, aging, and cataracts. Metal ion catalyzed oxidation of glycated proteins confirmed the fragmentation of proteins and the selective degradation of histidine residue (Cheng et al., 1991). To clarify whether or not these oxidations are induced by reactive oxygen radicals generated from the Amadori compound/copper(II) complex, the glycated Gly-Leu-Tyr/copper(II) system was chosen for the present study. Through the reaction of proteins or peptides with this system, the selective degradation of histidine residue in proteins (Figure 1) or peptides (Figure 2; Tables I, II) was observed depending on the incubation time. This observation was found to be similar to that of copper(II)-catalyzed oxidation of glycated proteins.

A marked decrease in N^{α}-benzoylhistidine and the formation of some oxidized derivatives (e.g., N^{α}-benzoyl-2-oxohistidine) were detected after incubation of either glycated Gly-Leu-Tyr or the glycated polylysine/copper(II) system with N^{α}-benzoylhistidine, a histidyl residue analog (Figures 2, 3). As shown in Figure 4, histidine residue was degraded via the 2-imidazolone intermediate to aspartate and its related derivatives. On the basis of the inhibition effects of the chelating agent (EDTA), radical scavengers and enzymes related to the elimination of the oxygen radical in this system, only EDTA and catalase completely suppress the degradation of histidine (data not shown). These results suggest that the copper ion strongly participates in the reaction and that the formation of hydrogen peroxide is required for the progress of this oxidation.

Previously, Wolff and Dean (1987) and Wolff et al. (1984, 1991) have suggested that α-ketoaldehydes such as glucosone could be derived from glucose by its autoxidation to contribute to protein modification. In our reaction system, glucosone was directly generated from copper(II)-catalyzed autoxidation of the Amadori compound [Kawakishi et al., 1991; and also in the present study (Figure 5)]. Glucosone is required to be very active to be further decomposed by copper ion and simultaneously induces the secondary oxidative damage of protein (Cheng et al., 1992). On the other hand, Ahmed et al. (1986) have confirmed that N^{ϵ}-(carboxymethyl)lysine was formed from the autoxidation of the Amadori compound in phosphate buffer. The differences in the results found between our and Ahmed's systems may be the result of the addition of transition metal ion to our reaction systems.

Although glucosone was a main generator of active oxygens in the Amadori compound/copper(II) system, the Amadori compound itself also has exhibited a strong activity for oxidizing histidine derivatives (Figures 3, 6). According to these facts, a possible mechanism for oxidation of protein mediated by the Amadori compound/copper(II) system was proposed:



The enaminol form of Amadori compounds would easily generate the complex with copper ion and oxygen to produce some active oxygen species which may attack the neighboring part of the protein. By such complex formation, the Amadori compound is degraded to glucosone and amino acid or original amino compounds. Glucosone

that is generated secondarily must then induce the oxidative damage of protein by the participation of a transition-metal ion.

Therefore, these results may be convenient to interpret the copper ion mediated oxidation of glycated protein. Recently, we have also confirmed a site-specific modification of histidine residue in glycated protein catalyzed by copper ion, which will be published in another paper.

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