Oxidative Damage of Glycated Protein in the Presence of Transition Metal Ion

Rong-zhu CHENG, Jun TSUNEHIRO, Koji UCHIDA
and Shunro KAWAKISHI

*Department of Food Science and Technology, Nagoya University,
Furo-cho, Chikusa-ku, Nagoya 464-01, Japan*

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Glycation of proteins was induced by incubation of high concentrations of D-glucose (1 M) and proteins (4 mg/ml) at 40°C for 7 days. The oxidative damage of glycated protein mediated by addition of traces of metal ion in phosphate buffer (1/15 M, pH 7.2) under aerated conditions was investigated. Free amino groups (mainly ϵ -NH₂ groups of lysine residues) of protein decreased during the glycation of protein but were regenerated in the oxidation of glycated protein induced with metal ion. Also in this process, an α -dicarbonyl compound was generated. Glycated protein was degraded to lower molecular weight fragments in the presence of Cu(II) but not in the absence of Cu(II). Moreover, the histidine residue of protein was selectively degraded in this oxidative process. The oxidative fragmentations of glycated protein were inhibited by EDTA or catalase, but not by urea, DMSO (scavengers of hydroxy radicals) or superoxide dismutase. A possible mechanism of oxidative fragmentation of glycated protein catalyzed by the metal ion is discussed.

Glycation, also known as nonenzymatic glycosylation, is the condensation reaction between glucose and the free amino groups of protein molecules. The reactive amino groups are either α -NH₂ groups of protein N-termini or ϵ -NH₂ groups of certain lysine residues, depending on their accessibility and environment.^{1–3)} Glycation proceeds through a Schiff's base intermediate, follows by Amadori rearrangement to yield relatively stable ketoamine adducts of proteins.^{4,5)} This product is called glycated protein. The reaction up to the formation of Amadori compounds is considered to be the initial stage of the Maillard reaction. Glycation has been found both *in vivo* and *in vitro* and the relationship of glycated proteins with several diseases and aging in the human body is also known. For example, in hemoglobin, plasma protein, collagen, hair, lens, and other long-lived proteins, the levels of fructoselysine formed by glycation of lysine residues in protein have been known to increase in proportion to the degree of hyperglycemia in diabetes.⁶⁾ The oxidation of fructoselysine (FL) in phosphate buffer under aerated con-

ditions generates two products, *N*-(carboxymethyl)lysine (CML) and 3-(*N*-lysino)lactic acid, and these two products have also been identified in human lens proteins, collagen, and urine.^{7–9)} Furthermore during the aging of lens proteins *in vivo*, Baynes¹⁰⁾ also confirmed that the *N*-(carboxymethyl)lysine (CML) rather than fructoselysine (FL) was the major age-dependent product of glycation in adult lens proteins.

On the other hand, oxygen radicals were generated from the browning solution of glucose and amino acids in the presence of copper ion and their oxidative action on protein have been investigated in detail.¹¹⁾ It has been reported that the active oxygen species generated from Amadori compounds under physiological conditions induced cleavage of nucleic acid and inactivation of viruses.^{12,13)} Furthermore, generation of superoxide (O₂⁻) and lipid peroxidation during the oxidation of glycated polylysine in the presence of iron-ADP have also been reported recently.¹⁴⁾

With this background, it is considered that glycated protein may generate active oxygen

radicals in the presence of traces of transition metal ion under physiological conditions and the radical species will oxidatively attack the protein itself to cause conformational change and structural damage. This paper is concerned with the autooxidative degradation and fragmentation of glycated protein and the oxidative damage of histidine residue in protein molecules in the presence of traces of transition metal ion under physiological conditions.

Materials and Methods

Materials. Bovine serum albumin (BSA) and chicken egg white lysozyme were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo). Ovalbumin was purchased from Sigma Chemical Co. Polylysine was obtained from Peptide Institute Inc. (Osaka). Other reagents were of the best grades commercially available.

Glycation of protein. Protein (4 mg/ml) was incubated with glucose (1 M) in phosphate buffer (1/15 M, pH 7.2) containing traces of gentamicin for 7 days at 40°C. Excess glucose in the reaction mixture was dialyzed against distilled water at 4°C for 3 days and the water was changed two times a day. The dialyzate was lyophilized and analyzed. Glycated polylysine was prepared under the same reaction conditions as glycated protein. *N*^ε-*t*-Boc-*N*^ε-Fructoselysine (FL) was prepared by reaction of *N*^ε-*t*-Boc-lysine with D-glucose as described by Monnier.¹⁵⁾

Incubation conditions. The incubation mixtures (10 ml) contained 0.04% (w/v) glycated proteins and 50 μM Cu(II) in phosphate buffer (1/15 M, pH 7.2). The reactions were started by adding Cu(II) at 40°C and stopped by the addition of an EDTA solution (2%, w/v).

Measurement of free amino groups and α-dicarbonyl compound. Free amino groups in proteins were measured by the TNBS method and the absorbance at 420 nm.¹⁶⁾ α-Dicarbonyl compounds were measured by the reaction with Girard-T reagent and the absorbance at 325 nm.¹⁷⁾

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE using 10% acrylamide gel was done by the method of Laemmli.¹⁸⁾ Gel sheets were stained with a solution of 0.25% Coomassie Brilliant Blue R-250 in water–2-propanol–acetic acid (5:5:1) and destained with 7% acetic acid containing 5% methanol.

High performance liquid chromatography (HPLC). Chromatographic measurement of glycated BSA was done by HPLC on a Develosil TSK-Gel 3000 SW column (7.5 × 600 mm). Samples were eluted at a rate of 1.0 ml/min

with 1/15 M phosphate buffer (pH 7.0) containing 0.1 M NaCl. The elution was monitored continuously by UV absorbance at 210 nm.

Amino acids analyses. The reaction solutions of glycated protein and Cu(II) were dialyzed against distilled water for 2 hr to remove metal ions and other low molecular weight products generated during the reaction and then reduced with NaBH₄ for 2 hr to prevent the decomposition of the Amadori type bond in the glycated protein by hydrolysis. The reduced solutions were treated with 6% TCA solution. The precipitated proteins were hydrolyzed in a sealed tube with 6 N HCl at 110°C for 24 hr. The hydrolysates were concentrated to dryness, dissolved in a dilute HCl solution (pH 2.2) and then put into an amino acid analyzer (JEOL Model JLC-6AH).

Results

Decomposition of glycated proteins induced with Cu(II)

Glycated BSA, ovalbumin, and lysozyme were incubated in the absence or presence of Cu(II) in phosphate buffer (1/15 M, pH 7.2) at 40°C. The changes of free amino groups in proteins measured by the TNBS method are shown in Table I. The α-dicarbonyl compound released from glycated proteins was measured by reaction with Girard-T reagent. This result is shown in Fig. 1. Similar to Amadori compounds prepared from glucose and amino acid,¹⁹⁾ glycated proteins were also degraded by the addition of Cu(II). The free amino groups of each glycated protein increased in the presence of Cu(II) but there were not any

Table I. THE CHANGES IN FREE AMINO GROUPS DURING GLYCATION AND OXIDATION OF GLYCATED PROTEINS

	Percentage of free amino groups (%)			
	Native protein	Glycated protein	Glycated protein 24 hr ^a	Glycated protein + Cu(II) 24 hr ^b
BSA	100	67	66	83
Ovalbumin	100	77	74	86
Lysozyme	100	71	67	80

^a Glycated protein (0.04%) was incubated in phosphate buffer at 40°C for 24 hr in the absence of Cu(II).

^b Glycated protein (0.04%) was incubated in phosphate buffer at 40°C for 24 hr in the presence of 0.05 mM Cu(II).

changes in the absence of Cu(II) after incubation for 24 hr (Table I). The α -dicarbonyl compound was also released from glycated proteins only in the presence of Cu(II) (Fig. 1). The major part of generated α -dicarbonyl compounds was estimated to be D-glucosone, similar to the autooxidation of Amadori compounds induced with Cu(II),¹⁹ but it was

not identified in the glycated protein-Cu(II) system.

Oxidative damage of glycated bovine serum albumin induced with Cu(II)

Time-dependent alteration of glycated BSA during incubation with or without Cu(II) was examined by SDS-PAGE as shown in Fig. 2. The major band of glycated BSA (about 67 kDa) gradually disappeared and the oxidized lower molecular fragments appeared under 14 kDa in the presence of Cu(II). After incubation for 24 hr, more than 80% of the glycated BSA was degraded (measured by HPLC, data not shown). However, no changes in the glycated BSA were observed in the absence of Cu(II). On the other hand, glycated ovalbumin and lysozyme were also degraded only in the presence of Cu(II). To investigate the site-specific damage of amino acid residues during the oxidation of glycated protein induced with Cu(II), the amino acids were analyzed. Glycated BSA, ovalbumin, lysozyme were incubated with Cu(II) in phosphate buffer

Fig. 1. Formation of α -Dicarbonyl Compounds in Glycated Protein-Cu(II) System.

The reaction mixtures of 0.04% glycated protein in phosphate buffer (1/15M, pH 7.2) were incubated in the presence (0.05 mM) and absence of cupric sulfate at 40°C. The mixtures were reacted with Girard-T reagent and the absorbance were measured at 325 nm. The quantity of α -dicarbonyl compounds was calculated as glucosone.

—●—, glycated protein/Cu(II) system; —▲—, glycated protein only.

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis of the Glycated Protein in the Presence (B) and Absence (A) of Cu(II).

The reactions were carried out at 40°C, the mixtures contained 0.04% glycated bovine serum albumin and 0.05 mM cupric sulfate in phosphate buffer (pH 7.2). Each portion (0.5 ml) of the reaction mixtures was sampling on time course (0, 2, 4, 8, and 24 hr) and treated with 0.5 ml of TCA solution (12%) for 24 hr at 4°C and the precipitates were submitted to SDS-PAGE.

Fig. 3. Time-Dependent Changes of Histidine and Lysine Residues in Glycated Protein/Cu(II) Reaction System.

The reaction mixtures contained 0.04% glycated protein and 0.05 mM cupric sulfate were incubated in phosphate buffer solution (pH 7.2) at 40°C. Each portion (2.5 ml) of the reaction mixtures was sampling on time course (0, 4, 8, and 24 hr), dialyzed, reduced with NaBH₄ and then, treated with TCA solution (12%) for 24 hr at 4°C. The precipitated samples were submitted to amino acids analyses.

—●—, glycated BSA/Cu(II); —▼—, glycated ovalbumin/Cu(II); —▲—, glycated lysozyme/Cu(II).

(1/15 M, pH 7.2) at 40°C. Samples were taken in time course and the proteins were precipitated by 6% TCA solution. Based on the amino acid analyses, only histidine and lysine residues changed with the oxidation of glycosylated proteins induced with Cu(II), while the other amino acids did not have any changes under the same conditions. Figure 3 shows the time-dependent changes in histidine and lysine residues of glycosylated proteins during the oxidation induced with Cu(II). According to incubation time, lysine residues increased and histidine residues decreased gradually.

Furthermore, to clarify the changes of lysine residue in glycosylated protein, *N*^α-*t*-Boc-*N*^ε-fructoselysine (FL) and glycosylated polylysine prepared from polylysine and glucose were used. Fructoselysine and glycosylated polylysine were incubated with the same reaction conditions mentioned above and followed by the reduction with NaBH₄ for 2 hr. The reduced sample prepared from the reaction mixture of *N*^α-*t*-Boc-*N*^ε-fructoselysine (FL) was concentrated and then the Boc-group was removed by reaction with BF₃ (CH₃CH₂O)₂/CH₃COOH for 30 min at room temperature²⁰⁾

Table II. AMINO ACIDS ANALYSES OF OXIDIZED *N*^ε-FRUCTOSELYSINE AND GLYCATED POLYLYSINE^a

	Area percent (%)		
	Lysine	Amadori comp.	NH ₄ OH
Polylysine	97.8	0	1.2
Glycated polylysine	51.9	33.5	4.0
Glycated polylysine + Cu(II) ^b	77.2	2.4	7.3
Fructoselysine	0	86.5	1.2
Fructoselysine + Cu(II) ^c	66	7.9	2.6

^a All samples were reduced with NaBH₄ for 2 hr before hydrolysis.

^b The reaction was done at 40°C for 24 hr, the solutions containing 0.04% glycosylated polylysine and 0.05 mM Cu(II) in phosphate buffer.

^c The reaction was also done at 40°C for 24 hr, the solutions containing 5 mM fructoselysine and 0.05 mM Cu(II) in phosphate buffer.

and concentrated again. The reduced sample was dissolved in dilute HCl (pH 2.2) and then analyzed using an amino acid analyzer. For glycosylated polylysine, the reduced sample was hydrolyzed by the same procedure with the glycosylated proteins. These data (Table II) showed that fructoselysine and glycosylated polylysine were also oxidatively degraded by the addition of Cu(II). After incubation with Cu(II) for 24 hr, most of the Amadori compounds were decomposed and lysine residues were re-generated similarly to the glycosylated proteins.

From these results, it shows that in the presence of Cu(II), glycosylated proteins were decomposed mainly at the site of ε-amino groups of lysine residues, releasing the dicarbonyl compound, and then caused selective oxidative degradation of its histidine residues (Fig. 3).

Effects of metal ions on the oxidation of glycosylated BSA

To evaluate the effects of Cu(II) on the specificity of oxidative damage of glycosylated protein, the effects of other metal ions, such as Fe(II), Fe(III), Co(II), Mn(II), Mg(II), and Ca(II) were examined (Fig. 4). Among of them,

Fig. 4. Effects of Metal Ions on Oxidation of Glycosylated Bovine Serum Albumin by SDS-Polyacrylamide Gel Electrophoresis.

The reactions were carried out at 40°C for 24 hr and the mixtures contained 0.04% glycosylated bovine serum albumin and 0.05 mM of each kind of metal ion in phosphate buffer. Ferric and ferrous ions were solubilized to EDTA (0.2 mM)-phosphate buffer solution prior to addition into the reaction mixture. After incubation for 24 hr, each portion of the reaction mixtures (0.5 ml) was followed by the same procedure in Fig. 2.

Table III. EFFECTS OF INHIBITORS ON OXIDATION OF GLYCATED BOVINE SERUM ALBUMIN WITH Cu(II) SYSTEM^a

Inhibitors	Conc. ^b	(%) Inhibition ^c
None	—	0
EDTA	10 mM	79.3
Urea	10 mM	20.7
DMSO	10 mM	10.3
SOD	500 units/ml	1.7
Catalase	500 units/ml	82.8

^a The reactions were done at 40°C for 24 hr, the solutions containing 0.04% glycated bovine serum albumin and 0.05 mM Cu(II) in phosphate buffer (1/15 M, pH 7.2). Oxidation of the protein was detected by HPLC on a TSK-GEL G3000SW column.

^b All concentrations shown are final reaction concentrations.

^c The percentage of inhibition is calculated as follow:

$$(\%) \text{ inhibition} = 100 - \frac{A - X}{A - B} \times 100$$

A: the peak height of glycated BSA (0.04%) in phosphate buffer on 24 hr incubation.

B: the peak height of glycated BSA (0.04%) and 0.05 mM Cu(II) in phosphate buffer on 24 hr incubation.

X: the peak height of [B + inhibitor] system.

Cu(II) had stronger activity for oxidative damage of glycated BSA. By HPLC, about 80% of the glycated BSA was cleaved with Cu(II) and only about 15% by Fe(II)/EDTA or Fe(III)/EDTA. Other metal ions had little activity toward the oxidation of glycated BSA.

Effects of several inhibitors on the fragmentation of glycated BSA induced with Cu(II)

Since these reactions might arise from some oxygen radicals formed through the interaction in the glycated protein/Cu(II)/O₂ system like that of the ascorbate/Cu(II)/O₂ system, a series of hydroxyl radical scavengers, enzymes relating to the degradation of active oxygen and chelating agent of metal ion were added to this system. As shown in Table III, catalase and EDTA completely depressed the oxidative degradation of glycated BSA induced with Cu(II), but the hydroxyl radical scavengers (dimethyl sulfoxide and urea) and superoxide

dismutase (SOD) only weakly suppressed the oxidative reaction.

Discussion

Glucose reacts with ε-amino groups of lysine residues on proteins *via* Schiff's base to form glycated proteins having an Amadori compound structure. In the preparation of glycated proteins, lysine residues on proteins decreased in the reaction with glucose. During the oxidation of glycated proteins, free amino groups on proteins increased (Table I) and the α-dicarbonyl compound was formed (Fig. 1) in the presence of Cu(II) but not in the absence of Cu(II). Also, only in the presence of Cu(II), the oxidative fragmentation of glycated BSA was observed and its histidine residues were markedly degraded (Fig. 3) while the lysine residues gradually increased (Fig. 3, Table II) during oxidation. Therefore these facts suggested that the glucose moieties bound to lysine residues of protein were oxidatively eliminated to form the free ε-amino group and D-glucosone by oxidative reaction.

Wolff and Dean reported that when protein were exposed to glucose, the cleavage and conformation changes of protein arose depending on the formation of hydroxyl radicals by glucose-autoxidation and ketoaldehyde was produced as an autoxidation-derived product of the glucose moiety.²¹⁻²³ Furthermore, we also demonstrated the formation of the α-dicarbonyl compound by oxidative degradation of the linkage between the ε-amino groups of lysine residues and glucose (Table I, Figs. 1 and 3). And the damage of protein, its fragmentation and selective degradation of histidine residues might be dependent upon some oxygen radicals or copper oxygen complex, similar to the ascorbate-Cu(II) system.^{24,25}

On the other hand, Nakano reported that the glycated polylysine-Fe(III)/ADP system produced O₂⁻ under physiological conditions and induced lipid peroxidation. This peroxidation reaction was slightly inhibited by 0.5 μM SOD but not by catalase. But in our glycated