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Lysine proximity significantly affects glycation of lysine-containing collagen model peptides

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ABSTRACT

Advanced glycation end products (AGE) are known to cause diabetes complications in hyperglycemia patients. In this study we prepared hetero-trimers of collagen model peptides comprising Ac-(Pro-Hyp-Gly)$_5$-Pro-Lys-Gly-(Pro-Hyp-Gly)$_5$-Ala-NH$_2$ (**4**) and Ac-(Pro-Hyp-Gly)$_{11}$-Ala-NH$_2$ (**5**) to investigate the clustering effect of lysine on AGE formation. The formation rate of carboxymethyllysine over several months was determined for the mixtures of peptides **4** and **5** at (3:0), (2:1) and (1:2) in the presence of glucose. The contents of carboxymethyllysine were significantly enhanced for (3:0) and (2:1) as compared with (1:2), suggesting that the proximity of lysine residues in the trimers accelerated formation of the AGE. Furthermore, a lysine dimerization moiety (GOLD) was identified for the first time from AGEs of glucose origin, which implied the significance of GOLD in oligomerization of collagens and other long-life proteins.

1. Introduction

Intravital proteins nonenzymatically react with reducing sugars such as glucose to produce advanced glycation end products (AGEs), which are often accumulated in diabetes patients. Recent studies have revealed that AGEs sometimes cause diabetes complications particularly through the glycation of long-lived proteins. Among these, collagen, the most abundant protein family in human tissues, is potentially implicated in AGE-related illnesses such osteoarthritis,[1,2] cardiovascular failures,[3,4] and renal disorders.[5,6] The structures of glycated moieties and their linkages between proteins have been extensively studied and greatly contributed to a better understanding of these complicated reactions and their resultant products. In addition to glucose, dicarbonyl compounds represented by glyoxal and methylglyoxal can be generated through the primary metabolic pathway of carbohydrates as well as the oxidative degradations of unsaturated fatty acids.[7,8] Among AGEs, carboxymethyllysine (CML, **1** in Fig. 1)[9] is often utilized as a diagnostic marker for protein glycation in hyperglycemia patients. Besides these N-substituted lysine and arginine, crosslinkers of proteins turned out to be clinically important since dimerization greatly damages physiological functions, and potentially causes AGE-related complications. There are several crosslinkers reported from glycated proteins such as crossline, DOGDIC, GODIC, GOLD, MOLD, pentosidine, and so on.[10–14] In particular, GOLD (**2**), glyoxal lysine dimer, which was reported to be derived from two molecules of lysine and glyoxal, has been found from human tissues as one of the aging-related AGEs.[15,16]

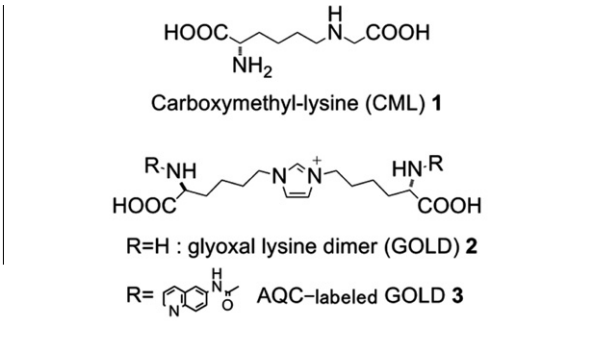

Figure 1. AGEs (**1**, **2**) generated from collagen model peptides, AQC-labeled GOLD (**3**) and collagen model peptides (**4**, **5**).

The structural elucidation of AGEs has largely been carried out by using the glycation products of N$^{\alpha}$-protected lysine and arginine

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since AGEs obtained from proteins are a complex mixture with diverse glycation modifications. Despite a great advance brought about by the model studies, little information is obtained as to how closely glycation reactions of proteins in human can be reproduced by these simplified systems. To know what exactly happens in protein glycation under physiological conditions, intact AGEs should be examined without chemical or enzymatic degradation. To address these problems, we have previously prepared a simple and small model peptide of collagen and successfully confirmed the presence of CML as an AGE product upon incubation with glyoxal and glucose.[17] When this model peptide forms a collagen-type triple helix,[18] however, the lysine residue of each peptide comes close together. The close alignment of lysine is not common in collagen since lysine constitutes only 3% of the total amino acids. Iberg et al reported that the basic amino acid in the vicinity of lysine residues accelerated formation glycation reactions.[19] Thus, we attempted to prepare a better collagen model to reproduce AGE formation occurring in vivo.

Collagens in certain tissues such as guts are known to last long with a half life over 7 months.[20] Therefore, long-term in vitro incubation of collagens in the presence of glucose has been often carried out.[17,21] In this report we examined the long-term glycation products of glucose origin obtained from mixed trimers of model peptides comprising Ac-(Pro-Hyp-Gly)$_5$-Pro-Lys-Gly-(Pro-Hyp-Gly)$_5$-Ala-NH$_2$ (**4**) and Ac-(Pro-Hyp-Gly)$_{11}$-Ala-NH$_2$ (**5**), and quantified CML as the most abundant AGE. We also succeeded in confirming the presence of GOLD as a protein linker in glucose-derived AGEs.

2. Materials and methods

2.1. Glycation reactions

Collagen model PKG **4** (320 μM, 1.0 mg/mL) was incubated in 50 mM phosphate buffer (pH 7.5) in the presence of 3 mM NaN_3 with D-glucose (0.2 M) or $^{13}C_6$-D-glucose (0.2 M) at 37 °C for 3 months. The glycated **4** in these preparations was then passed through a PD-10 column to remove glucose. In the separate experiment, PKG **4** or POG **5** was dissolved in 50 mM phosphate buffer (pH 7.5) at concentration of 1.0 mg/mL. Subsequently, PKG and POG solutions were mixed, that is, PKG–POG = 3:0, 2:1 and 1:2. Then samples were incubated in the presence of 3 mM NaN_3 with D-glucose (0.2 M) at 37 °C for 9 months and later for 21 months. A small aliquot of the glycated sample was passed through a Sephadex G-10 gel filtration to remove glucose before hydrolysis.

2.2. Detection and quantification of CML and lysine in glycated collagen model by MS and HPLC

Mixtures of collagen models **4**/**5** (3:0), (2:1) and (1:2) were incubated with glucose for a long period. MALDI-TOF mass spectra were recorded on a AXIMA-CFR (Shimadzu) in a positive ion mode with insulin (*m*/*z* 5734) as an internal standard. Glycated peptides were dissolved in aqueous CH_3CN containing 0.1% trifluoroacetic acid and sinapic acid as a matrix and loaded on the sample plate for MALDI-TOF measurements. For determination of glycation products, an aliquot of each mixture was hydrolyzed with 6 M HCl at 110 °C, 24 h and subjected to ninhydrin-based amino acid analysis (Hitachi LaChrom Elite L-200).

2.3. Detection of GOLD in glycated collagen model by HPLC

GOLD **2** was identified in the hydrolysate by fluorescent LC after fluorescence-labeling with 6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate (AQC) (Accq·Fluor reagent kit) purchased from Waters (Bedford, MA, USA). The collagen models after incubation with glucose were hydrolyzed in 6 M HCl at 110 °C for 24 h. Hydrolysates were dissolved in sodium borate buffer (0.2 M, pH 8.8), vortexed several seconds and dissolved in 10.5 mM AQC acetonitrile solution.

The sample was heated at 55 °C for 10 min. The resulting solution was lyophilized for HPLC determinations. The HPLC conditions for separation of AQC derivatized hydrolysates are as follows; column: COSMOSIL 5C$_{18}$-ARII (4.6 × 150 mm), flow rate: 1.0 mL/min, eluent: A 10 mM ammonium acetate (pH 6.9), B methanol, linear gradient: B 5–50% (0–60 min) in A, column temperature: 40 °C, fluorescence detector: E_x 250 nm and E_m 395 nm. The retention times for CML and GOLD were 33–34 and 41–42 min, respectively. Then, the fraction containing AQC-labeled GOLD was subjected to another HPLC in the following conditions; column: COSMOSIL 5C$_{18}$-ARII (4.6 × 150 mm), flow rate: 1.0 mL/min, eluent: A 10 mM ammonium acetate (pH 6.9), B acetonitrile, linear gradient: B 5–20% (0–20 min), 20% (20–40 min) in A, column temperature: 40 °C, fluorescence detector: E_x 250 nm and E_m 395 nm.

2.4. LC–MS/MS analysis for GOLD

A Thermo Scientific TSQ Vantage tandem mass spectrometer equipped with an electrospray ionization (ESI) source was used for LC–MS/MS analysis. AQC-labeled GOLD fractions after first HPLC separations were loaded onto the instrument. HPLC separations were achieved at 40 °C using a Hypersil GOLD C$_{18}$ (50 × 2.0 mm, 5 μm). The mobile phase consisting of a mixture of solvent A (5 mM ammonium acetate) and solvent B (acetonitrile) was delivered at a flow-rate of 0.3 mL/min. The gradient started at 2% solvent B and changed linearly to 60% B in first 4.0 min, and maintained at 60% for 0.1 min. A subsequent re-equilibration time (2.9 min) was allowed before the next injection. The selected reaction monitoring chromatograms were recorded on a MS instrument of LTQ Orbitrap Velos (Thermo Scientific). Analysis was carried out using transitions of *m*/*z* 334.3 → 497.3 and 334.3 → 171.0. The collision induced dissociations for these transitions were carried out using collision energy at 12 eV and 21 eV, respectively.

3. Results and discussion

3.1. Detection of CML as abundant AGE in glycated collagen model by mass spectrometry

Following the previous report,[17] model peptide **5** comprising the repeated tripeptides (Pro-Hyp-Gly)$_n$ was designed to stabilize the triple helix characteristic of collagen;[18] under the experimental conditions, most of peptides **4** and **5** should take a triple helix structure.[17] The *N*- and *C*-termini of the peptide are blocked by *N*-acetyl and amide groups, respectively, to reduce ionic repulsion. For the glycation site, one lysine residue of model peptide **4** was introduced in the place of Hyp 17. These peptides were prepared by the combined use of liquid-phase and solid-phase chemical syntheses in an essentially similar manner to that in the previous report.[17] In the MALDI-TOF-MS spectra of glycated products of PKG **4** after 17 months incubation, an ion peak corresponding to CML-containing PKG was observed at *m*/*z* 3145 (Fig. 2). It is noteworthy that CML is generated as a dominant product as compared to **4** with an intact lysine residue at *m*/*z* 3087 by a long-term glycation reaction with glucose. In a separate experiment where $^{13}C_6$-glucose was used for glycation reactions, an ion peak corresponding to CML-containing **4** was shifted by two mass units, indicating the incorporation of C2 unit from glucose (see Supplementary data).

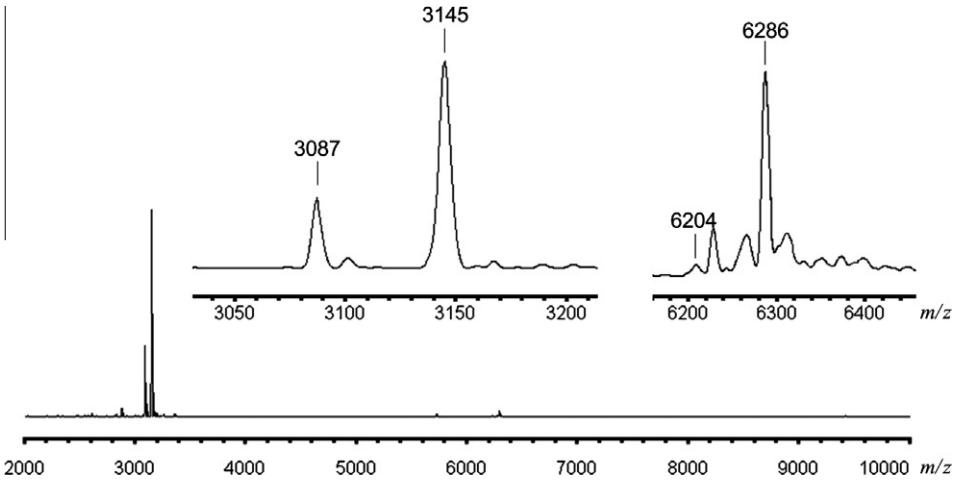

Figure 2. MALDI-TOF-MS spectra of PKG peptide **4** after 17 months of glucose treatment. An ion peak at m/z 3087 in the inset spectra corresponds to $[M+H]^+$ of intact (non-glycated) PKG **4** while that at m/z 3145 agrees with CML-containing **4**. A weak ion peak at m/z 6204 match M^+ of a dimer of **4** that is linked with GOLD **2**, and that at m/z 6286 is probably assignable to a 2 mol cluster of CML-containing **4** with no covalent linkage.[22] Mass values were calibrated with insulin taken as m/z 5734.

3.2. Time-dependent changes of CML and lysine contents in glycated collagen model

To investigate the influence of the average number of lysine residues per triple helix on glycation reactions, the two model peptides were mixed at three ratios (PKG–POG = 3:0, 2:1 and 1:2). Since mass spectrometry is not suitable for quantifying the CML-containing peptides and intact peptide **4** due to significant difference in ionization efficiency. Thus, we applied HPLC determination methods for the acid hydrolysates of the peptides. Figures 3 and 4 depict increase of CML, a representative AGE, and decrease of lysine, a sole glycation site in the peptide, over 7 months. It is worth noting that 40–60% of lysine of the model peptide was converted to CML in the presence of glucose. When the reduced lysine contents were taken into account, most of glycated lysine has been converted to CML in these experimental conditions. Regarding the effect of PKG ratios on AGE production in the peptide mixtures, formation of CML is significantly higher in PKG and PKG–POG 2:1 as compared with PKG–POG 1:2. These collagen model peptides are known to align both ends in the triple helix, where the lysine residues of each peptide occur at the same position in the trimeric helices. This neighboring effect of basic amino acid residues on glycation reactions is reported by Iberg et al.[19] to enhance formation AGEs. Most likely explanation for this effect is that the Amadori reaction after condensation between lysine ε-amine and glucose C1 is accelerated in weakly basic conditions.[23] On the other hand, after 21 months, CML contents of PKG–POG 3:0, 2:1 and 1:2 were 55.5%, 55.3% and 52.7%, respectively, on the basis of the initial lysine (see Supplementary data for details); the values are quite consistent regardless of the PKG–POG ratios. This result may be accountable by substitution of a carboxylate moiety to the ε-amino group of lysine in CML, which neutralized the basic environment. The CML contents of PKG–POG 3:0 grossly dropped after incubation for 17–21 months (Supplementary data); this may be accounted for by further conversion of CML to the other AGEs, which were not characterized in this study except for GOLD. Moreover, this conversion of CML seems to be slower than production of CML from lysine, and independent on PKG–POG ratios since the CML content was reduced for the 3:0 or 2:1 mixture after 17 months due to a less amount of intact lysine residues, whereas being significantly increased for the 1:2 mixture due to a significantly higher lysine content in earlier months. This notion may partly account for the consistent CML contents after 21 months.

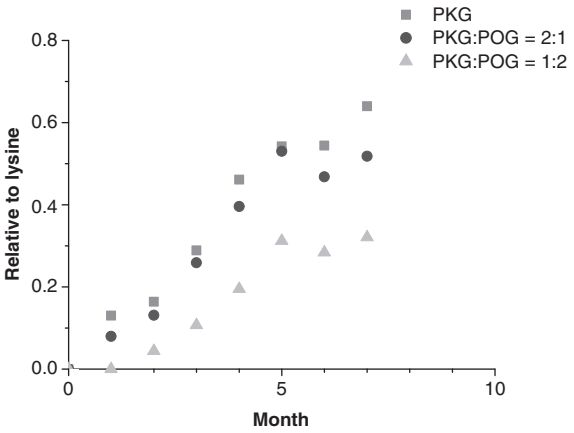

Figure 3. CML (**1**) contents in glycated PKG by glucose in mixed peptides.

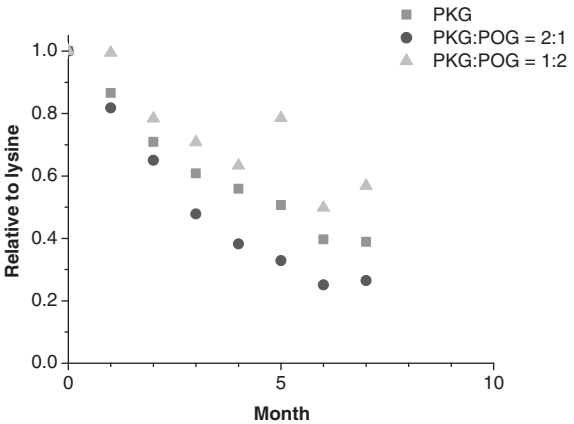

Figure 4. Lysine contents in glycated PKG–POG by glucose in mixed peptides.

As was the case with CML contents, decrease in lysine content was significantly greater in PKG–POG 2:1 than that of PKG–POG 1:2. On the other hand, reduction in lysine content of PKG–POG 3:0 was lower than that of 2:1. This contradictory observation may be explainable as follows: the effect of the three-lysine cluster in a PKG trimer on early-stage glycation is not significant or even inhibitory as compared with the two-lysine one in PKG–POC 2:1.

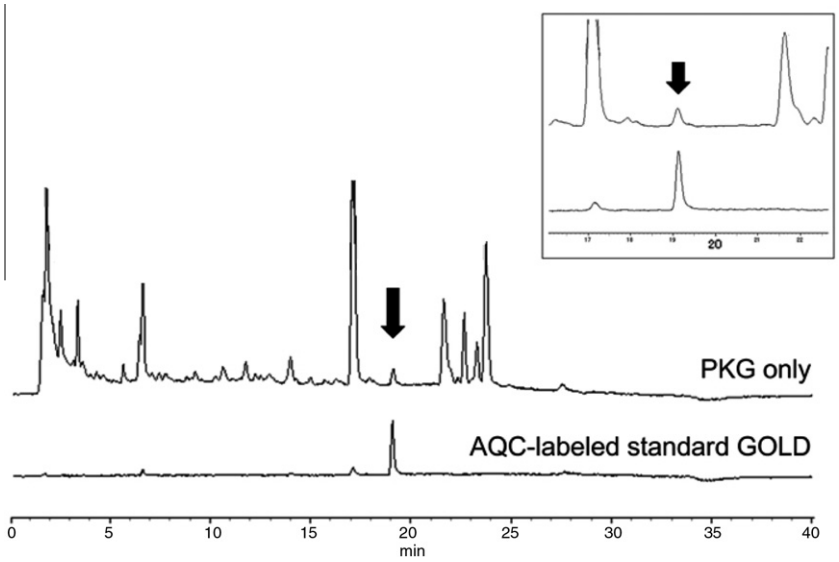

Figure 5. HPLC profiles of glycation products from collagen models and standard GOLD after AQC derivatization. An inset traces are expansion of regions at retention time of AQC-labeled standard GOLD **3**. Filled arrows indicate the retention time of **3**, which is confirmed by infusion ESI-S measurement. The peak of **3** from PKG corresponds to 1.6 pmol.

On the other hand, formation of CML from Amadori products is promoted in the PKG only trimer since oxidation reactions, which are necessary to generate CML, is generally known to be accelerated in basic conditions.

3.3. Detection of GOLD in glycated collagen model by glucose

A weak ion peak at *m*/*z* 6204 observed in the mass spectrum of the glycated PKG **4** (Fig. 2) suggested the formation of **4** dimer linked with GOLD **2** as we reported for glycation reaction with glyoxal,[17] which is a highly reactive dicarbonyl compound frequently used for producing potential AGEs. Thus, for confirming the presence of a trace amount of **2** in the hydrolysates of glucose-derived AGEs, amino acids obtained from peptide **4** (and **5**) were converted to fluorescent derivatives. We first established a simple HPLC method to detect **2**. Hydrolysates obtained from 21 months incubated model peptides were derivatized with 6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate (AQC) for fluorescent HPLC analysis. GOLD **2** in the hydrolysates was first purified by HPLC separation, and then a fraction containing **3** was rechromatographed on reversed-phase HPLC under a different mobile phase (Fig. 5). A peak detected in the hydrolysates from the glycated collagen model (PKG–POG 3:0) were identical with the standard **3** as depicted by filled arrows in Figure 5.

In the next step, LC–MS/MS analysis on a triple-quadrupole instrument (QqQ) in a selected reaction monitoring (SRM) mode was adopted for quantifying GOLD in the glycated peptides. Target ions were monitored at *m*/*z* 334.3 for $[M+2H]^{2+}$ of **3** and an ion chromatogram was recorded for the product ions at *m*/*z* 497.3, which was generated by a lose of one AQC group from **3**, in a positive mode. Linearity was established for the concentration range from 0.3 to 30 fmol with a correlation coefficient (*r*) of 0.9973. Figure 6 shows the SRM chromatograms of AQC–GOLD fractions obtained from the hydrolysates of PKG–POG 3:0 and 1:2 after incubation with glucose for 21 months. Hydrolysates from the glycated peptides, after fractionation with the first HPLC, gave rise to a peak showing the identical retention time with that of standard AQC-labeled GOLD. Contents of GOLD in the hydrolysates from PKG–POG 3:0 and 1:2 were around 0.18% and 0.19%, respectively, based on the initial content of a lysine residue. Thus, there was no significant difference by *dilution* of PKG with POG. This result

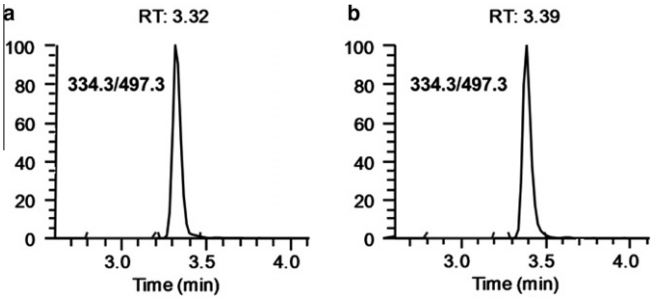

Figure 6. Selected reaction monitoring (SRM) chromatograms of AQC–GOLD fractions obtained from the hydrolysates of the glycated collagen models PKG–POG 3:0 (**a**) and 1:2 (**b**). Double charged target ions at *m*/*z* 334.3 were selected for fragmentation to monitor LC chromatogram at *m*/*z* 497.3.

may imply that crosslinking reactions to form a GOLD moiety occur not in an intra-triple helix manner but rather in an inter-triple helix way.

In summary, the present study has disclosed that long-term glycation with glucose leads to markedly high generation of CML. The dilution of PKG with POG attenuated the formation of CML, supporting the local pH effects on acceleration of AGE formation.[19] The LC–MS/MS analysis first demonstrated that GOLD, as one of important AGEs in terms of protein cross-linking, was formed in vitro under the long-term glycation not only with dicarbonyl compounds but with glucose. AGEs acting as a crosslinker of proteins have been attracting much attention since protein dimerization heavily deteriorates physiological functions and potentially causes AGE-related complications in human. Formation of dimers from small model peptides and glucose, as demonstrated by the present study, may open up a way to gain a better understanding of the molecular basis of generation mechanisms of human AGEs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.02.048.

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