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Identification of N^{ε}-(carboxyethyl)lysine, one of the methylglyoxal-derived AGE structures, in glucose-modified protein: mechanism for protein modification by reactive aldehydes

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Abstract

We have developed a separation system for N^{ε}-(carboxyethyl)lysine (CEL) and N^{ε}-(carboxymethyl)lysine (CML) by HPLC equipped with a styrene–divinylbenzene copolymer resin coupled with sulfonic group cation-exchange column and examined whether CEL is formed from proteins modified by glucose via the Maillard reaction. CEL was generated by incubating bovine serum albumin (BSA) with glucose, a reaction inhibited by aminoguanidine, but enhanced by phosphate. Although several aldehydes were detected during incubation of N^{α}-acetyllysine with glucose, incubation of BSA with methylglyoxal alone generated CEL. These results indicate that methylglyoxal is responsible for CEL formation on protein in vitro.

Keywords: Maillard reaction; N^{ε}-(Carboxyethyl)lysine; N^{ε}-(Carboxymethyl)lysine; Proteins; Aldehydes

1. Introduction

Long-term incubation of proteins with glucose leads to the formation of advanced glycation end products (AGE) which are derived from reactive intermediates of the Maillard reaction through Schiff base and Amadori product. AGEs are characterized by fluorescence, a brown color and intra- or intermolecular cross-linking. Immunohistochemical studies using anti-AGE antibodies have demonstrated the presence of AGE-modified proteins in several human tissues under pathological conditions including kidneys of patients with diabetic nephropathy [1] and chronic renal failure [2], atherosclerotic lesions of arterial walls [3], amyloid fibrils in hemodialysis-related amyloidosis [4,5], and actinic elastosis of the photoaged skin [6]. These findings suggest the potential involvement of AGE-modification in the pathogenesis of age-related diseases.

Mclellan et al. [7] demonstrated that the concentration of plasma methylglyoxal (MG), which is believed to be generated from the Embden–Meyerhof and polyol pathways, in insulin-dependent diabetic patients were about seven times higher than in non-diabetic individuals. Modification of protein

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with MG is known to generate MG-derived AGE-structures such as CEL [8,9], argpyrimidine [10], imidazolone [11] and imidazolium salt cross-links like MG-lysine dimer (MOLD) [12]. These MG-derived AGE-structures have been demonstrated in vivo. For example, CEL was identified in human lens protein at a concentration similar to that of N^{ε}-(carboxymethyl)lysine (CML), and its accumulation increased with age [9]. Shamsi et al. [13] recently prepared a polyclonal antibody against MG-modified RNase A and identified its epitope as argpyrimidine. This antibody significantly reacted with the collagen prepared from human cornea [13]. Furthermore, 5-methylimidazolone was detected in granular cytoplasmic elements of foam cells in human atherosclerotic lesions [14]. Nagaraj et al. [15] quantified MOLD in human plasma proteins by reversed-phase high-performance liquid chromatography (RP-HPLC) and showed that cross-linking of plasma proteins by MOLD was increased in diabetes.

Chemistry between protein and glucose forms a number of AGE-structures through complex reactions in which reactive intermediates are formed by enolization, dehydration, cyclization, fragmentation and oxidation reactions. Recently MG has been shown to be generated not only via the Embden–Meyerhof and polyol pathway, but also from the Maillard reaction [16]. Furthermore, 10.7 μ*M* MG was detected when 50 m*M* glucose was incubated for 3 weeks with 50 m*M* N^{α}-*tert.*-butyloxycarbonyl-L-lysine and it was enhanced by an increase in phosphate concentration [16]. These results indicate that MG is generated not only via the Embden–Meyerhof and polyol pathways but also via the Maillard reaction. However, it remains unclear whether CEL is formed during the incubation of protein with glucose.

Previous determination of CEL in glucose-modified bovine serum albumin (AGE-BSA) by our HPLC system was unsuccessful due to co-elution of CEL with CML. This co-elution is because of the similar chemical structure of CEL and CML, which makes separation of these compounds difficult [17]. In the present study, we have developed a new HPLC system in which CEL and CML are measured simultaneously in a single HPLC run. This system was used to quantify CEL in aldehyde-modified protein in order to elucidate the formation pathway of CEL. These results are the first to show the role of MG, which was generated during incubation of protein with glucose, in the formation of CEL on protein.

2. Experimental

2.1. Chemicals

β-D-Glucose and MG were purchased from Sigma (St. Louis, MO, USA). Upon analysis using the Hantzsch reaction, the amount of formaldehyde in the MG solution was less than detectable levels. Fatty acid-free bovine serum albumin (BSA), aminoguanidinium chloride, 40% glyoxal and sodium pyruvate were purchased from Wako (Osaka, Japan). All other chemicals were of the best grade available from commercial sources.

2.2. Modification of BSA with MG, glucosone, 3-deoxyglucosone and glucose

MG-modified BSA (MG-BSA) was prepared by incubating 2 mg/ml of BSA (1.5 m*M* lysine residues) with 33 m*M* MG at 37 °C for 7 days. Glucosone [18] and 3-deoxyglucosone (3-DG) [19] were prepared according to previously published methods. Glucosone and 3-DG-modified BSA were prepared by incubating 200 m*M* glucosone or 3-DG with 2 mg/ml BSA. Glucose-modified AGE-BSA (AGE-BSA) was prepared by incubating 50 mg/ml BSA with 1 *M* glucose at 37 °C for up to 40 weeks as described previously [20]. These samples were incubated with different concentrations of sodium phosphate buffer (pH 7.4) as were previously published [16,21,22]. Aliquots of samples were taken from each reaction mixture and dialyzed against phosphate-buffered saline (PBS) (pH 7.4).

2.3. Preparation of CEL and CML

CEL was purified from hippuryl-CEL using a modified method of Ahmed et al. [8]. Briefly, hippuryl-CEL was prepared by overnight incubation of 40 mg/ml hippuryllysine (benzoylglycyllysine, Peptide Institute, Osaka, Japan) with 0.13 *M* pyruvate in the presence of 0.45 *M* $NaCNBH_3$ in distilled

water at room temperature, followed by acid hydrolysis with 6 *M* HCl for 24 h at 110 °C. The extent of CEL-modification of hippuryllysine was determined to be 100% by HPLC. The pH of the hydrolyzed hippuryl-CEL was adjusted to 2 using 0.005 *M* HCl and was loaded on a Dowex-50-H^+ cation-exchange column (5 ml). The column was washed with five column volumes of 0.05% pyridine, then eluted with five column volumes of 10% pyridine. The eluate was lyophilized and its structure was confirmed by fast atom bombardment (FAB) spectroscopy. The FAB (negative)–MS spectrum for CEL showed an $(M–H)^-$ ion at $m/z=217$. CML was prepared by overnight incubation of 0.26 *M* N^{α}-acetyllysine with 0.13 *M* glyoxylic acid in the presence of 0.65 *M* $NaCNBH_3$ in 0.5 ml of 0.1 *M* sodium carbonate buffer (pH 10.0) at room temperature as described previously [23].

2.4. Determination of CEL and CML by HPLC

CEL-contents of modified BSA preparations were quantified by amino acid analysis after acid hydrolysis with 6 *M* HCl for 24 h at 110 °C in an amino acid analyzer (L-8500A, Hitachi, Tokyo, Japan) equipped with a styrene–divinylbenzene copolymer resin coupled with sulfonic group cation ($–SO_3H$) exchange column (#2622 SC, 4.6×80 mm, Hitachi) followed by a ninhydrin post-column derivatization and detection by fluorescence (ex. 271 nm/em. 503 nm). High resolution analysis of the separation of CML and CEL was conducted using four different buffers as listed in Table 1, and eluted at 100% buffer 1 from 0 to 9.0 min, 100% buffer 2 from 9.1 to 28.0 min, linear gradient from 100% buffer 2 to 100% buffer 3 (28.1 to 60.9 min), 100% buffer 3 from 61.0 to 74.0 min and buffer 4 from 74.1 to 93 min. High-resolution analysis was achieved by the modification of buffer condition, column temperature, flow-rate and time program based on our previously published HPLC method [17]. Hydrolyzed BSA samples (10 μg each) were injected on the HPLC system and CEL content was normalized to the amount of CEL/BSA molecule (mol of CEL/mol of BSA) based on the value of alanine content because alanine is highly stable against acid hydrolysis and its side chain does not react with aldehydes. Since BSA contains 46 alanines, the CEL content on BSA was normalized by the following Eq. (1)

$$\text{detected CEL content (nmol)} \times 46/\text{detected alanine content (nmol)} \quad (1)$$

Furthermore, the detection limit of CEL on BSA (mol of CEL/mol of BSA) was calculated by Eq. (2)

$$\text{detection limit of CEL (nmol)} \times 46/\text{detected alanine content (nmol)} \quad (2)$$

2.5. Detection of α-oxoaldehydes by 2,3-diaminonaphthalene

Generation of aldehydes during incubation of lysine with glucose was determined by 2,3-diaminonaphthalene (DAN) as described previously [24]. Briefly, 50 m*M* N^{α}-acetyllysine with 50 m*M* glucose were incubated in PBS in the presence of

Table 1
Chemical composition of elution buffers used in CEL and CML analysis

Materials	Buffer 1	Buffer 2	Buffer 3	Buffer 4
Sodium citrate (m*M*)	10.5	26.3	90.7	–
Sodium hydroxide (m*M*)	–	–	–	200
Sodium chloride (m*M*)	48.5	121.1	930.6	–
Citric acid	51.5	114.5	31.8	–
Ethyl alcohol (ml)	130.0	20.0	–	100.0
Benzyl alcohol (ml)	–	–	5.0	–
Thiodiglycol (ml)	5.0	5.0	–	–
BRIJ-35 (ml)	4.0	4.0	4.0	4.0
Caprylic acid (ml)	0.1	0.1	0.1	0.1
pH	3.3	3.2	4.9	–
Total sodium conc. (*M*)	0.08	0.2	1.2	0.2

DAN (10 m*M*) at 37 °C for 7 days. The generation of α-oxoaldehydes such as glucosone, 3-DG, glyoxal and MG were measured by DAN as α-oxoaldehyde–DAN adducts using a HPLC system (L-7100, Hitachi) with a reversed-phase column (Mightysil, RP-18 GP 150–3.0, 3 μm; Cica-Reagent). Effluents were monitored by the fluorescence at ex. 271 nm/em. 503 nm. All analyses were performed at 40 °C at an elution rate of 0.4 ml/min using an elution buffer composed of 70% phosphoric acid (50 m*M*), 15% methanol and 15% acetonitrile. The authentic MG–DAN adducts were prepared by incubating MG with DAN.

3. Results

3.1. Detection of CEL and CML by HPLC

To demonstrate the detection limit of CEL, 5–50 pmol of CEL was measured by HPLC. As shown in Fig. 1A, different concentrations of CEL were eluted at the same retention time of 50.2 min. Furthermore, CEL was measured linearly up to 2 nmol (Fig. 1B), indicating that 5 pmol–2 nmol of CEL can be determined quantitatively by our HPLC system. CEL (5–500 pmol) was also analyzed in the presence of an amino acid standard composed of 19 of the 20 amino acids excluding cysteine (500 pmol each). As shown in Fig. 2, CEL was detected linearly between 5 and 500 pmol in the presence of the amino acid standard mixture, indicating that neighboring peaks did not affect the determination of CEL. Since 7–10 nmol of alanine (eluted retention time at 35.1 min) was detected in each hydrolyzed BSA sample, the detection limit of CEL on BSA was 0.03 mol of CEL/mol of BSA.

CEL that was obtained after hydrolysis of hippuryl-CEL eluted at a retention time of 50.2 min (Fig. 3A) whereas CML eluted at 50.7 min (Fig. 3B). Elution of a mixture of CEL, CML and standard amino acids (0.5 nmol each) resulted in a clear separation of CEL from CML (Fig. 3C). Although the retention time of a mixed solution of CEL and

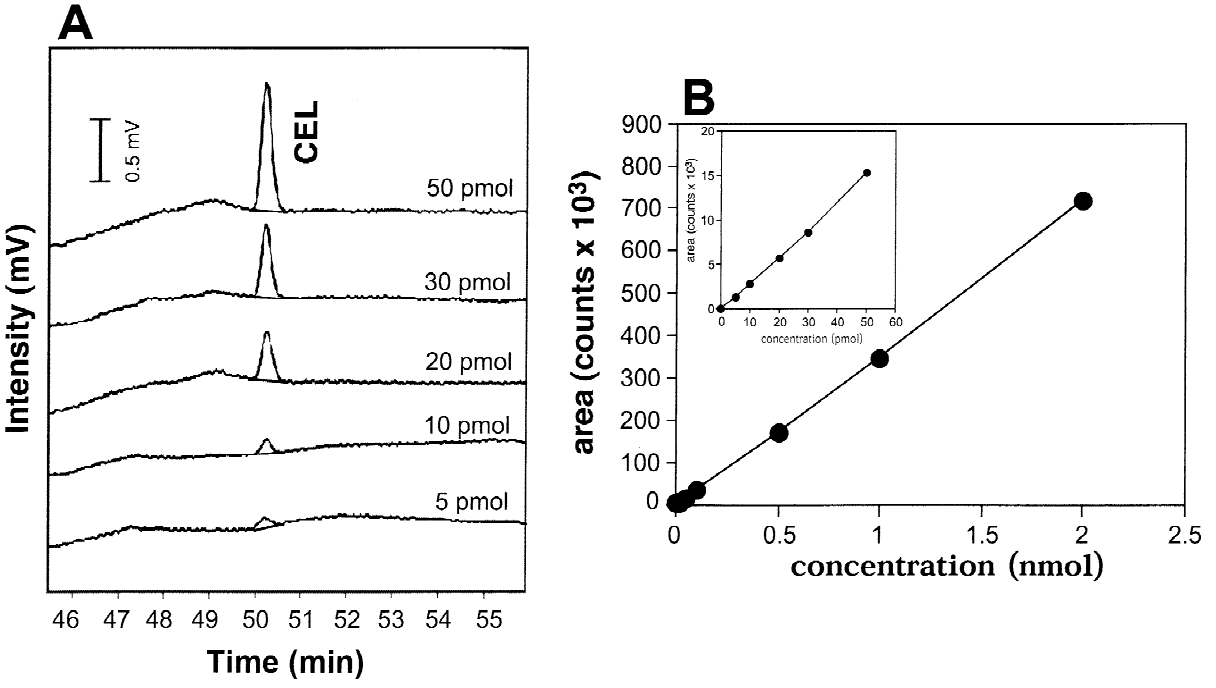

Fig. 1. Determination of the detection limit of CEL by HPLC. (A) CEL (5–50 pmol) was injected on an amino acid analyzer equipped with a cation-exchange HPLC column followed by elution with gradient buffers and detection by a ninhydrin post-column system. (B) CEL (5 pmol–2 nmol) was injected on the same HPLC system for standard curve determination ($R^2 = 0.999$).

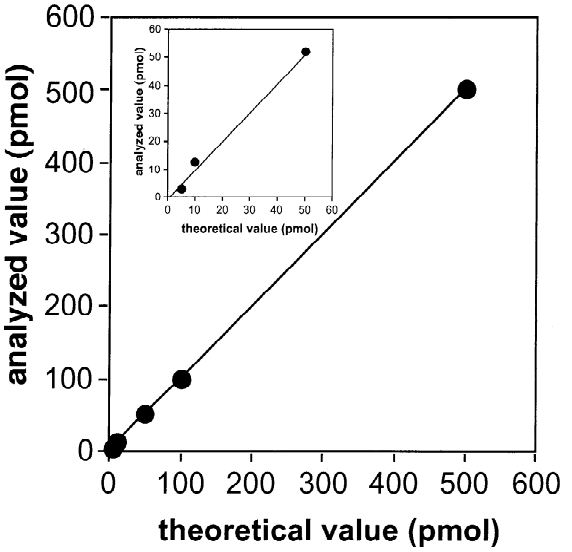

Fig. 2. Accuracy of quantification of CEL by HPLC. CEL (5–500 pmol) was analyzed in the presence of 500 pmol methionine, the nearest peak from CEL, and a theoretical and analyzed value (based on peak area) of CEL was compared. The theoretical value of CEL positively correlated with the analyzed value between 5 and 500 pmol CEL ($R^2 = 0.999$).

CML was the same with Fig. 3C, any injection above 30 nmol had a diminished resolution between CML and CEL. According to this system, the amount of CEL in AGE-BSA (obtained by incubation at 37 °C for 62 days) was determined to be 0.35 mol of CEL/mol of BSA (Fig. 3D). In time-course studies where 50 mg/ml BSA were incubated with 1 *M* glucose in 500 m*M* sodium phosphate buffer at 37 °C, the amount of CEL became detectable on day 20, and increased progressively up to day 62 (Fig. 4).

3.2. Detection by DAN of α-oxoaldehydes formed in the Maillard reaction

To determine the contribution of α-oxoaldehydes on CEL formation during the Maillard reaction, we incubated N^{α}-acetyllysine with glucose (50 m*M* each) at 37 °C for 7 days in the presence of DAN, a dicarbonyl trapping reagent, followed by determination of α-oxoaldehyde content by HPLC. As shown in Fig. 5A, four separated peaks emerged at 9.5, 13.1, 37.2 and 52.4 min in the reaction of

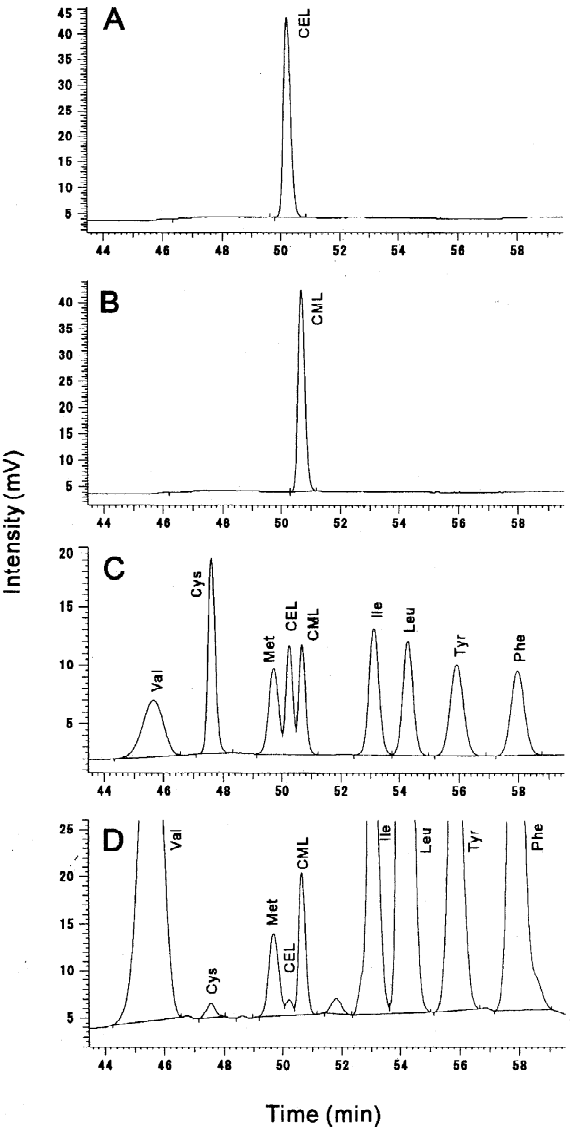

Fig. 3. Detection of CEL and CML in modified proteins by HPLC analysis. Two nanomoles of hydrolyzed-hippuryl CEL (A) and CML (B) were injected on the amino acid analysis system. CEL eluted at 50.2 min and CML at 50.7 min. CML and hydrolyzed-hippuryl CEL were mixed with a mixture of standard amino acids (0.5 nmol each), followed by HPLC analysis under the identical system (C). This system provided the resolution of 78.9% for Met-CEL and 75.2% for CEL-CML. AGE-BSA was hydrolyzed with 6 *M* HCl for 24 h at 110 °C as described in Section 2, followed by HPLC analysis (D).

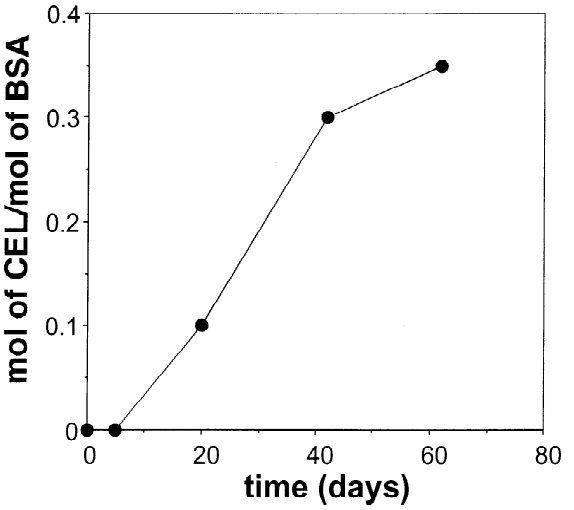

Fig. 4. Time course study of CEL formation in the Maillard reaction. BSA (50 mg/ml) was incubated at 37 °C for 62 days with 1 *M* glucose in 500 m*M* sodium phosphate buffer. Aliquots were taken from the reaction mixture and dialyzed against PBS. CEL-contents were determined by the HPLC system described in Fig. 1. CEL-contents in modified BSA are expressed as mol of CEL/mol of BSA.

N^{α}-acetyllysine and glucose for 7 days, whereas these aldehydes were undetectable before incubation (data not shown). These peaks were identified by authentic samples at 9.5 min with glucosone, at 13.1 min with 3-deoxyglucosone, at 37.2 min with glyoxal and at 52.4 min with MG. The time course study demonstrated that the generation of each aldehyde increased in a time-dependent manner (data not shown). Although 3.1 μ*M* MG was detected in the day-7 sample (Fig. 5A), no MG was detected after incubation in the presence of aminoguanidine (90 m*M*) under the identical conditions (Fig. 5B).

3.3. Formation of CEL by incubation of BSA with α-oxoaldehydes

Since reactive α-oxoaldehydes such as glucosone, 3-DG, MG and glyoxal are generated by the incubation of N^{α}-acetyllysine with glucose, we needed to determine which α-oxoaldehydes play an important role in CEL formation. As shown in Table 2, when BSA (2 mg/ml) was incubated with 33 m*M* MG in 50 m*M* sodium phosphate buffer, the CEL-content was 1.15 mol CEL/mol of BSA. Although BSA

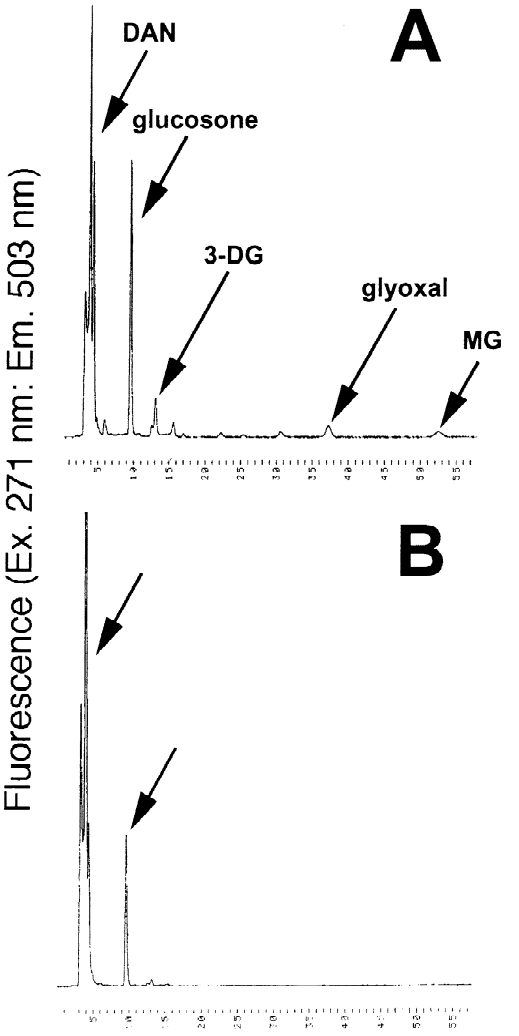

Fig. 5. Detection by 2,3-diaminonaphthalene of α-oxoaldehydes formed in the Maillard reaction. (A) Fifty m*M* N^{α}-acetyllysine and 50 m*M* glucose were incubated at 37 °C for 7 days in the presence of 10 m*M* DAN followed by identification of α-oxoaldehydes–DAN adducts by RP-HPLC. The sample was injected on to the column, eluted in 70% phosphoric acid, 15% acetonitrile, 15% methanol at a flow-rate of 0.4 ml/min (40 °C) and monitored at ex. 271 nm/em. 503 nm. The α-oxoaldehyde concentration was 79.6 μ*M* for glucosone, 5.3 μ*M* for 3-DG, 9.3 μ*M* for glyoxal and 3.1 μ*M* for MG. (B) N^{α}-Acetyllysine and glucose were incubated under identical conditions with the exception of 90 m*M* aminoguanidine.

incubations with 33 m*M* glyoxal generated 14.4 mol of CML/mol of BSA, CEL was not detected (Table 2). Furthermore, glucosone and 3-DG were synthesized and upon incubation of BSA with 200 m*M*

Table 2
CEL levels in modified BSA incubations containing α-oxoaldehydes

Sample	Incubation period (week)	Na-PB (m*M*)	CEL/BSA
MG-BSA	1	50	1.15
Glyoxal-BSA	1	50	ND
3DG-BSA	4	500	ND
Glucosone-BSA	4	500	ND

BSA was incubated with 33 m*M* MG, 33 m*M* glyoxal, 200 m*M* glucosone and 200 m*M* 3-DG as described in Section 2. CEL-contents of modified BSA preparations were quantitated by the HPLC method after acid hydrolysis with 6 *M* HCl for 24 h at 110 °C. MG-BSA, MG-modified BSA; glyoxal-BSA, glyoxal-modified BSA; glucosone-BSA, glucosone-modified BSA; 3-DG-BSA, 3-DG-modified BSA. CEL-contents were expressed as ''mol of CEL/mol of BSA (CEL/BSA)''.

of these α-aldehydes, the CEL content was under the detection limit (Table 2) (<0.03 mol of CEL/mol of BSA). Since 3-DG shows preferential reactivity for lysine residues and might block all the lysine residues on the protein, a parallel experiment was performed with a lower concentration of 3-DG (50 m*M*). However, the resultant CEL-content also remained undetectable (data not shown). These results demonstrated that only incubation of BSA with MG give rise to CEL formation, and not incubation with glyoxal, 3-DG and glucosone.

3.4. Effects of phosphate and aminoguanidine on CEL formation

Since Thornalley et al. demonstrated that MG formation by the Maillard reaction was enhanced in a dose-dependent manner in the presence of phosphate [16], we decided to examine the effects of phosphate concentration on CEL formation. As shown in Table 3, BSA (50 mg/ml) incubated for 40 weeks with 1 *M* glucose in 500 m*M* sodium phosphate buffer resulted in a CEL-content of 0.60 mol of CEL/mol of BSA. In contrast, when BSA was incubated under identical conditions, except for a 10-fold lower sodium phosphate concentration (50 m*M*), the CEL-content revealed undetectable amounts (<0.03 mol of CEL/mol of BSA) (Table 3). These results suggested that phosphate has an enhancing effect on CEL formation from BSA modified with glucose. Furthermore, we also examined the effect of aminoguanidine on CEL formation during incubation of BSA with glucose. Aminoguanidine was used as a trapping reagent for α-oxoaldehydes such as glyoxal and MG [25], which are thought to react with aminoguanidine to form aminotriazine derivatives [26]. Incubation of BSA for 62 days with 1 *M* glucose in 500 m*M* sodium phosphate buffer resulted in the production of 0.35 mol of CEL/mol of BSA (Table 3). Parallel incubations with 90 m*M* aminoguanidine resulted in a markedly reduced level (<0.03 mol of CEL/mol of BSA) (Table 3).

4. Discussion

Recent studies have indicated that MG, which is believed to be generated in the Embden–Meyerhof and polyol pathway, reacts with proteins to form CEL in vivo. In the present study, we developed a separation system for CEL and CML by HPLC. This system was used for the measurement of CEL

Table 3
Effects of phosphate and aminoguanidine on CEL formation

Sample	Incubation period (week)	Na-PB (m*M*)	Aminoguanidine (90 m*M*)	CEL/BSA
AGE-BSA	40	50	−	ND
AGE-BSA	40	500	−	0.60
AGE-BSA	9	500	−	0.35
AGE-BSA	9	500	+	ND

AGE-BSA was prepared by incubation of BSA with 1 *M* glucose in 50 or 500 m*M* phosphate buffer as described in Section 2. CEL-contents in AGE-BSA were quantitated by the HPLC method and expressed as ''mol of CEL/mol of BSA (CEL/BSA)''

content in AGE-BSA in order to elucidate the formation pathway of CEL. Our results made it clear that CEL was generated during incubation of BSA not only with MG but also with glucose. Furthermore, our results demonstrated that MG was generated by glucose-mediated AGE formation and acts as a reactive aldehyde to enhance further CEL formation because (1) formation of CEL was enhanced in accordance with phosphate (Table 3), (2) aminoguanidine inhibited CEL formation (Table 3), (3) α-oxoaldehydes such as MG, glyoxal, glucosone and 3-DG were detected during the incubation of lysine with glucose and were trapped by aminoguanidine (Fig. 5), and (4) CEL was detected from the incubation of BSA with MG but not from glyoxal, 3-DG nor glucosone (Table 2).

Ahmed et al. [8] demonstrated that CEL was generated by the incubation of N^{α}-acetyllysine or collagen with glucose. However, the pathway for CEL formation in the Maillard reaction has not yet been demonstrated. There are two possible pathways for CEL formation in the Maillard reaction. Firstly, CEL is generated directly by cleavage of Schiff base (the so-called Namiki pathway) or Amadori product which is the same as oxidative CML formation [27]. Secondly, MG generated by cleavage of Schiff base reacts with proteins to form CEL. In the present study, glucosone, 3-DG, glyoxal and MG were detected during incubation of 50 m*M* N^{α}-acetyllysine with 50 m*M* glucose. Therefore, we further determined the possibility of glucosone and 3-DG contribution to CEL formation. However, formation of CEL in glucosone and 3-DG-modified BSA was not detected (Table 2). Furthermore, we demonstrated a time-dependent CEL -formation during the Maillard reaction, which was inhibited by aminoguanidine but enhanced by increasing concentration of phosphate, an enhancer for MG production during the Maillard reaction. These results strongly demonstrated that MG generated from the Maillard reaction plays an important role in CEL formation (Fig. 6).

Although accumulation of CEL was demonstrated to occur in human lens proteins [8,9], human and rat skin [9], rat aorta [9], human plasma [9] and articular cartilage [28], the pathway for CEL formation in vivo has been poorly understood. Three pathways have been proposed for CEL formation in vivo. The first is the highly site-specific carboxyethylation of Lys-238 in $NADP^{+}$-dependent prostaglandin dehydrogenase, isolated from human placenta, by reduction of Schiff base adduct of pyruvate, which is associated with enzymatic activity [29]. The second is the protein modification by MG [8] and the third is the production of reactive intermediates during lipid peroxidation such as arachidonate [30] that may be further oxidized after adduction to lysine. The formation of MG by degradation of glyceraldehyde-3-phosphate (G3P) [31,32], which was enzymatically derived from the Embden–Meyerhof and polyol pathways, is known to play an important role in the formation of MG-derived AGE structure. Phillips et al. [31] showed that 90 μ*M* MG was generated when 100 μ*M* G3P was incubated with red blood cells at 37 °C for 2 h. Although the exact formation ratio of MG from G3P in vivo hasn't been demonstrated, G3P has been thought to be an important precursor for MG under physiological conditions because of the high production of G3P by red blood cells. Regarding lipid peroxidation, 0.3 mmol of CEL/mol of Lys was generated by incubating RNase (13.7 mg/ml) with 100 m*M* arachidonate at 37 °C for 6 days [30], suggesting that lipid peroxidation might contribute to CEL formation in vivo [30]. The present study provided the first evidence that MG, which is generated during incubation of protein with glucose, is directly involved in protein modification by CEL. However, further studies are required to elucidate the formation pathways of CEL in vivo.

5. Nomenclature

AGE(s)	advanced glycation end products
BSA	bovine serum albumin
AGE-BSA	glucose-modified AGE-bovine serum albumin
MG-BSA	methylglyoxal-modified BSA
3-DG	3-deoxyglucosone
3DG-BSA	3-deoxyglucosone-modified BSA
CML	N^{ε}-(carboxymethyl)lysine
CEL	N^{ε}-(carboxyethyl)lysine
PBS	phosphate-buffered saline
RP-HPLC	reversed-phase high-performance liquid chromatography
MOLD	imidazolium salt cross-links like MG-lysine dimer

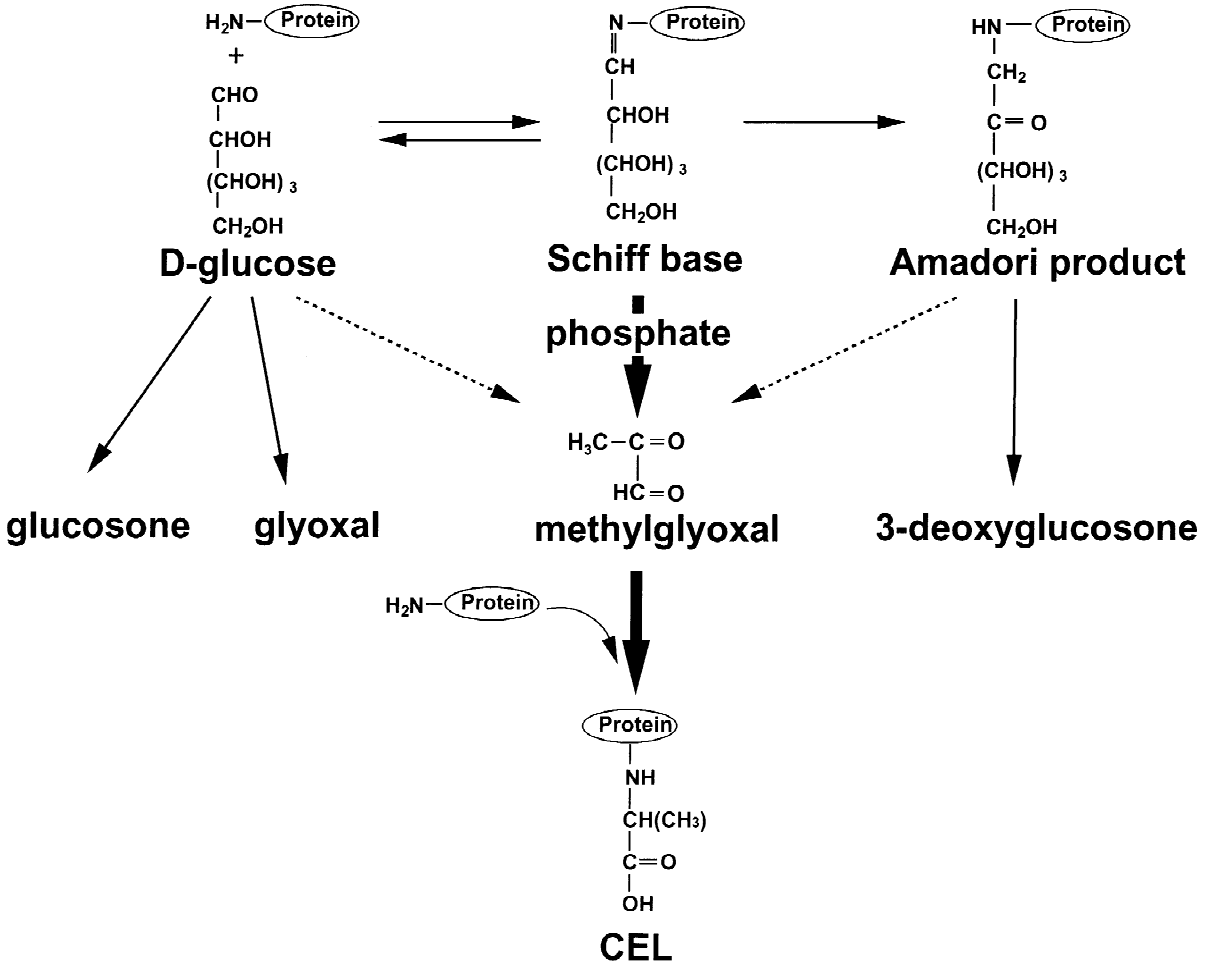

Fig. 6. Possible mechanisms for CEL -formation in the Maillard reaction.

DAN 2,3-diaminonaphthalene
G3P glyceraldehyde-3-phosphate

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