Protein Cross-linking by the Maillard Reaction

ISOLATION, CHARACTERIZATION, AND IN VIVO DETECTION OF A LYSINE-LYSINE CROSS-LINK DERIVED FROM METHYLGLYOXAL*

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The Maillard reaction, initiated by nonenzymatic glycosylation of amino groups on proteins by reducing sugars, has been studied for its potential role in aging and the complications of diabetes. One of the major consequences of the advanced Maillard reaction in proteins is the formation of covalently cross-linked aggregates. The chemical nature of the cross-linking structures is largely unknown. Recently, methylglyoxal has been shown to be a potential glycating agent in vivo and suggested to be a common intermediate in the Maillard reaction involving glucose. Methylglyoxal can form enzymatically or nonenzymatically from glycolytic intermediates and by retro-aldol cleavage of sugars. Its elevation in tissues in diabetes and its high potency to glycate and cross-link proteins led us to investigate the chemical nature of its advanced Maillard products. Using an approach in which a synthetic model peptide was reacted with methylglyoxal, we isolated and purified a cross-linked peptide dimer. Characterization of this dimer revealed that the peptides are linked through ε-amino groups of lysine residues. The actual cross-link was shown to be a methylimidazolium, formed from the reaction of two lysines and two methylglyoxal molecules. We have named this cross-link imidazolysine. Imidazolysine was detected in proteins by high performance liquid chromatography using a postcolumn derivatization method. Proteins incubated with methylglyoxal showed a time-dependent formation of imidazolysine. Quantification of imidazolysine in human serum proteins revealed a significant increase (p < 0.05) in diabetic samples (mean ± S.D., 313.8 ± 52.7 pmol/mg protein) when compared with normal samples (261.3 ± 50.4). These values correlated with glycohemoglobin (p < 0.05). These results provide chemical evidence for protein cross-linking by dicarbonyl compounds in vivo.

The Maillard reaction, initiated by the nonenzymatic reaction of reducing sugars with proteins, is proposed to play a significant role in protein aging and the complications of aging and diabetes. The reaction is initiated with the reversible formation of a Schiff base between a reducing sugar and the amino group of a protein. The relatively unstable Schiff base undergoes rearrangement to form a more stable Amadori product. The Amadori product, over a period of several months to years, undergoes a series of further reactions through dicarbonyl intermediates to form advanced glycation end products (AGEs) that are also known as advanced Maillard products. The AGEs are a heterogeneous group of structures formed as both cross-linking and noncross-linking adducts on proteins. The long-lived proteins in the body like lens crystallins and collagen accumulate AGEs because of their negligible or slow turnover. Immunochemical and chemical methods have clearly shown the progressive accumulation of AGEs in tissues in vivo in aging (1–3). In diabetes, AGE accumulation in general is accelerated and linked to atherosclerosis, nephropathy, neuropathy, retinopathy, and cataract (4–8). The binding of AGEs to specific receptors on macrophages has been shown to result in the synthesis of cytokines and growth factors and enhanced oxidative stress (9–12). These events are proposed to play a major role in age- and diabetes-associated vascular complications.

Considerable interest has focused recently on the structural characterization of AGEs. Attempts are being made to isolate major products by using specific antibodies and by chromatographic methods. A consistent difficulty in these procedures has been the isolation of individual AGEs in a reaction where numerous products are formed with highly cross-linked structures. Although protein cross-linking is a major end result of the Maillard reaction, only two minor protein cross-linking compounds, pentosidine and fluorophore LM-1, have been detected in tissues (13–15). Therefore, to better understand the impact of the Maillard reaction in aging and diabetes and to develop effective therapeutic methods to prevent AGE accumulation in tissues, it is necessary to elucidate the chemical nature of the major protein cross-links derived from this reaction.

In addition to glucose, a number of other sugars and sugar metabolites are known to initiate the Maillard reaction. Among them methylglyoxal (MG) has attracted considerable attention recently. MG is formed by enzymatic and nonenzymatic routes from glycolytic intermediates as well as from autoxidation of sugars (16, 17). It has been shown that in diabetes the concentration of MG increases in the lens, blood, and kidney (18–20). In Type I diabetic patients the blood MG levels increased 5–6-fold and in Type II 2–3-fold compared with normal control.

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¶The abbreviations used are: AGEs, advanced glycation end products; MG, methylglyoxal; HSA, human serum albumin; HPLC, high performance liquid chromatography; t-BOC, butoxycarbonyl; TFA, trifluoroacetic acid; HFBA, heptfluorobutyric acid; GK-peptide, N′-acetyl-glycyl-lysine-methyl ester; OPA, O-phthalaldehyde; LC/MS, liquid chromatography/mass spectrometry; FAB, fast atom bombardment.
subjects (18, 19). In vitro experiments have shown that MG undergoes a rapid Maillard reaction with proteins to form covalently cross-linked aggregates (21). Such reactions have been shown to occur even at physiological concentrations of MG (22) and form fluorescent products, characteristics of which resemble those occurring in proteins in aging and diabetes (23).

The high reactivity of MG with proteins and its relatively high concentration (0.1 mM) in the plasma (24) suggest that it is one of the major glycation agents present in vivo. A specific receptor on macrophages has been identified for proteins that are modified by the Maillard reaction with MG (25). Papoulis et al. (26) have suggested that MG is a common intermediate in the Maillard reaction by glucose and its derivatives.

This study was undertaken to understand the chemical nature of cross-linking structures derived from the advanced Maillard reaction initiated by MG. Using a model synthetic peptide in our reaction, we have isolated a lysine-lysine cross-link and developed an assay for its detection and quantification in proteins. We report the isolation, characterization, and detection in human serum proteins of this novel lysine-lysine cross-link.

**EXPERIMENTAL PROCEDURES**

Materials—Methylglyoxal (40% aqueous solution), human serum albumin (HSA, essentially fatty acid free), hexafluorobutyric acid (HFBA), trifluoroacetic acid (TFA) were obtained from Sigma. Bio-Gel P-2 was from Bio-Rad. O-Phthalaldehyde (OPA) was from Aldrich. N-\text{t-BOC}-lysine and N-\text{t-BOC-arginine} and N-\text{\alpha-acyetyl-glycol-lysine} methyl ester were from Bachem, CA. N-\text{Carboxymethyllysine} was a kind gift from Dr. J. John Baynes, University of South Carolina, Columbia, SC. All other chemicals used were of highest quality commercially available. Bovine lenses were obtained from a local slaughterhouse. Human blood was collected from the University Hospitals of Cleveland, Cleveland, OH, and from volunteers at this University. Glycosylated hemoglobin was determined by boronate affinity chromatography with the Glycogel B column system (Pierce).

Reaction of \text{N-\text{t-Gly-Gly-Lysine Methyl Ester}} (\text{GK-Peptide})—To isolate cross-links derived from the advanced Maillard reaction initiated by methylglyoxal, the reaction was carried out with 1 \text{ g of \text{GK-peptide}, 500 mg of \text{N-\text{t-BOC arginine}, and 100 mM methylglyoxal (78 \mu M) in 4.0 ml of 0.2 M sodium phosphate buffer, pH 7.4, at 37 °C in the dark}. The pH of the reaction mixture was adjusted to 7.4 using 5 N NaOH. The reaction mixture was incubated for 24 h. The resulting deep brown mixture was then subjected to gel filtration on a Bio-Gel P-2 column.

Gel Filtration of the Reaction Mixture—The reaction mixture was applied onto a Bio-Gel P-2 column (20 × 30 cm) equilibrated with 0.02 M sodium phosphate buffer, pH 7.4, and 0.01 M NaCl. The column was eluted at a flow rate of 18 ml/h, and fractions of 4.0 ml were collected. All fractions from the collected fractions were diluted with distilled water for absorption measurement at 220 nm. The fractions were also analyzed by thin-layer chromatography (TLC) on silica gel plates (Kieselgel 60, 0.2 mm, E. Merck, Germany) using butanol:acetic acid:water:pyridine (2:5:1:25:25, v/v/v/v/v) as solvent. The TLC plates were developed with ninhydrin (5% in ethyl alcohol). Based on the absorbance at 220 nm and the TLC profile, the fractions were combined into four pools. The pooled fractions were dried in a speed vacuum concentrator (Savant Instruments, Farmingdale, NY).

High Performance Liquid Chromatography (HPLC)—The dried pools from the Bio-Gel column were reconstituted in 3.0 ml of 0.1% trifluoroacetic acid (TFA) in water and subjected to HPLC on a C18 reversed phase semi-preparative column for further purification. The solvent system and other conditions were the same as above, except that a longer solvent gradient was used (0–50 min, 0–100% B). The major peaks were collected and lyophilized. Using this procedure, purified \text{GK-peptide} was obtained. Characterization of this peptide revealed that it is a dimer and cross-linked by a Maillard reaction product. This product was named imidazolysine. Other details of purification and characterization are described under the “Results.”

Purification of \text{Imidazolysine from the Incubation of \text{N-\text{t-BOC Lysine with MG}}—Five hundred mg of \text{N-\text{t-BOC lysine} and 200 mg of MG were dissolved in 5.0 ml of 0.2 M sodium phosphate buffer, pH 7.4, and incubated at 37 °C for 16 h. The resulting reaction mixture was subjected to cation exchange chromatography on a AG-50W-X4 (Bio-Rad) (2.5 × 7 cm) column equilibrated with 0.02 M sodium acetate buffer, pH 5.0. The column was washed with 250 ml of buffer and then eluted with a gradient of 0–1 M NaCl in 100 ml. This was followed with 100 ml of buffer containing 1 M NaCl. Fractions of 3.0 ml were collected at a flow rate of 20.0 ml/min. To each fraction 100 \mu l of 1 M HCl was added (to release the BOC group) and allowed to stand overnight. The presence of imidazolysine was determined by TLC on silica (butanol:water:acetic acid:pyridine, 5:2.5:2.5:2.5, v/v/v/v, solvent system A). The plates were visualized with ninhydrin.

The fractions that showed the presence of imidazolysine were pooled and lyophilized. The sample was then extracted twice with 20 ml of methanol to remove salt and finally dissolved in 5.0 ml of water and subjected to HPLC on a C18 reversed phase semi-preparative column with the following solvents and program: solvent A, 0.1% TFA in water, and solvent B, 50% acetonitrile in water + 0.1% TFA; 0–100% linear gradient of B in 0–35 min at a flow rate of 2.0 ml/min. The column effluent was collected and lyophilized. The fractions were analyzed by TLC using the solvent system A and visualized with ninhydrin. The fractions that showed the presence of imidazolysine (Rf, 0.06) were pooled and dried in a speed vac concentrator. This fraction was further purified by HPLC using similar conditions as described above except that the gradient of solvent B was 0–300% over 10 min. Fractions of 1.0 ml were collected, and imidazolysine in the fractions was detected by TLC. Fractions with imidazolysine were pooled and concentrated by lyophilization.

Incubation of Proteins with MG—Formation of imidazolysine in proteins incubated with MG was investigated. Bovine lens crystallins were prepared as described previously (27). The water-soluble fraction was used in this experiment. Lens crystallins and HSA (100 mg/50.0 ml) in phosphate-buffered saline were incubated with 50 mM MG at 37 °C, and aliquots (0.5 ml) were withdrawn at specified time intervals. Protein was precipitated by the addition of 0.5 ml of 10% trichloroacetic acid (w/v in water), and the precipitate was separated by centrifugation at 3500 × g for 15 min and hydrolyzed at 110 °C for 20 h in the presence of trichloroacetic acid (w/v in water), and the precipitate was separated by centrifugation at 3500 × g for 30 min. This fraction was then lyophilized and stored at −80 °C until use. Ten mg of protein was hydrolyzed with 2.0 ml of 6 N HCl at 110 °C for 20 h. The acid was evaporated in a speed vac concentrator, and the final residue was redissolved with 200 μl of water.

Imidazolysine was detected and quantified by HPLC with post-column derivatization by OPA. Initially, the retention time of purified imidazolysine (purified from the reaction of N-\text{t-BOC lysine and MG, 15 μl} = 1.06 nmol) was determined. A C18 reversed phase column was used. The column was eluted with a linear gradient of 0–25% acetonitrile in water with 0.01 M HCl at a flow rate of 1.0 ml/min. The effluent from the column was mixed with OPA (0.5 ml) in a three-way static mixing apparatus (Upchurch Scientific, Oak Harbor, WA). The OPA reagent was prepared by the following procedure. 2.43% boronic acid was taken in water and the pH was adjusted to pH 9.7 with KOH. To this, 0.08% OPA dissolved in 10.0 ml of ethanol and 0.2% \text{\beta-mercaptoethanol} (w/v) were added and filtered through a 0.45-μm filter. To the filtrate, 0.1% Brij 35 was added. The volume was then adjusted to 1.0 liter with water. The OPA-derivatized proteins were detected with an on-line fluorescence detector (Jasco, Inc., MD) which was set at excitation/emission wavelengths of 340/455 nm. Under these conditions imidazolysine standard eluted between 29 and 30 min. The acid-hydrolyzed protein samples were injected (50 μl = 500 μg of protein in the case of proteins incubated with MG and 65 μl = 1.3 mg of protein in the case of human serum proteins) under the conditions described above but without postcolumn derivatization. The effluent from the column from 27 to 33 min was collected, dried in a speed vac concentrator, and reconstituted in 200 μl of water. Aliquots of 75 and 50
from plasma proteins and proteins incubated with MG, respectively, were subjected to HPLC using a C18 column (5 μm, 201HS54). A concave gradient of acetonitrile was used (0–8% acetonitrile in water + 0.1% HFBA in 40 min). Under these conditions imidazolysine standard eluted between 34 and 35 min. Quantification was performed by comparison of peak area in serum proteins to that of a purified imidazolysine standard. Results were expressed as picomoles of imidazolysine per milligram of protein.

Spectroscopy—1H NMR (400 MHz), 13C NMR (100 MHz, acetone as external reference), 1H-1H COSY and DEPT analysis were performed on a Bruker MSL-400 spectrometer. Chemical shifts are expressed in δ scale. The samples were exchanged three times with D2O and taken in 5-mm tube (5–12 mg) for analysis. All mass spectrometry analyses were performed at the Department of Chemistry, Washington University, St. Louis, MO. Molecular weight was determined by FAB spectroscopy with a KRATOS MS-50 double focusing mass spectrometer. Analysis was initially conducted at low resolution (1000) at an accelerating voltage of 8 KeV. Sample was mixed with 3-nitrobenzyl alcohol/sodium iodide (NBA/NaI). Ions were formed by FAB with a 6-KeV argon beam. FAB high resolution mass analysis was performed at a resolution of 10,000 by peak matching with CsI/glycerol matrix ion at m/z 484.7625 and 576.8099.

RESULTS

The reaction of GK-peptide and MG was carried out in the presence of arginine with the objective of obtaining not only lysine-lysine cross-links but also lysine-arginine cross-links. The reaction mixture was passed through a Bio-Gel column to separate modified peptides from the unreacted peptides. The aim was to separate peptide dimers and peptides having arginine cross-links from the unreacted peptides and MG. The chromatogram is shown in Fig. 1. Most of the peptide remained in the native state (fractions 51–59). The TLC profile of the fractions also showed a strong ninhydrin positive spot in fractions just before the native peptide (Rf = 0.18, Rf for the native peptide = 0.5). HPLC analysis and mass spectral determinations showed that this material corresponds to the deesterified peptide. On the other hand, deep brown products were observed in fractions ahead of the deesterified peptide (fractions 38–50). Since these fractions were in a higher molecular weight region than the native peptide, it was assumed that these contained peptide polymers, dimers, and peptides carrying AGEs. Based on the TLC results and absorption values at 220 nm, fractions 38–44, 45–50, 51–59, and 60–65 were combined and designated as pools 1, 2, 3, and 4, respectively.

The pools from the Bio-Gel column were concentrated to dryness, dissolved in 2.0 ml of water, and subjected to HPLC. The native peptide eluted in 13.7 min and the deesterified peptide in 9.3 min. The other major peaks (Fig. 1) detected by monitoring the effluent at 220 nm (retention time between 17 and 30 min) were collected, dried, and reconstituted in 0.5 ml of water and further purified on a C18 reversed phase column by HPLC. By this procedure four peaks from pool 1 (labeled 1a–1d), six from pool 2 (labeled 2a–2f), five from pool 3 (labeled 3a–3e), and four from pool 4 (labeled 4a–4d) were separated and collected. The peaks with identical retention times from adjacent pools were mixed.

The collected peaks were dried, and a portion of the material was hydrolyzed with 6 N HCl at 110 °C for 20 h. The acid-hydrolyzed material was subjected to TLC on a silica plate with the solvent system butanol:water:acetic acid:pyridine (4:2:3:3, v/v/v/v). Glycine, arginine, and lysine were also spotted. In the peak at 19.3 min retention time (1a and 2a), a distinct ninhydrin positive spot with an Rf value of 0.13 was present. Glycine...
was present in this material but lysine was absent. Based on this observation, it was concluded that the material was a modified peptide. No other HPLC peak showed the presence of glycine, which suggested the absence of the peptide. The isolated modified peptide was characterized by $^1$H, $^{13}$C, NMR, and FAB-MS along with the native peptide (purified from pool 3 by HPLC).

$^1$HNMR (in D$_2$O) for the native peptide and for the modified peptide are shown in Fig. 2. The following signals were observed for the native peptide; the first letter within the parentheses represents proton position on the peptide: $\delta$H 1.42 (f, 2H, m), 1.67 (g, 2H, m), 1.77 (e, 2H, m), 2.05 (a, 3H, s), 2.97 (h, 2H, t, $J_{h,g} = 7.6$ Hz), 3.75 (d, 3H, s), 3.92 (b, 2H, q, $J_{b,b} = 16.9$ Hz), and 4.46 (c, 1H, q, $J_{c,e} = 5$ Hz). The signal at $\delta$ 1.92 (3H, s) was from acetate (the peptide was supplied as the acetate salt). For the modified peptide the signals were $\delta$H 1.38 (f, 2H, m), 1.78 (g, 4H, m), 1.87 (e, 4H, m), 2.05 (a, 6H, s), 2.33 (j, 3H, s), 3.76 (d, 6H, s), 4.14 (h, 4H, q, $J_{h,g} = 7.2$ Hz, $J_{h,h} = 14.8$ Hz), 4.45 (e, 2H, q, $J_{c,e} = 4$ Hz), 7.26 (k, 1H, s) and 8.69 (l, 1H, s). In the case of the modified peptide, no signals corresponding to BOC- or free arginine were observed ruling out the presence of arginine. The number of protons for lysine (between 1 and 2 ppm) were 12 for the modified peptide (6 in the case of native peptide). This suggested the presence of two peptides. This was further evidenced by the following observations: 1) the presence of 6 protons from the acetyl group ($\delta$ 2.05 ppm) and 2) two protons corresponding to $\alpha$-position in lysine ($\delta$ 4.45 ppm). The two singlets in the aromatic region ($\delta$ 7.26 and 8.69 ppm) suggested the presence of 2 uncoupled protons in the aromatic ring and the signal at $\delta$ 2.33 ppm suggested the presence of a methyl group on the ring. $^1$H-$^1$H COSY analysis showed spin-spin interaction between protons on C2 and C4 (5) carbons (data not shown). Protonation of carbons was determined by DEPT analysis, which showed the presence of a single proton on C2 and C4 carbons (data not shown).

$^{13}$C NMR spectroscopy showed the following signals for the native peptide: $\delta$C 21.67 (CH$_3$-acetyl), 23.34 (g-CH$_2$-lys), 26.08 (d-CH$_2$-lys), 29.82 (b-CH$_2$-lys), 39.10 (e-CH$_2$-lys), 42.32 (CH$_2$-gly), 52.32 (a-CH-lys), 52.81 (CH$_3$-ester), 171.41 (CO-acetyl), 173.82 (CO-gly), and 174.45 (CO-lys). The spectrum for the modified peptide showed four new signals. The signals at $\delta$C 8.23, 49.01, 119.28, and 134.55 corresponded to methyl group, C4, C5, and C2 carbonson the aromatic ring, respectively, and were fully compatible with the structure in Fig. 2.

The FAB-mass spectrum on 3-nitrobenzyl alcohol (NBA) matrix showed a molecular ion at m/z of 567.3 (M$^+$, not shown). High resolution FAB-MS in the positive ion mode showed the molecular mass of 567.314238. This corresponded to molecular formula C$_{26}$H$_{43}$N$_6$O$_8$ and was in agreement with the proposed structure. Based on these characteristics, it was determined that the purified product was a cross-link of peptides joined by a methylimidazolium ring. The structure of the peptide cross-link is shown in Fig. 2.

Imidazolysine was then synthesized by the reaction of N$^\alpha$-t-BOC-lysine with MG. Cation exchange chromatography followed by HPLC resulted in the isolation and purification of...
imidazolysine. This was evident by a single spot on TLC (solvent: butanol:water:acetic acid:pyridine, 5:2:2:5:2.5 (v/v/v/v), detection by ninhydrin). The Rf value of this preparation (0.06) was identical to that of the ninhydrin-positive spot observed with the acid hydrolysate of the isolated peptide under the same condition suggesting that they were the same compound. Furthermore, the results establish that the sample was different than carboxymethyllysine ($R_f$ 0.09), an advanced Maillard reaction product that can be formed by the reaction of glyoxal with lysine (28). This was further confirmed by $^1H$ NMR spectroscopy (Fig. 3) which showed the following signals: $\delta_H$ 1.45 (c, 4H, m), 1.91 (b, d, 8H, m), 2.3 (f, 3H, s), 3.94 (a, 2H, q, $J_{a,b}$ = 5 Hz), 4.17 (e, 4H, q, $J_{e,d}$ = 14.2 Hz), 7.24 (g, 1H, s), and 8.68 (h, 1H, s). These signals were compatible with that of the modified peptide (Fig. 2). FAB-MS (glycerol matrix) showed m/z at 341.3 (Fig. 3) which corresponded to the expected structure with a molecular formula of $C_{16}H_{29}O_4N_6$. Acid hydrolysis of this preparation (110 °C, 20 h with 6N HCl) did not change its $R_f$ value on TLC suggesting that it was an acid-stable compound. Imidazolysine is likely to be formed from the reaction of two molecules of MG with two molecules of GK-peptide lysines. The reaction may involve first the formation of a diimine, with two lysine residues, followed by the addition of another molecule of MG. Subsequently, acetic acid and water may be removed to form imidazolysine.

Since imidazolysine was found to be an acid-stable compound, its detection and quantification were performed after acid hydrolysis of proteins. Human serum albumin and lens crystallins incubated with MG showed a time-dependent formation of imidazolysine. A two-step HPLC method was used for better resolution of the imidazolysine peak. Imidazolysine was formed within a short period of incubation. After 3 h 18 nmol/mg protein was formed in HSA, and after 2 h 14 nmol/mg protein was formed in lens crystallins. The concentration reached 146.6 nmol/mg protein in HSA and 28 nmol/mg protein in lens crystallins after 3 days of incubation (Fig. 4). This accounted for 15 and 8.5% modification of lysine residues on HSA and lens crystallins, respectively. These values corresponded to 7–8 imidazolysines per HSA molecule (molecular mass 65 kDa and 59 lysines) and one imidazolysine per 5–6 $\alpha$-crystallin subunits (average molecular mass 20 kDa and 7–8 lysines). In the case of lens crystallins, saturable amounts of imidazolysine formed within a relatively short period (24 h). This suggests that in the native crystallins, the reactive lysine residues are positioned favorably for imidazolysine formation. The formation of imidazolysine in proteins under physiological conditions suggested that it could form on proteins in vivo and led us to investigate the formation of imidazolysine in serum proteins.

HPLC of the sample with postcolumn derivatization displayed a peak with a retention time identical to that of purified imidazolysine in serum proteins (Fig. 5). A single homogeneous peak was observed when purified imidazolysine mixed with serum protein hydrolysate was subjected to HPLC (data not shown). Further confirmation for the presence of imidazolysine in serum proteins was obtained by its partial purification and characterization. A pool of serum protein hydrolysate corresponding to 120 mg of protein was subjected to HPLC using a Vydac C18 semi-preparative column (0–60% acetonitrile in water and 0.01 $\mu$HFBA in 0–40 min, flow rate 1.5 ml/min). The
In diabetic samples, and comparison between groups showed a weak but significant difference (p = 0.049, Student's t test). Imidazolysine expressed as a function of glycosylated hemoglobin showed a statistically significant positive correlation (r = 0.57, p < 0.05) (Fig. 8). For this study, diabetic samples were chosen in which the glycohemoglobin values were ≥10%.

**DISCUSSION**

Protein cross-linking and insolubilization are among the major biochemical changes in proteins during aging. This is especially prominent in long-lived extracellular proteins and the proteins of the eye lens. In general, diabetes accelerates cross-linking and insolubilization of proteins. Enhancement of protein cross-linking in extracellular matrix is implicated in a number of age- and diabetes-associated complications, e.g. atherosclerosis and cataract formation. The observation that non-enzymatic glycation of proteins results in covalent cross-linking and insolubilization led to the suggestion that AGEs may in part be responsible for age- and diabetes-associated protein cross-linking. Thus, in recent years, much effort has focused on the chemical characterization of cross-linking structures derived from the Maillard reaction.

In the method adopted in the present study, a synthetic peptide containing a lysine residue (GK-peptide) was reacted with MG in the presence of α-t-BOC-arginine. The objective was to isolate peptide dimers in which lysines are bridged by an advanced Maillard product. This procedure was also expected to generate peptides with advanced Maillard product without dimerization through lysine-arginine cross-linking. The major advantage of using a peptide instead of amino acids is that Maillard products that lack chromophoric or fluorophoric properties can be isolated by monitoring the HPLC effluent for peptide bond absorption (220 nm). In fact, the cross-link purified in the present study, imidazolysine, is neither a chromophore nor a fluorophore. The second advantage is that the cross-links of Maillard products (peptide dimers) could be separated from non-cross-linking Maillard products by gel filtration (employed in the present work), which is difficult to accomplish using amino acids.

Dicarbonyl compounds like MG are known modifiers of arginine residues in proteins (23, 29, 30). One of our objectives was to investigate whether the reaction product of MG with lysine on the peptide can further react with arginine to form lysine-arginine cross-links. This was the reason for including arginine along with the peptide for incubation with MG. However, our results showed no major lysine-arginine cross-linking structures.

Compounds similar to imidazolysine have been isolated by food chemists from the reaction of glycine or glycol glycine with glyoxal in the presence of formaldehyde at high temperature (31, 32). However, imidazolysine was isolated from the reaction mixture obtained under physiological conditions, and its mechanism of formation does not seem to involve the participation of formaldehyde. Recently, Wells-Knecht et al. (33) and Brinkmann et al. (34) have described the formation of imidazolium cross-links from the reaction of hippuryllysine with glyoxal and MG. One of the cross-links, MOLD, is identical to imidazolysine. Our study thus confirms the formation of lysine-lysine cross-links in the Maillard reaction by MG under physiological conditions.

Effective defense systems may exist intracellularly to reduce MG toxicity through the Maillard reaction. The GSH-dependent glyoxalase converts it to D-lactate (35). It is therefore conceivable that in tissues where the GSH level is decreased in aging and diabetes, MG concentration would increase. In fact, the MG concentration is reported to be elevated in lens, red blood cells, and kidney in diabetes, where GSH levels have
been determined to decrease (36–41). Another potentially important metabolic pathway for decreasing MG’s toxicity is by its conversion to acetol by aldose reductase (23). MG is one of the best substrates for aldose reductase. In addition, aldehyde reductase, sorbitol dehydrogenase, and dihydrodiol dehydrogenase are all shown to metabolize MG (42–44). Weakening of these defenses by inactivation by glycation or oxidation, as reported for aldose reductase (45) and aldehyde reductase (46), may promote the Maillard reaction by MG. Alternatively, the Maillard reaction by MG would enhance if the concentration of MG, together with other endogenous sugars, overwhelms the capability of the enzymes to metabolize them. In the lens, these events may account for accelerated protein cross-linking and cataract formation in diabetes.

The in vivo detection of imidazolysine in serum proteins and its positive correlation with glycosylated hemoglobin is consistent with enhanced formation of MG as a function of glycemia in diabetes. In a recent paper Westwood et al. (25) have described a macrophage receptor for MG-modified proteins. This receptor has been shown to recognize glucose-modified proteins also, suggesting the formation of structurally similar AGEs from these glycating agents. The dicarbonyl intermediates in the glucose-initiated Maillard reaction, like 3-deoxyglucosone, are likely to form imidazolysine. Thus, imidazolysine may represent a common structure in advanced Maillard reactions. It remains to be seen whether imidazolysine is recognized by the receptor described by Westwood et al. (25) and whether such an interaction could induce synthesis of growth factors and cytokines similar to glucose-derived AGEs (47).

In summary, this study demonstrates the formation of lysine-lysine cross-linking by a physiological α-dicarbonyl compound, methylglyoxal, and for the first time provides evidence for its formation in vivo. Formation of imidazolysine in other tissues and relationship to complications of diabetes remain to be established. Since protein cross-linking is a major end result of the Maillard reaction, imidazolysine may serve as a useful marker for assessing the role of the Maillard reaction in aging and pathogenesis of diabetic complications.

![Liquid chromatography/mass spectrometry analysis of imidazolysine standard (A) and partially purified imidazolysine from human serum proteins (B). The molecular mass for the peak in serum proteins (at the retention time of purified imidazolysine, 15–16 min) was identical to the imidazolysine standard (m/z 341). Background signals were subtracted to remove the mass ions from the matrix and solvent.](image-url)
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Fig. 7. Imidazolysine in normal and diabetic human serum proteins. Serum proteins were acid-hydrolyzed and analyzed for imidazolysine as described for Fig. 5. Imidazolysine level in normal controls was 261.3 ± 50.4 pmol/mg protein (mean ± S.D.) and in diabetics was 313.8 ± 52.7 (p < 0.05).

Fig. 8. Relationship between serum protein imidazolysine and glycohemoglobin. Glycohemoglobin was measured by boronate affinity column method. The data include values for samples from both normal and diabetic subjects.

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