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N-Terminal pyrazinones: a new class of peptide-bound advanced glycation end-products*

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Summary. The reaction of the peptide Gly-Ala-Phe with the α-dicarbonyl compounds glyoxal and methylglyoxal was studied under physiological conditions (pH = 7.4, 37°C). Using HPLC with UV and fluorescence detection, a rapid derivatization of the peptide and the concomitant formation of well-defined products were observed. The products, which showed characteristic UV absorbance ($\lambda_{max} = 320$ to 340 nm) and fluorescence ($\lambda_{ex} = 330$ to 340 nm, $\lambda_{em} = 395$ to 405 nm), were identified by ESI-MS and NMR spectroscopic analysis as the N-terminally pyrazinone-modified peptides I (N-[2-(2-oxo-2*H*-pyrazin-1-yl)-propyl]-phenylalanine) and II (N-[2-(5-methyl-2-oxo-2*H*-pyrazin-1-yl)-propionyl]-phenylalanine). Model experiments revealed that the reactivity of the N-termini of peptides towards a derivatization by glyoxal is in the same order of magnitude as that of arginine, which generally is attributed as main target for α-dicarbonyl compounds in proteins. Incubation of insulin with glyoxal proved the protein-bound formation of pyrazinones, with the N-terminus of the B-chain as the main target. According to these results, we conclude that N-terminal pyrazinones represent a new type of advanced glycation end-products (AGEs) with significance for biological systems and foods.

Keywords: Glycation – Maillard reaction – Pyrazinone – Advanced glycation end-product (AGE) – Glyoxal – Methylglyoxal – Insulin

Abbreviations: AGE, advanced glycation end-product; CEL, N^{ε}-[1-(2-carboxy)ethyl]lysine; CMA, N^{ω}-(carboxymethyl)arginine; CMC, N^{ε}-(carboxymethyl)cysteine; CML, N^{ε}-carboxymethyllysine; GDP, glucose degradation product; Gly-Ala-Phe, glycyl-L-alanyl-L-phenylalanine; GOLD, glyoxal-lysine dimer; N^{α}-hippurylarginine, N^{α}-benzoyl-glycyl-L-arginine; N^{α}-hippuryllysine, N^{α}-benzoyl-glycyl-L-lysine; MOLD, methylglyoxal-lysine dimer

Introduction

Nonenzymatic glycation reactions between proteins and carbohydrates or there degradation products leading to protein-bound advanced glycation end-products (AGEs). These are discussed to play an important role for pathophysiological processes like diabetes or uraemia (Lyons, 2002; Miyata et al., 2000a; Niwa et al., 1997; Singh et al., 2001; Raj et al., 2000; Thornalley, 1999). A large number of individual AGEs have been isolated from model systems, resulting from the reaction of the ε-amino group of lysine or the guanidino side chain of arginine (Ahmed et al., 1986; Glomb and Lang, 2001; Grandhee and Monnier, 1991; Nakayama et al., 1980; Niwa et al., 1997). Examples for compounds, which have been quantified in biological systems using chromatographic or immunological means, are N^{ε}-carboxymethyllysine (CML), pyrraline, pentosidine, 3DG-Arg-imidazolone and argpyrimidine (Henle et al., 1999; Niwa et al., 1997; Odani et al., 1999a; Weiss et al., 2000; Wilker et al., 2001).

In addition to glucose, highly reactive α-dicarbonyl compounds, the so called glucose degradation products (GDPs), have been described to initiate the formation of AGEs (Miyata et al., 2000b). Among these GDPs, glyoxal and methylglyoxal reached particular attention.

Glyoxal is formed during glycation reaction of proteins from the degradation of the Schiff base and the Amadori rearrangement product (Glomb and Monnier, 1995; Thornalley et al., 1999) as well as from autoxidation of glucose (Glomb and Monnier, 1995; Thornalley et al., 1999; Wells-Knecht et al., 1995). Additionally, glyoxal is generated in lipid peroxidation (Fu et al., 1996) and shall be also formed by enzymatic degradation of serine by myeloperoxidase (Anderson et al., 1997).

The major source of methylglyoxal may be the nonenzymatic fragmentation of triosephosphates, which are key metabolites in the Embden-Meyerhof and Polyol pathway. Additionally, it is also a by-product in the catabolism

* This paper is dedicated to Prof. Dr. U. Freimuth on the occasion of his 90th birthday.

of threonine and the ketone body acetone. Beside these metabolic origins, it is formed nonenzymatically in the course of glycation reaction from glucose (Thornalley et al., 1999).

Increased levels of these α-dicarbonyl compounds have been measured especially for uraemic and diabetic patients (Lapolla et al., 2003; Odani et al., 1999b). Considerable levels also have been found in heat sterilized fluids used for peritoneal dialysis (Nilsson-Thorell et al., 1993).

The high reactivity of glyoxal towards the side chains of arginine and lysine is well documented. According Al-Abed and Bucala (1995), CML is one of the quantitatively most important AGE structure which can be formed directly from lysine and glyoxal. Glomb and Pfahler (2001) could show that crosslinking of proteins by glyoxal involving lysine side chains may be possible. Schwarzenbolz et al. (1997) has described a protein-bound iminohydantoine structure designated as ''glarg'' resulting from the reaction of glyoxal with arginine. Recently, Glomb and Lang (2001) discussed that this reaction under physiological conditions may lead predominately to a dihydroxyimidazolidine derivative.

Important glyoxal-related AGEs quantified *in vivo* are CML (Weiss et al., 2000), glyoxal-lysine dimer (GOLD, Odani et al., 1999a), N^{ω}-(carboxymethyl)arginine (CMA, Odani et al., 2001) and N^{ε}(carboxymethyl)cysteine (CMC, Thorpe and Baynes, 2003). Corresponding products of methylglyoxal from *in vivo* sources are N^{ε}-[1-(2-carboxy)ethyl]lysine (CEL, Ahmed et al., 1997), methylglyoxal-lysine dimer (MOLD, Odani et al., 1999a) and the blue fluorophore argpyrimidine (Wilker et al., 2001).

Despite the fact that the Maillard-reaction *in vivo* was first described for N-terminally glycated haemoglobin HbA_{Ic} (Bookchin and Gallop, 1968), glycation reactions at the N-terminal amino groups of proteins have not reached particular attention up to now. The purpose of our study, therefore, was to investigate the reaction between α-amino groups of peptides and the protein insulin with glyoxal and methylglyoxal under physiological conditions.

Materials and methods

Chemicals

Gly-Ala-Phe (glycyl-L-alanyl-L-phenylalanine), N^{α}-hippuryllysine (N^{α}-benzoyl-glycyl-L-lysine), N^{α}-hippurylarginine (N^{α}-benzoyl-glycyl-L-arginine) were obtained from Bachem (Heidelberg, Germany). HPLC gradient grade methanol and acetonitril were from Riedel de Haen (Seelze, Germany). Insulin from bovine pancreas (no. I5500) as well as glyoxal and methylglyoxal solution (each 40% in water) were from Sigma (Taufkirchen, Germany). All other chemicals used were p.a. and purchased from Fluka (Taufkirchen, Germany). The water used for the preparation of buffers and solutions was obtained with a Purelab plus purification system (USFilter, Ransbach-Baumbach, Germany).

Incubation of peptides with glyoxal or methylglyoxal

Peptides (Gly-Ala-Phe, N^{α}-hippuryllysine, N^{α}-hippurylarginine, 5 mM each) alone or in combination (for competitive experiments) were incubated in the presence of glyoxal or methylglyoxal (5 mM) in 100 mM sodium phosphate buffer pH = 7.4, at 37°C and up to 14 d. Control incubations were made without the α-dicarbonyl compound. After certain time intervals, incubations were terminated and samples were prepared for HPLC analysis by addition of 100 mM formic acid (1 + 1 dilutions for samples containing Gly-Ala-Phe, 1 + 3 dilutions for N^{α}-hippuryl-derivatives).

For testing the stability of the isolated compound **I**, the isolate was dissolved to a final concentration of 5 mM in 100 mM sodium phosphate buffer, with pH of 2.0, 4.0, 7.4 or 9.1 (adjusted with 1 M or 4 M hydrochloric acid), respectively and incubated for 3 d at 60°C.

*Isolation of compound **I***

235 mg (0.72 mmol) Gly-Ala-Phe × 1.9 H_2O dissolved in 100 ml sodium phosphate buffer pH = 7.4 were incubated with glyoxal at 37°C for 7 d. The addition of the glyoxal solution was performed in portions of 0.08 mmol (9.1 μl) twice a day over the first five days. If necessary, the pH-value was corrected with 4 M sodium hydroxide solution. Following incubation, the solution was evaporated to about 10 ml under reduced pressure, acidified with 1.0 ml acetic acid and membrane filtered (0.45 μm, regenerated cellulose).

Semi preparative isolation of compound **I** was performed with a high pressure gradient pump system from Knauer (Berlin, Germany) with online degasser, K1500 solvent organizer, two K1001 pumps with 50 ml pump heads, dynamic mixing chamber and column oven. The separation was done on a stainless steel column, 250 × 16 mm with guard column 30 × 16 mm, both filled with Knauer Eurospher 100 RP18-material of 15 μm particle size (Knauer, Berlin, Germany). Flow rate was set at 6.0 ml/min, temperature was set at 20°C and ultraviolet detection was done at 320 nm. 1000 μl-samples were applied and elution was achieved isocratically with 0.1% acetic acid in 30% methanol (v/v). The desired fractions were pooled, lyophilised and the resulting powder was stored at −20°C.

Compound **I**: ESI-MS, positive mode: $[M+H]^+$ m/z = 315.7, negative mode: $[M-H]^-$ m/z = 313.7, UV and fluorescence data (100 mM PB, pH = 7.0): $\lambda_{UVmax} = 322$ nm, $\varepsilon_{322} = 49001 \cdot mol^{-1} \cdot cm^{-1}$, $\lambda_{ex,max} = 330$ nm, $\lambda_{em,max} = 395$ nm, elemental analysis, $C_{16}H_{17}N_3O_4 \times 0.05$ $CH_3COOH \times 0.45$ H_2O (MW = 326.43) calculated: C = 59.24%, H = 5.59%, N = 12.87%, found: C = 59.75%, H = 5.49%, N = 12.86%; yield: 112 mg (molar yield 47.9%).

*Isolation of compound **II***

470 mg (1.44 mmol) Gly-Ala-Phe × 1.9 H_2O dissolved in 200 ml sodium phosphate buffer pH = 7.4 were incubated with methylglyoxal at 37°C for 12 d. The addition of the methylglyoxal solution was performed in portions of 0.16 mmol (24.6 μl) once a day over the first ten days. If necessary, the pH-value was corrected with 4 M sodium hydroxide solution. Following incubation, the solution was evaporated to about 20 ml under reduced pressure, acidified with formic acid to pH = 4.7 and membrane filtered (0.45 μm, regenerated cellulose).

Semi preparative separation of compound **II** was performed with analytical gradient pump system from Knauer (Berlin, Germany). A stainless steel column, 250 × 8 mm with guard column 30 × 8 mm, both filled with Knauer Eurospher 100 RP18-material of 10 μm particle size (Knauer,

Berlin, Germany) was used. Detection was achieved using two UV detectors set at 220 and 320 nm as well as a fluorescence detector ($\lambda_{ex} = 320$ nm, $\lambda_{em} = 400$ nm). Flow rate was set at 2.1 ml/min, and the temperature was set at 20°C. 400 μl-samples were applied and elution was achieved isocratically with 0.1% acetic acid in 35% methanol (v/v). The desired fraction was identified using characteristic UV absorbance at 320 nm and fluorescence. After lyophilisation, the resulting powder was stored at −20°C.

Compound **II**: ESI-MS, positive mode: $[M+H]^+$ m/z = 330.1, negative mode: $[M-H]^-$ m/z = 328.2, UV and fluorescence data (100 mM PB, pH = 7.0): $\lambda_{UVmax} = 336$ nm, $\varepsilon_{336} = 46001 \cdot mol^{-1} \cdot cm^{-1}$, $\lambda_{ex,max} = 340$ nm, $\lambda_{em,max} = 405$ nm, elemental analysis, $C_{17}H_{19}N_3O_4 \times 0.13\ CH_3COOH \times 0.50\ H_2O$ (MW = 346.17) calculated: C = 59.87%, H = 5.97%, N = 12.14%, found: C = 59.72%, H = 5.90%, N = 12.17%; yield: 29 mg (molar yield 5.8%).

Incubation of insulin with glyoxal

Incubation of insulin were made in phosphate-buffered saline pH = 7.4 (PBS, containing 10 mM phosphate). Bovine insulin was freshly dissolved in 2 mM hydrochloric acid (1.0 mg/ml). Final concentrations in PBS were 17 μM (0.1 mg/ml) for insulin and 50 μM for glyoxal. Control incubations were made without glyoxal. Preservation was achieved by sterile filtration (0.2 μm, regenerated cellulose) and adding a crystal of thymol. The samples were incubated at 37°C for 15 d. For direct HPLC analysis, the reaction was terminated by addition of 33 μl 2.65 M formic acid per 1000 μl incubation mixture.

Separation of the A- and B-chain was achieved by reduction of the disulfide bridges and blocking the sulfhydryl groups according to a modified protocol of Gray (1997). For HPLC analysis, 240 μl of the incubation mixture (without addition of formic acid) was mixed with 10 μl of 750 mM dithiothreitol (DTT)-solution and kept in the dark for 50 min. For alkylation, 100 mg iodacetamide was dissolved in 200 μl 0.5 M tris-acetat buffer pH = 8.2 (containing 2 mM Na_2EDTA) at 60°C. After cooling to about 30°C, this blocking solution was mixed with the reduced sample for 35 s. Alkylation was terminated with 400 μl 0.5 M citric acid solution. Samples were immediately analysed by HPLC.

For LC-MS analysis the protocol was modified as follows: 576 μl insulin incubation mixture, 24 μl DTT-solution, 400 mg iodacetamide in 400 μl tris-acetat buffer were used and as acid quench solution 40 μl formic acid were added. After vigorous shaking, samples were immediately injected in the HPLC system.

High pressure liquid chromatography (HPLC) analysis

HPLC was performed with an analytical gradient pump system from Knauer (Berlin, Germany) with online degasser, K1500 solvent organizer, 10 ml-K1001 pump, dynamic mixing chamber and column oven. For detection, the effluent was monitored by two K2501 Knauer variable wavelength detectors ($\lambda_1 = 220$ nm, $\lambda_2 = 320$ nm) and a La Chrom L-7485 Merck Hitachi (Darmstadt, Germany) fluorescence detector ($\lambda_{ex} = 320$ nm, $\lambda_{em} = 400$ nm).

Analytical separation of the peptide incubation mixtures was achieved using a stainless steel column, 250 × 4.6 mm, filled with Knauer Eurospher 100, RP18-material of 5 μm particle size, with integrated guard column 5 × 4 mm filled with the same material (Knauer, Berlin, Germany). The injection volume was 20 μl and the temperature was set at 20°C.

Samples containing Gly-Ala-Phe were run with a mobile phase consisting of 0.1% triethylamine, 0.05% acetic acid and 0.05% formic acid in water (solvent A), and 0.1% triethylamine, 0.05% acetic acid and 0.05% formic acid in methanol (solvent B). For analysis of samples resulting from incubation of glyoxal with Gly-Ala-Phe, a linear gradient from 12% B to 57% B in 35 min and a flow rate of 0.5 ml/min was used. Methylglyoxal-Gly-Ala-Phe-incubations were analysed with a linear gradient from 25% B to 55% B in 35 min at a flow rate of 0.7 ml/min.

Separation of N^{α}-hippuryl-derivatives was performed with a mobile phase consisting of 0.01 M sodium phosphate buffer, pH = 7.0 (solvent A), and methanol (solvent B). A linear gradient from 6% B to 13% B in 25 min (N^{α}-hippuryllysine) or from 12% B to 19% B in 25 min (N^{α}-hippurylarginine) at a flow rate of 0.7 ml/min was used. Formation of N^{ε}-carboxymethyl-N^{α}-hippuryllysine after incubating N^{α}-hippuryllysine with glyoxal was checked by co-injection of sample and reference material for N^{ε}-carboxymethyl-N^{α}-hippuryllysine, which had been synthesised as described previously (Krause et al., 2003).

Separation of the incubation mixtures of glyoxal with insulin was performed on a PLRP-S column (150 × 4.6 mm, 300 Å, 8 μm, Polymer Laboratories, Darmstadt, Germany) with a PLRP-S guard column (5 × 3 mm, 300 Å, 8 μm, Polymer Laboratories, Darmstadt, Germany). The eluent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitril (solvent B). The flow rate was set at 1.0 ml/min, and the temperature was set at 20°C. Direct analysis of incubation mixtures were made by applying 100 μl-samples and separation was achieved with a linear gradient from 5% B to 50% B in 30 min. For analysis of the reduced and blocked samples, 500 μl were applied and a linear gradient from 15% B to 40% B in 30 min was used.

LC-ESI-MS analysis was performed on a agilent 1100 series HPLC system (agilent technologies, Palo Alto, USA) consisting of a high pressure gradient pump system, column oven and diode array detector, which was coupled to a mariner ESI-TOF-MS instrument (see below). Samples were applied manually via injection valve. Separation was achieved using the same column, temperature and solvents. For direct analysis of incubation mixtures, 500 μl samples were applied and a linear gradient from 5% B to 50% B in 30 min at a flow rate of 1.0 ml/min was used. To study the reduced and blocked samples, 4000 μl were applied. For desalting as well as separation, the column was eluted with 5% B for 25 min at a flow rate of 1.0 ml/min and then the flow rate was set at 0.5 ml/min and a linear gradient was started to reach 42% B in 30 min.

Mass spectrometry

For mass-spectrometric analysis, a PerSeptive Biosystems Mariner time of flight mass spectrometry (TOF-MS) instrument equipped with an electrospray ionisation source (ESI) in general in the positive mode was used (Applied Biosystems, Stafford, USA). Calibration of the mass scale was established using a mixture of bradykinin, angiotensin I and neurotensin.

For direct ESI-TOF-MS analysis after appropriate dilution with 1% acetic acid in 50% methanol, the sample was infused at a flow rate of 5 μl/min into the ESI source using a syringe pump. In LC-ESI-MS experiments the outlet of the HPLC system was coupled to the ESI interface. Flow rates above 0.5 ml/min, the flow was split at a ratio 1 + 1 prior the interface.

The relative molecular masses (monoisotopic) were determined using the peak with the lowest m/z-ratio (monoisotopic peak) from prominent multiple charged ions and the equation $M_r = z \times M_z - 1.0078 \times z$, where M_r is the monoisotopic molecular mass, M_z is the m/z-ratio, z is the number of charges, and 1.0078 is the mass of a proton.

Ultraviolet and fluorescence spectroscopy

Absorption spectra were recorded with a Specord S100 diode array spectrophotometer (Carl Zeiss Jena, Germany) and fluorescence spectra were obtained with a f-4500 spectrofluorimeter (Hitachi, Tokyo, Japan). Measurements were performed in 100 mM formic acid (pH = 2.4), 100 mM sodium phosphate buffer (PB) pH = 7.0 and 100 mM sodium borate buffer (pH = 8.3) at a concentration of 0.03 mg/ml (compound **I**: 101 μM, compound **II**: 95 μM).

Nuclear magnetic resonance spectroscopy

NMR spectra were recorded on a Bruker DRX 500 instrument (Reinstetten, Germany) with 500 MHz for ^{1}H- and 125 MHz for ^{13}C-experiments. Chemical shifts (δ) are given in parts per million (ppm) relative to solvent signals as the internal reference, for measurements in dimethyl sulfoxide-

d6 $\delta_H = 2.50$ ppm and $\delta_C = 39.56$ ppm as well as for measurements in methanol-d4 $\delta_H = 3.38$ ppm and $\delta_C = 49.00$ ppm. Assignments of ^1H and ^{13}C signals were based on ^1H–^1H COSY (correlation spectroscopy), HSQC (heteronuclear single quantum coherence), HMBC (heteronuclear multiple bond correlation) and DEPT (distortionless enhancement by polarization transfer) experiments. The integrals of the proton signals of the methyl group of the alanine moiety in compound **I** and **II** and that of the residual acetic acid were used to determine their molar ratio and interpret the elemental analysis.

Elemental analysis

Elemental analysis data were obtained on Euro EA 3000 elemental analyser (Eurovector, Milano, Italy).

Statistical analysis

Quantitative determinations were made in triplicate and the results were expressed as mean values ± standard deviations, unless otherwise indicated.

Results and discussion

Reaction of peptides with glyoxal and methylglyoxal

The reaction of the α-amino group of the model peptide Gly-Ala-Phe with the glucose degradation product glyoxal under physiological conditions (up to 14 days at pH = 7.4 and 37°C) was investigated using RP-HPLC with UV-detection (Fig. 1). A time dependent rapid decrease of the peptide together with the formation of one well-defined new reaction product (compound **I**) was observed. After 8 h, 53% of the peptide was converted to the unknown product. Using semipreparative RP-HPLC, we were able to isolate 112 mg of compound **I** in a chromatographically pure form. A direct injection electrospray ionisation mass spectrometry analysis of the isolate revealed a relative molecular mass of $M_r = 314.7$ (monoisotopic), which is equivalent to the addition of one molecule of glyoxal to the peptide-moiety and a loss of two molecules of water.

As can be seen from Fig. 2A, UV-spectrum of the isolate showed a characteristic maximum at 322 nm. The compound was fluorescent with wavelength maxima of excitation at 330 nm and emission at 395 nm (Fig. 2B).

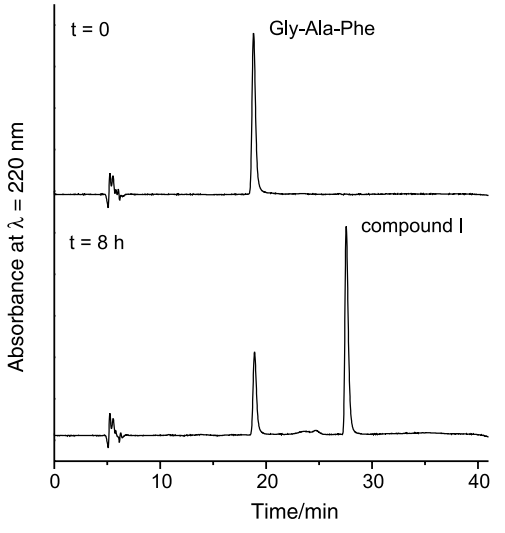

Fig. 1. HPLC analysis of the incubation mixture of Gly-Ala-Phe with glyoxal (5 mM each) in 100 mM PB (pH = 7.4, 37°C)

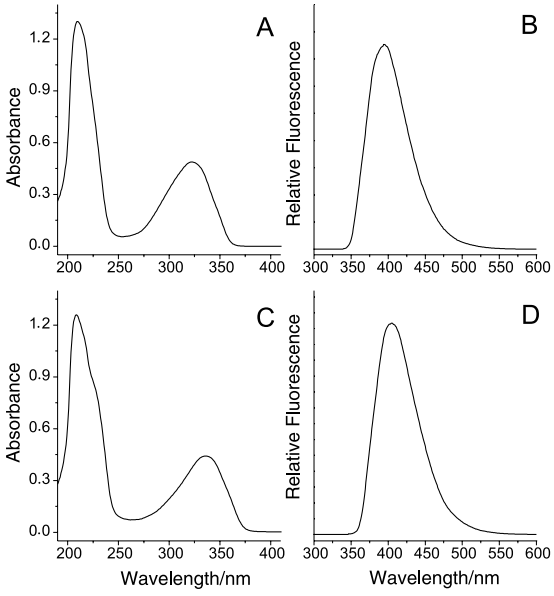

Fig. 2. Absorption and fluorescence emission spectra, recorded in 100 mM PB, pH = 7.0. Compound **I**: absorption (**A**), fluorescence emission ($\lambda_{ex} = 330$ nm) (**B**). Compound **II**: absorption (**C**), fluorescence emission ($\lambda_{ex} = 340$ nm) (**D**)

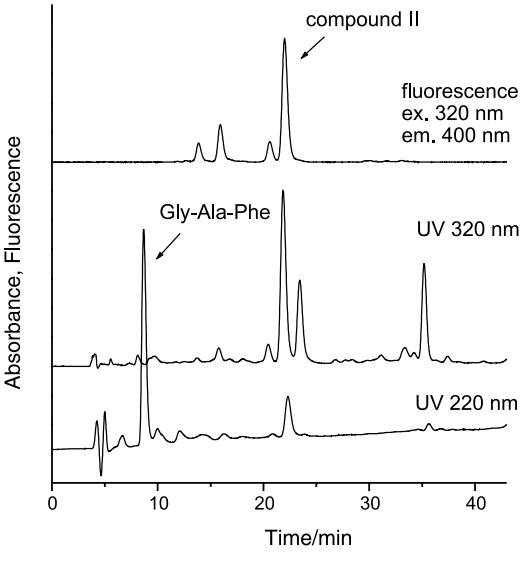

Fig. 3. HPLC analysis of the incubation mixture of Gly-Ala-Phe with methylglyoxal (5 mM each) in 100 mM PB (pH = 7.4, 37°C, 8 h) with UV and fluorescence detection as indicated

The chromatographic traces of the reaction mixture consisting of methylglyoxal and Gly-Ala-Phe were more complex (Fig. 3). Again, the dominating product designated compound **II** showed characteristic absorption at 320 nm and fluorescence (Fig. 3). The total conversion of the peptide was after 8 h with 50% similar to that of the mixture with glyoxal. After isolation using semipreparative RP-HPLC, 29 mg of compound **II** were obtained in chromatographically pure form. The mass spectrometric analysis gave a relative molecular mass of $M_r = 329.1$ (monoisotopic), indicating a reaction of one peptide and one methylglyoxal molecule with the loss of two water molecules similar to the glyoxal product mentioned above.

For compound **II**, the maxima in the UV spectrum at 336 nm and in the fluorescence emission spectrum at 405 nm ($\lambda_{ex,\ max} = 340$ nm) are slightly red-shifted compared to the glyoxal product (Fig. 2C, D). It is interesting to note, that the position of the UV maximum and the molar extinction coefficient as well as the fluorescence spectroscopic properties of both compounds did not change significantly within the checked pH-range from 2.4 to 8.3 (data not shown). According to these results, it was expected that no easily ionisable group is a part of the chromophor or fluorophor.

The structural elucidation of compound **I** and **II** is based on one and two-dimensional NMR spectroscopy.

Table 1. ^{13}C- and ^{1}H-NMR spectroscopic data of N-[2-(2-oxo-2*H*-pyrazin-1-yl)-propyl]-phenylalanine (**I**) and N-[2-(5-methyl-2-oxo-2*H*-pyrazin-1-yl)-propionyl]-phenylalanine (**II**)

Atom assignment[a]	Compound **I** (DMSO-d_6)		Compound **II** (Methanol-d_4)	
	δ_C/ppm	δ_H/ppm	δ_C/ppm	δ_H/ppm
Pyrazinone-moiety				
2	155.03 (s)		156.63 (s)	
3	147.56 (d)	7.99 (1H, d, $^5J = 1.0$ Hz)	147.72 (d)	8.06 (1H, s)
5	122.97 (d)	7.30 (1H, d, $^3J = 4.5$ Hz)	134.06 (s)	
6	127.40 (d)	7.52 (1H, dd, $^3J = 4.5$ Hz, $^5J = 1.0$ Hz)	124.74 (d)	7.36 (1H, s)
5-Me			19.51 (q)	2.32 (3H, s)
Alanine-moiety				
1	167.50 (s)		170.87 (s)	
2	52.50 (d)	5.38 (1H, q, $^3J = 7.2$ Hz)	54.67 (d)	5.51 (1H, q, $^3J = 7.2$ Hz)
3	16.29 (q)	1.45 (3H, q, $^3J = 7.2$ Hz)	16.45 (q)	1.61 (3H, d, $^3J = 7.2$ Hz)
Phenylalanine-moiety				
1	172.71 (s)		174.32 (s)	
2	55.50 (d)	4.08 (1H, m)	55.20 (d)	4.73 (1H, dd, $^3J = 8.9$ Hz, $^3J = 4.9$ Hz)
3	37.14 (t)	H3A 2.87 (1H, dd, $^2J = 13.3$ Hz, $^3J = 6.5$ Hz) H3B 3.02 (1H, dd, $^2J = 13.3$ Hz, $^3J = 4.9$ Hz)	38.27 (t)	H3A 3.02 (1H, dd, $^2J = 14.0$ Hz, $^3J = 8.9$ Hz) H3B 3.27 (1H, dd, $^2J = 14.0$ Hz, $^3J = 8.9$ Hz)
4	138.77 (s)		138.17 (s)	
5, 5′	129.35 (d)	7.04 (2H, m)	130.37 (d)	7.19–7.26 (2H, m)
6, 6′	127.63 (d)	7.07–7.10 (2H, m)	129.43 (d)	7.19–7.26 (2H, m)
7	125.65 (d)	7.07–7.10 (1H, m)	127.73 (d)	7.19–7.26 (1H, m)
Ala-N<u>H</u>-Phe	–	7.81 (1H, d, $^3J = 7.1$ Hz)		–

[a] For atom numbering, see Fig. 3

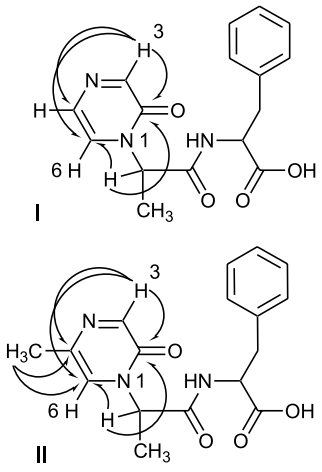

Fig. 4. Structural formulae of N-[2-(2-oxo-2*H*-pyrazin-1-yl)-propyl]-phenylalanine (compound **I**) and N-[2-(5-methyl-2-oxo-2*H*-pyrazin-1-yl)-propionyl]-phenylalanine (compound **II**) (arrows indicating the characteristic carbon-proton long range coupling connectivities from HMBC spectra)

The ^{1}H- and ^{13}C-NMR data are shown in Table 1 and confirmed structures are shown in Fig. 4.

In the spectra of the glyoxal-derived product **I**, beside the signals of the alanine and phenylalanine of the peptide-moiety additional signals in the olefinic region were observed. The signal of the methine proton of alanine showed a notable downfield shift from 4.37 ppm in the starting peptide to 5.38 ppm in the product, indicating the exposition to a new-formed electron-withdrawing group. In the HMBC experiment, correlations between the α-proton of alanine and C-2 and C-6 of the new part of the molecule were observed. This result showed that C-2 and C-6 are adjusted to the nitrogen atom. ^{1}H–^{1}H COSY and HSQC revealed the neighbouring of the methine group CH-5 to C-6. The methine proton, which appeared as small doublet at 7.99 ppm in the proton spectrum, was assigned to H-3 and showed cross peaks to C-5 and to the quaternary C-2 as well as a weak correlation to C-6 in HMBC spectra. In summary, the results of the two-dimensional NMR-experiments established the pyrazinone structure N-[2-(2-oxo-2*H*-pyrazin-1-yl)-propyl]-phenylalanine (compound **I** in Fig. 4). The deduced chemical structure is consistent with the results of mass spectrometry.

For the methylglyoxal-derived product, the pyrazinone N-[2-(5-methyl-2-oxo-2*H*-pyrazin-1-yl)-propionyl]-phenylalanine could be identified in the same manner (compound **II** in Fig. 4). In the case of methylglyoxal, the formation of two products is possible, which differ in the position of the methyl group (position 5 or 6 on the pyrazinone ring). Elucidation was achieved using the carbon-proton long range coupling connectivities from HMBC spectrum as shown in Fig. 4, clearly indicating the methyl group at position 5.

Formation of pyrazinone-modified peptides can be explained according to the reaction scheme shown in Fig. 5. Addition of glyoxal to the primary amino group may lead to a Schiff base. Following a tautomerization, the amido nitrogen of the first peptide bond nucleophile attacks the remaining carbonyl C-atom, followed by an elimination of a second molecule of water. This reaction is postulated in earlier work occurring under drastic and non-physiological conditions (Prey and Petershofer, 1968). Van Chuyen et al. (1973) discussed this reaction beside the well-known Strecker degradation in the context of peptide modifications occurring during food processing and heating at 100°C.

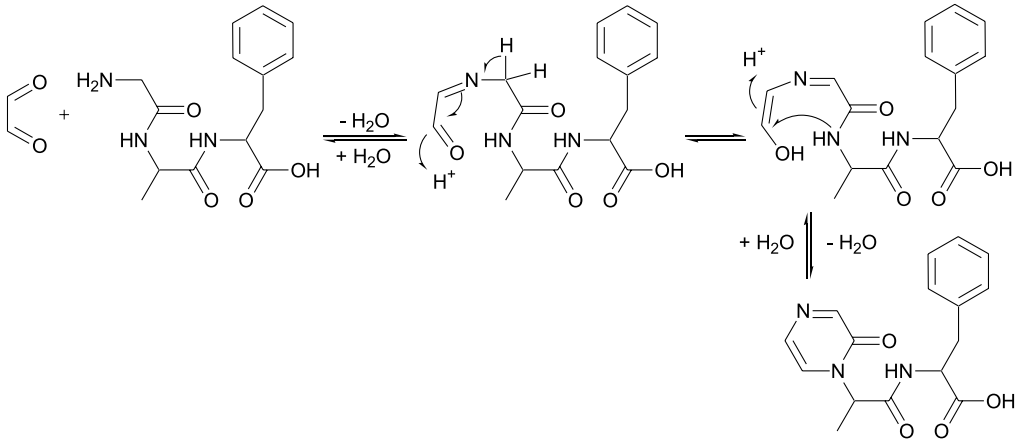

Fig. 5. Proposed reaction pathway for the formation of N-[2-(2-oxo-2*H*-pyrazin-1-yl)-propyl]-phenylalanine (compound **I**) from glyoxal and Gly-Ala-Phe

Our results clearly proof the formation of pyrazinone-moiety at the N-terminus of peptides under physiological conditions. Especially in the case of glyoxal, the reaction is very rapid and very specific, meaning that only one product is formed.

For further characterization, compound I was incubated in a wide pH-range from 2.0 to 9.1 at a elevated temperature of 60°C for 3 d. Using HPLC, no degradation was observed. This indicates that the glyoxal-related modification of the peptide is fairly stable.

Due to the fact that the 5-methyl-pyrazinone compound **II** is the main fluorescent product resulting from the reaction of Gly-Ala-Phe with methylglyoxal, pyrazinone formation must be highly regioselective. In general, an aldehyde function is more reactive than a ketonic carbonyl group. In the case of methylglyoxal, the aldehyde group is hydrated in aqueous solution (Thornalley, 1996). According to this, the nucleophilic attack of the primary amino group is targeted on the ketone function in the first step, which can explain the observed regioselectivity.

The remarkable high reactivity of the α-dicarbonyl compounds glyoxal and methylglyoxal raised the question about the relevance of the observed reaction for biological systems. As mentioned in the introduction, the guanidino side chain of arginine and the ε-amino group of lysine are generally discussed as the primary targets for α-dicarbonyl induced protein modification in foods and *in vivo*. To compare the reactivity of these side-chains with the N-terminal amino group and the first peptide bond, competitive incubation experiments with N^{α}-hippurylarginine and N^{α}-hippuryllysine as model-peptides for the protein-bonded amino acids were made. As shown in Fig. 6, the N-terminus of the peptide Gly-Ala-Phe and the guanidino function of arginine showed almost the same reactivity for a derivatization by glyoxal in single incubations with glyoxal as well as under competitive conditions. The reactivity of lysine in this experimental design was negligible. Corresponding data were obtained for methylglyoxal (data not shown). Based on these results, it can be concluded that the N-termini of peptides are of similar importance as arginine for reactions with α-dicarbonyl compounds.

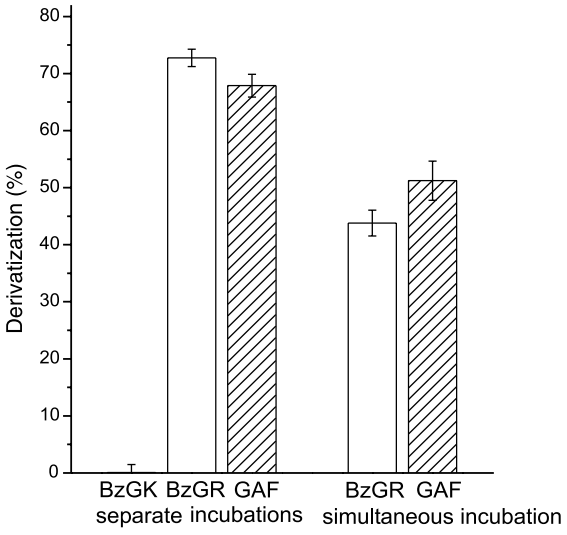

Fig. 6. Derivatization yield after separate incubation of BzGK (N^{α}-hippuryllysine), BzGR (N^{α}-hippurylarginine, open bars) or Gly-Ala-Phe (striped bars) with glyoxal and after simultaneous incubation of BzGR (open bars) and Gly-Ala-Phe (striped bars) with glyoxal (peptide(s) and glyoxal: 5 mM each, 100 mM PB pH = 7.4, 37°C, 24 h). Mean value ± standard deviation of three separate determinations

Reaction of insulin with glyoxal

To determine whether the new pyrazinone structure is also formed at the N-terminus of proteins under physiological conditions, the reaction of bovine insulin with glyoxal was investigated. For this purpose, insulin (17 μM) was incubated with glyoxal (50 μM) in PBS at 37°C for 15 d. The chosen molar ratio results in one molecule glyoxal for each potential reaction site of the insulin molecule, namely the N-terminus of the A- and B-chain as well as the single arginine residue within the B-chain. The samples were analysed using HPLC directly after the incubation as well as after reducing the disulfide bridges and blocking of the thiols for separation of the chains.

Figure 7 shows the chromatograms of the reaction mixture obtained via detection at 220 and 320 nm as well

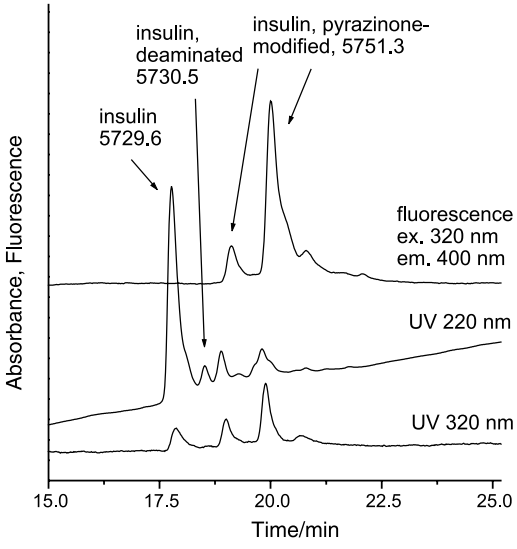

Fig. 7. HPLC analysis of incubation mixture of bovine insulin with glyoxal (17 μM and 50 μM, respectively, PBS pH = 7.4, 37°C, 15 d) with UV and fluorescence detection as indicated. Relative molecular masses (monoisotopic) given were determined via LC-ESI-MS

as fluorescence detection at excitation/emission wavelengths of 320/400 nm. It can be seen, that peaks with UV absorbance and fluorescence characteristic for pyrazinones are formed. Coupling of the LC system with mass spectrometric detection indicated a monoisotopic mass of $M_r = 5751.3$ for the fluorescent components eluting with retention times between 18.7 and 20.2 min in the chromatogram. The mass difference of 21.7 compared to the non-modified insulin molecule is consistent with the formation of a pyrazinone structure and substantiates the formation of mono-pyrazinone-modified insulin molecules. Additionally, a deaminated product was observed, eluting at 18.5 min in the chromatogram.

In the particular case of insulin, two N-termini are possible reaction sites for glyoxal. From non-enzymatic glycation reactions of proteins with glucose, it is known, that certain N-terminal α-amino groups or ε-amino groups of lysine show a higher reactivity. For instance, the α-amino group of terminal valine residue at the β-chain of haemoglobin (Bunn et al., 1979) or the ε-amino group of lysine-525 in human serum albumin (Garlick and Mazer, 1983) are preferential glycation sites *in vivo*. To answer the question, whether modification by glyoxal is also a site-specific process, the A- and B-chain of insulin were separated after incubation, by reducing the disulfide bridges and blocking of the thiols. HPLC analysis revealed the formation of two fluorescent peaks. Mass spectrometric detection (LC-MS) enabled identification of this peaks as the pyrazinone-modified A- and B-chain (Fig. 8). Comparing peak areas of the unmodified and modified chains obtained after detection at 220 nm showed that $(2.0 \pm 0.4)\%$ of the A-chain and $(5.9 \pm 0.6)\%$ of the B-chain were pyrazinone-modified. Consequently, glycation by glyoxal should be site-specific in the used experimental system.

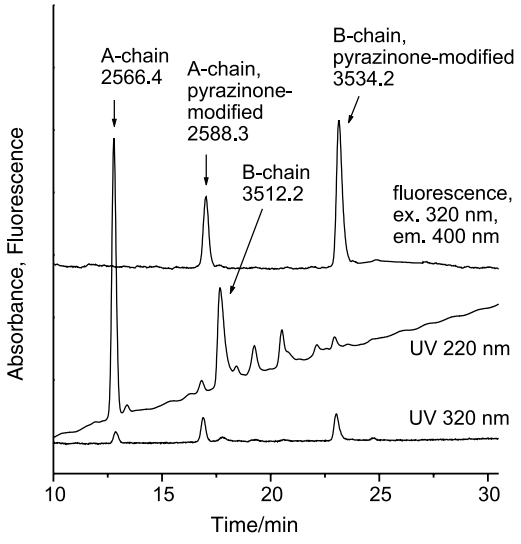

Fig. 8. HPLC analysis of incubation mixture of bovine insulin with glyoxal (17 μM and 50 μM, respectively, PBS pH = 7.4, 37°C, 15 d) with UV and fluorescence detection as indicated. Disulfide bridges reduced by dithiothreitol and sulfhydryl groups blocked by iodacetamide. Relative molecular masses (monoisotopic) given were determined via LC-ESI-MS

The same order of reactivity was observed for the modification of insulin by glucose in dry state at a relative humidity of approximately 70% and at 37°C (Schwartz and Lea, 1952; Amaya-F et al., 1976) as well as in solution under physiological conditions (O'Harte et al., 1996, 2000). To clarify a possible derivatization of Lys(B29) or Arg(B22), the LC-MS spectra were checked for possible products, like CML, 5-(4,5-Dihydroxy-2-imino-1-imidazolidinyl)-norvaline, 5-(2-imino-5-oxo-1-imidazolidinyl)-norvaline ("Glarg") and N^7-carboxymethyl-arginine. None of these modifications could be detected.

In order to explain the preferential formation of the pyrazinone structure at the B-chain, the pK_S-value of the two terminal amino groups must be considered. As the formation of pyrazinones starts by a nucleophilic attack of the amino group and the formation of the Schiff base, the reaction rate should increase with increasing amounts of unprotonated amino groups. The lower pK_S-value of the α-amino group of N-terminal phenylalanine at the B-chain ($pK_S = 7.1$) compared to glycine at the A-chain ($pK_S = 8.4$, Gao et al., 1995) is a possible reason for its higher reactivity. Additional factors like the effect of neighbouring groups during the next intramolecular steps and the accessibility of the individual N-Termini may play also a role. To discuss the accessibility, it is important to note that insulin is present in a dimeric state at a concentration of 17 μM used in our experiments (Kaarsholm and Ludvigsen, 1995). Based on the crystal structure (Blundell et al., 1972) as well as the structure in solution (Kaarsholm and Ludvigsen, 1995) neither of the N-Termini is known to be involved in the dimer forming part of the molecule. We therefore assume that both reaction sites should be accessible for glyoxal.

Taken together, our model studies have shown a high reactivity of the N-terminus of peptides for modification by the α-dicarbonyl compounds glyoxal and methylglyoxal. This reaction leads to well-defined and stable products, showing characteristic UV absorbance and fluorescence. Structural elucidation revealed the formation of a pyrazinone-moiety at the N-terminus of the starting peptide. Further experiments with the model protein insulin and glyoxal unequivocally demonstrated the formation of the corresponding protein-bound N-terminal pyrazinones under physiological conditions.

AGE-specific fluorescence is often measured for the evaluation of the extent of the Maillard reaction in foods and *in vivo*, but the structure of the fluorophores is only partially known (Kollias et al., 1998; Avendano et al., 1999; Birlouez-Aragon et al., 2001). In this context, the formation of pyrazinones can contribute to the observed fluorescence in thermally treated foods and *in vivo*.

We conclude that the pyrazinone derivatization of peptides and proteins represents a new class of posttranslational modifications, which have to be taken into account as advanced glycation end-products (AGEs). Since the precursors glyoxal and methylglyoxal are present ubiquitously in biological systems as well as in thermal-treated and fermented foods, the formation of N-terminal pyrazinones in these systems is very likely. Corresponding studies about the occurrence of pyrazinone-modified peptides and proteins in foods and biological systems as well as concerning physiological and nutritional consequences are underway in our laboratory.

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References

Ahmed MU, Baynes JW, Thorpe SR (1986) Identification of N^{ε}-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. J Biol Chem 261: 4889–4894

Ahmed MU, Brinkmann Frye E, Degenhardt TP, Thorpe SR, Baynes JW (1997) N^{ε}-carboxyethyllysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. Biochem J 324: 565–570

Al-Abed Y, Bucala R (1995) N^{ε}-carboxymethyllysine formation by direct addition of glyoxal to lysine during the Maillard reaction. Bioorg Med Chem Lett 5: 2161–2162

Amaya-F J, Lee T-C, Chichester CO (1976) Biological inactivation of proteins by the Maillard reaction: Effect of mild heat on the tertiary structure of insulin. J Agric Food Chem 24: 465–467

Anderson MM, Hazen SL, Hsu FF, Heinecke JW (1997) Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to convert hydroxy-amino acids into glycolaldehyde, 2-hydroxypropanal and acrolein. J Clin Invest 99: 424–432

Avendano GF, Agarwal RK, Bashey RI, Lyons MM, Soni BJ, Jyothirmayi GN, Regan TJ (1999) Effects of glucose intolerance on myocardial function and collagen-linked glycation. Diabetes 48: 1443–1447

Birlouez-Aragon I, Leclere J, Quedraogo CL, Birlouez E, Grongnet J-F (2001) The FAST method, a rapid approach of the nutritional quality of heat-treated foods. Nahrung/Food 45: 201–205

Blundell TL, Dodson GG, Hodgkin DMC, Mercola DA (1972) Insulin: the structure in the crystal and its reflection in chemistry and biology. Adv Protein Chem 26: 279–402

Bookchin RM, Gallop PM (1968) Structure of hemoglobin A_{Ic}: nature of the N-terminal beta chain blocking group. Biochem Biophys Res Comm 32: 86–93

Bunn HF, Shapiro R, McManus M, Garrick L, McDonald MJ, Gallop PM, Gabbay KH (1979) Structural heterogeneity of human hemoglobin A due to nonenzymatic glycosylation. J Biol Chem 254: 3892–3898

Fu MX, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR (1996) The advanced glycation end product, N^{ε}-(carboxymethyl)lysine, is a product of both lipid peroxydation and glycoxydation reactions. J Biol Chem 271: 9982–9986

Gao J, Mrksich M, Gomez FA, Whitesides GM (1995) Using capillary electrophoresis to follow the acetylation of the amino groups of insulin and to estimate their basicities. Anal Chem 67: 3093–3100, correction Anal Chem 68: 2287

Garlick RL, Mazer JS (1983) The principal site of nonenzymatic glycosylation of human serum albumin *in vivo*. J Biol Chem 258: 6142–6146

Glomb MA, Lang G (2001) Isolation and characterization of glyoxal-arginine modifications. J Agric Food Chem 49: 1493–1501

Glomb MA, Monnier VM (1995) Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. J Biol Chem 270: 10017–10026

Glomb MA, Pfahler C (2001) Amides are novel protein modifications formed by physiological sugars. J Biol Chem 276: 41638–41647

Grandhee SK, Monnier VM (1991) Mechanism of formation of Maillard protein cross-link pentosidine. Glucose, fructose and ascorbate as pentosidine precursors. J Biol Chem 266: 11649–11653

Gray WR (1997) Disulfide bonds between cysteine residues. In: Creighton TE (ed) Protein structure. A practical approach. IRL Press, Oxford, pp 164–186

Henle T, Deppisch R, Beck W, Hergesell O, Haensch GM, Ritz E (1999) Advanced glycated end-products (AGE) during haemodialysis treatment: discrepant results with different methodologies reflecting the heterogeneity of AGE compounds. Nephrol Dial Transplant 14: 1968–1975

Kaarsholm NC, Ludvigsen S (1995) The high resolution solution structure of the insulin monomer determined by NMR. Receptor 5: 1–8

Krause R, Knoll K, Henle T (2003) Studies on the formation of furosine and pyridosine during acid hydrolysis of different Amadori products of lysine. Eur Food Res Technol 216: 277–283

Kollias N, Gillies R, Moran M, Kochevar IE, Anderson RR (1998) Endogenous skin fluorescence includes bands that may serve as quantitative markers of aging and photoaging. J Invest Dermatol 111: 776–780

Lapolla A, Flamini R, Vedova AD, Senesi A, Reitano R, Fedele D, Basso E, Seraglia R, Traldi P (2003) Glyoxal and methylglyoxal levels in diabetic patients: quantitative determination by a new GC/MS method. Clin Chem Lab Med 41: 1166–1173

Lyons TJ (2002) Glycation, carbonyl stress, EAGLEs, and the vascular complications of diabetes. Vasc Med 2: 175–189

Miyata T, Kurokawa T, VanYpersele DeStrihou C (2000a) Relevance of oxidative and carbonyl stress to long-term uremic complications. Kidney Int 58 [Suppl 76]: 120–125

Miyata T, Kurokawa T, VanYpersele DeStrihou C (2000b) Advanced glycation and lipoxidation end products: role of reactive carbonyl compounds generated during carbohydrate and lipid metabolism. J Am Soc Nephrol 11: 1744–1752

Nakayama T, Hayase F, Kato H (1980) Formation of ε-(2-formyl-5-hydroxymethyl-pyrrol-1-yl)-L-norleucine in the Maillard reaction between D-glucose and L-lysine. Agric Biol Chem 44: 1201–1202

Nilsson-Thorell CB, Muscalu N, Andren AHG, Kjellstrand PTT, Wieslander AP (1993) Heat sterilization of fluids for peritoneal dialysis give rise to aldehydes. Perit Dial Int 13: 208–213

Niwa T, Katsuzaki T, Ishizaki Y, Hayase F, Miyazaki T, Uematsu T, Tatemichi N, Takei Y (1997) Imidazolone, a novel advanced glycation

end product, is present at high levels in kidneys of rats with streptozotocin-induced diabetes. FEBS Lett 407: 297–302

Odani H, Matsumoto Y, Shinzato T, Usami J, Maeda K (1999a) Mass spectrometric study on the protein chemical modification of uremic patients in advanced Maillard reaction. J Chrom B 731: 131–140

Odani H, Shinzato T, Matsumoto Y, Usami J, Maeda K (1999b) Increase in three α,β-dicarbonyl compound levels in human uremic plasma: specific *in vivo* determination of intermediates in advanced Maillard reaction. Biochem Biophys Res Comm 256: 89–93

Odani H, Iijima K, Nakata M, Miyata S, Kusunoki H, Yasuda Y, Yoshiyuki H, Irie S, Maeda K, Fujimoto D (2001) Identification of N^{ω}-carboxymethylarginine, a new advanced glycation end-product in serum proteins of diabetic patients: possibility of a new marker of aging and diabetes. Biochem Biophys Res Comm 285: 1232–1236

O'Harte FPM, Højrup P, Barett CR, Flatt PR (1996) Identification of the site of glycation of human insulin. Peptides 17: 1323–1330

O'Harte FPM, Boyd AC, McKillop AM, Abdel-Wahab YHA, McNulty H, Barett CR, Conlon JM, Højrup P, Flatt PR (2000) Structure, antihyperglycemic activity and cellular actions of a novel diglycated human insulin. Peptides 21: 1519–1526

Prey V, Petershofer G (1968) Zur Kenntnis von Melanoidinvorprodukten. Z Zuckerind 18: 63–65

Raj DSC, Choudhury D, Welburne TC, Levi M (2000) Advanced glycation end products: a nephrologist's perspective. Am J Kidney Dis 35: 365–380

Schwartz HM, Lea CH (1952) The reaction between proteins and reducing sugars in the dry state. Relative reactivity of the α- and ε-amino groups of insulin. Biochem J 50: 713–716

Schwarzenbolz U, Henle T, Haessner R, Klostermeyer H (1997) On the reaction of glyoxal with proteins. Z Lebensm Unters Forsch A 205: 121–124

Singh R, Barden A, Mori T, Beilin L (2001) Advanced glycation end-products: a review. Diabetologia 44: 129–146

Thornalley PJ (1996) Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification – a role in pathogenesis and antiproliferative chemotherapy. Gen Pharmac 27: 565–573

Thornalley PJ (1999) The clinical significance of glycation. Clin Lab 45: 263–273

Thornalley PJ, Langborg A, Minhas HS (1999) Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. Biochem J 344: 109–116

Thorpe SR, Baynes JW (2003) Maillard reaction products in tissue proteins: new products and new perspectives. Amino Acids 25: 275–281

Van Chuyen N, Kurata T, Fujimaki M (1973) Studies on the reaction of dipeptides with glyoxal. Agric Biol Chem 37: 327–334

Weiss MF, Erhard P, Kader-Attia FA, Wu YC, DeOreo PB, Araki A, Glomb MA, Monnier VM (2000) Mechanism for the formation of glycoxidation products in end-stage renal disease. Kidney Int 57: 2571–2585

Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, Baynes JW (1995) Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. Biochemistry 34: 3702–3709

Wilker SC, Chellan P, Arnold BM, Nagaraj RH (2001) Chromatographic quantification of argpyrimidine, a methylglyoxal-derived product in tissue proteins: comparison with pentosidine. Anal Biochem 290: 353–358

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