Communications

Characterization of an Imidazolium Salt Formed from Glyoxal and N^{α}-Hippuryllysine: A Model for Maillard Reaction Crosslinks in Proteins

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The Maillard reaction is a complex series of reactions between reducing sugars and amino groups on proteins which leads to browning, fluorescence, and crosslinking of protein.[3,4] In long-lived tissue proteins, these chemical modifications accumulate with age and may contribute to pathophysiologies associated with aging[5] and long-term complications of diabetes.[6] α-Dicarbonyl compounds, such as glyoxal (GO), methylglyoxal, and deoxyglucosones, are important intermediates in this reaction. They may be formed by reverse aldol, dehydration, and intramolecular rearrangement reactions or by oxidation of sugar or sugar adducts to proteins. We have shown previously that oxidation reactions are rate-limiting for the browning of proteins by glucose[7,8] and have identified GO as the dicarbonyl compound formed on autoxidation of glucose in physiological buffers.[9] We also showed that GO was a precursor of N^{ϵ}-(carboxymethyl)lysine, a characteristic modification of lysine residues, formed during the Maillard reaction.[9] Since GO is also a potent crosslinker of protein, we studied the reaction of GO with the peptide mimic, N^{α}-hippuryllysine (N^{α}-(benzoylglycyl)-lysine) (BGL), under physiological conditions (pH 7.4, 37 °C). We report here the isolation and structural characterization of an imidazolium salt, 1,3-bis-N^{α}-hippuryllysine–imidazolium salt, or glyoxal–lysine dimer (GOLD) (Figure 1), a proposed model for crosslinks formed in protein during the Maillard reaction.

When GO (0.125 M) was incubated with BGL (0.25 M) in 0.20 M phosphate buffer at 37 °C under air or N_2, the reaction mixture darkened rapidly and GO disappeared with a half-life of ~3 days. The pH was adjusted daily to 7.4 by addition of 5 M NaOH. GO was also readjusted to 0.125 M at 3 day intervals, and maximum product yield was obtained at 14 days. The reaction was monitored for products by C_{18} reversed-phase HPLC (RP-HPLC) using a photodiode array detector; BGL-containing products were distinguished by characteristic spectra with $\lambda_{max} = 228$ nm. Two major products were identified, accounting for ~65% of initial BGL. The first product (33% yield), in order of elution during RP-HPLC, was identified as the N^{ϵ}-(carboxymethyl) derivative of the lysine residue of BGL (BG-CML), structurally analogous to N^{ϵ}-(carboxymethyl)lysine formed on reaction of GO with the N^{ϵ}-amino group of lysine residues in protein.[9] The structure of BG-CML was confirmed by its molecular weight ($m/z = 375$) on electrospray mass spectrometry (ESI-MS) and by the recovery of equal amounts of glycine and N^{ϵ}-(carboxymethyl)lysine on hydrolysis and amino acid analysis. The second major product, eventually identified as GOLD (32% yield), gave a molecular ion of $m/z = 649$ by ESI-MS, suggesting that it was a crosslink between two BGL molecules (molecular weight of BGL is 307).

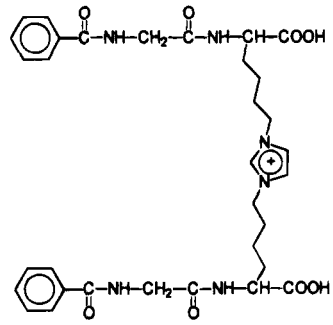

Figure 1. Structure of GOLD.

GOLD (60 mg) was isolated from a 6 mL reaction mixture by preparative RP-HPLC and characterized by NMR spectroscopy (^{1}H, ^{13}C, INEPT, ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY, and INADEQUATE) and mass spectrometry (ESI, low, and high-resolution FAB). GOLD had no appreciable UV absorbance other than the maximum at 228 nm characteristic of the hippuryl moiety. In addition to resonances characteristic of BGL, ^{1}H NMR[10] of GOLD in D_2O showed a singlet resonance at 8.61 ppm and a closely spaced doublet at 7.30 ppm ($J = 1.1$ Hz)[11] with a 0.74:2 area ratio corresponding to the C-2 and C-4(5) protons on the imidazolium ring, respectively. These resonances are consistent with previously reported values for a series of imidazolium compounds.[12] Two-dimensional ^{1}H–^{1}H COSY NMR analysis confirmed that the C-2 and C-4(5) protons were coupled.[11] Proton-decoupled ^{13}C NMR of GOLD in D_2O showed two new resonances

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(10) **BGL**: NMR spectroscopy were acquired on a Bruker AM-500 spectrometer running at 125 and 500 MHz for ^{1}H and 13C, respectively. Samples were in 100 mM phosphate buffer, pH 7.4. ^{1}H NMR (D_2O) δ 1.21–1.89 (6H, m), 2.8–3.1 (2H, t), 3.98–4.14 (2H, q), 4.16–4.24 (1H, q), 7.38–7.80 (5H, m); ^{13}C NMR (D_2O) δ 22.52 [γ-CH_2 (Lys)], 26.81 [β-CH_2 (Lys)], 31.58 [δ-CH_2 (Lys)], 39.72 [ε-CH_2 (Lys)], 43.60 [α-CH_2 (Gly)], 55.28 [α-CH (Lys)], 127.68 [*m*-CH], 129.32 [*o*-CH], 132.99 [*p*-CH], 133.18 [*ipso*-C], 171.22 [CO (benzoyl)], 171.35 [CO (Gly)], 179.18 [COOH (Lys)]. **GOLD**: ^{1}H NMR (D_2O) δ 1.21–1.89 (12H, m), 3.98–4.31 (10H, m), 7.25–7.37 (2H, s), 7.38–7.80 (10H, m), 8.57–8.65 (1H, s); ^{13}C NMR (D_2O) δ 22.33 [γ-CH_2 (Lys)], 29.18 [β-CH_2 (Lys)], 31.46 [δ-CH_2 (Lys)], 43.66 [α-CH_2 (Gly)], 49.75 [ε-CH_2 (Lys)], 55.18 [α-CH (Lys)], 122.71 [4,5-CH (imidazolium)], 127.68 [*m*-CH], 129.35 [*o*-CH], 132.99 [*p*-CH], 133.26 [*ipso*-C], 135.44 [2-CH (imidazolium)], 171.08 [CO (benzoyl)], 171.21 [CO (Gly)], 179.05 [COOH (Lys)].

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at 135.4 and 122.7 ppm corresponding to the C-2 and C-4(5) carbons of the imidazolium ring.[12,13] Further evidence for modification of the ϵ-amino group of BGL were shifts downfield of the ϵ-CH_2 proton (~2.9–4.1 ppm) and ^{13}C (39.7–49.8 ppm) resonances. Proton-coupled ^{13}C NMR of GOLD caused the 135.7 ppm signal to split into two with a J_1 coupling value of 221 Hz. The 122.7 ppm signal was split into two sets of doublets (J_1 = 203 Hz, J_2 = 11.5 Hz) that were further split into triplets (J_3 = 3.2 Hz) from the methylene group attached to the nitrogens in the ring. These coupling constants are in close agreement with published values for 1,3-dialkylimidazolium compounds.[13] Protonation of the carbons was confirmed by INEPT analysis, revealing that the C-2 and C-4(5) carbons of the imidazolium ring were singly protonated. The C-2 and C-4(5) carbons were not connected to any other carbon atoms, as assessed by two-dimensional INADEQUATE analysis.

FAB-MS using 3-nitrobenzyl alcohol (NBA) matrix gave ions of m/z = 649 [M^+], 671 [$(M + Na - H)^+$], and 693 [$(M + 2Na - 2H)$] in the positive ion mode. In the negative ion mode, ions of m/z = 647 [$(M - 2H)^-$] and 669 [$(M - 3H + Na)^-$] were observed. High-resolution FAB-MS in the positive mode gave a mass of 649.2974. The predicted formula for the observed mass was $C_{33}H_{41}O_8N_6$, in excellent agreement (1.8 ppm error) with the proposed structure of GOLD. There were seven exchangeable protons in GOLD when analyzed by FAB-MS using deuterated NBA.[14] In addition to the exchangeable protons located at the amide bonds (4) and carboxylic acid groups (2), there was one located on the imidazolium ring which is consistent with published observations on the lability of the C-2 proton.[15] The ability of the C-2 proton to exchange with D_2O also explains the less than expected area obtained for the signal by ^1H NMR. To obtain fragmentation data on GOLD, ESI-MS/MS by collision-induced dissociation using the m/z = 649 as the parent ion gave daughter ions of m/z = 488 [100%, loss of benzoylglycine (BzGly)], 442 [53%, loss of BzGly and COOH], 359 [83%, BGL + imidazolium ring], 315 [17%, BGL + imidazolium ring − CO_2], 105 [17%, loss of Bz], further supporting the structure of GOLD. The imidazolium nucleus of GOLD was stable to conditions used for acid hydrolysis of proteins (6 N HCl for 24 h at 110 °C), yielding a product with m/z = 327 by ESI-MS, consistent with the structure of the 1,3-dilysine–imidazolium salt.

A proposed mechanism for the formation of GOLD is shown in Figure 2. One molecule of GO reacts with two molecules of BGL, forming a diimine adduct, which then reacts with another molecule of GO to form a cyclic intermediate. This intermediate undergoes a Cannizzaro-type rearrangement after nucleophilic attack by hydroxide, yielding a five-membered ring structure which loses a hydroxyl group to form GOLD. We have previously proposed a role for the Cannizzaro reaction in the formation of N^{ϵ}-(carboxymethyl)lysine during reaction of glyoxal with lysine residues in protein.[9] The recent observation of dilysine–glyoxal diimine adducts in glyoxal-treated proteins[16] now suggests a role for diimines and Cannizzaro chemistry in the formation of GOLD and related imidazolium salt crosslinks in protein during the Maillard reaction. The synthesis of GOLD by this pathway represents a previously undescribed route for synthesis of imidazolium salts from α-dicarbonyl compounds and primary amines in aqueous solution under mild conditions, i.e., neutral pH at body temperature. Our results suggest that imidazolium compounds, such as GOLD, may be formed from other dicarbonyl compounds *in vivo*, including glucosones and deoxyglucosones, and may constitute an important class of crosslinks formed in tissue proteins during the Maillard reaction.

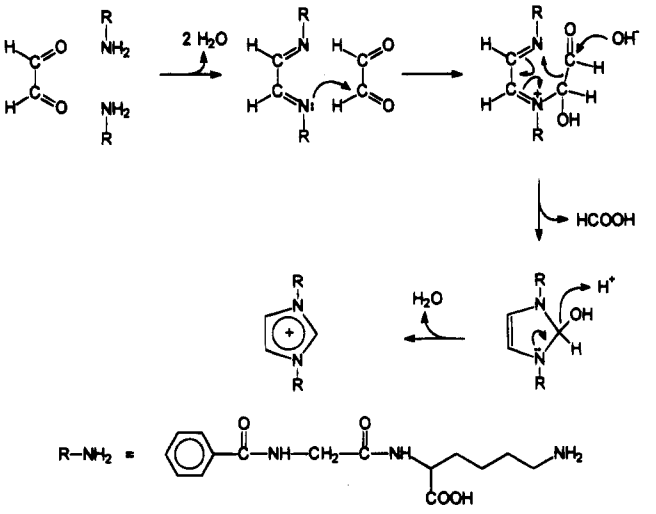

Figure 2. Proposed mechanism for formation of GOLD.

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Supporting Information Available: Experimental procedures, chromatograms, and spectra (6 pages).

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