Pathways of Formation of Glycoxidation Products during Glycation of Collagen†

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ABSTRACT: Glycoxidation products (GOPs), such as N^{ϵ}-(carboxymethyl)lysine (CML) and pentosidine, are formed during reaction of glucose with protein under oxidative conditions *in vitro*. It is uncertain whether these GOPs are derived from oxidation of Amadori adducts on protein or from oxidation of glucose or intermediates formed prior to the Amadori rearrangement. To address this question, we reacted collagen with 250 mM glucose in 200 mM phosphate buffer, pH 7.4, under antioxidative conditions, yielding a protein rich in Amadori adducts, but with only traces of GOPs. This "preglycated" collagen was then exposed to [$^{13}C_6$]glucose under oxidative conditions, producing both natural and [$^{13}C_2$]-CML. At 200 mM phosphate buffer, [$^{13}C_2$]-CML was the major product, even at low (5 mM) [$^{13}C_6$]glucose concentration, indicating a limited role for Amadori compounds in formation of CML in high phosphate. The relative yields of natural and [$^{13}C_2$]-CML varied with phosphate concentration, becoming similar at more physiological (10 mM) phosphate. We conclude that during glycation of proteins at high phosphate concentrations *in vitro*, GOPs are formed primarily by oxidation of free glucose or rapidly-formed intermediates preceding the Amadori rearrangement, such as carbinolamine or Schiff base adducts. In contrast, at lower phosphate and glucose concentrations *in vivo*, the Amadori adduct may be the more significant precursor of GOPs. The fact that glycoxidation reactions proceed by multiple routes must be considered in the development of therapeutic approaches for inhibiting the Maillard reaction in diabetes.

Both glycation and oxidation chemistry are involved in Maillard or browning reactions of proteins with reducing sugars. Glycation and oxidation reactions lead to formation of advanced glycation end-products (AGEs),[1] including the glycoxidation products (GOPs) N^{ϵ}-(carboxymethyl)lysine (CML) and pentosidine. These GOPs accumulate naturally in tissue proteins with age and at an accelerated rate during hyperglycemia in diabetes (Dunn *et al.*, 1989, 1991; Dyer *et al.*, 1991, 1993; Monnier *et al.*, 1992; Sell *et al.*, 1993). Increases in age-adjusted levels of both CML and pentosidine in tissue proteins are correlated with the severity of retinopathy and nephropathy in diabetes (Sell *et al.*,1992; Dyer *et al.*, 1993; McCance *et al.*,1993; Beisswenger *et al.*, 1993), suggesting a role for glycoxidation reactions and oxidative stress in the pathogenesis of diabetic complications (Baynes, 1991).

There is controversy about the source of GOPs and oxidative damage to proteins during the Maillard reaction. GOPs may be formed by oxidation of free glucose in solution (autoxidative glycosylation) (Wolff & Dean, 1987) followed by reaction of the oxidation products with protein; by oxidation of protein-bound Amadori adducts; by oxidation of intermediates between free glucose and the Amadori adduct, such as carbinolamine and Schiff base adducts (Hayashi & Namiki, 1986; Glomb & Monnier, 1995); or by reaction of proteins with a number of other non-glucose carbohydrates, including ascorbate, dehydroascorbate, aldoses, ketoses, glycolytic intermediates, and 3-deoxyglucosone (Dunn *et al.*, 1990; Grandhee & Monnier, 1991; Dyer *et al.*, 1991). The controversy regarding the source of GOPs is not merely academic because there is considerable interest in the design of drugs, such as aminoguanidine (Brownlee *et al.*, 1986), to inhibit the Maillard reaction *in vivo* and retard or prevent the development of diabetic complications.

The focus of this study is on determining the mechanism of formation of GOPs during reaction of protein with glucose. Ahmed *et al.* (1986, 1988) originally described CML as a product of metal ion-catalyzed, oxidative fragmentation of the Amadori compound, N^{ϵ}-(1-deoxy-D-fructos-1-yl)lysine (fructoselysine, FL). Smith and Thornalley (1992) later reported that N^{ϵ}-(carboxymethyl)hippuryllysine was formed on oxidative degradation of N^{ϵ}-(1-deoxy-D-fructos-1-yl)-hippuryllysine. Dyer *et al.* (1991) also noted that the Amadori compound N^{α}-formyl-N^{ϵ}-fructoselysine reacted with arginine to form pentosidine. The formation of GOPs in these oxidation reactions was catalyzed by transition metal ions and accompanied by the production of reactive oxygen species (ROS: $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$) which cause collateral damage to proteins, lipids, and DNA. Thus, Kawakishi *et al.* (1990) showed that, in the presence of copper ions, the Amadori compound 1-deoxy-1-β-alanino-D-fructose caused oxidative degradation of histidine residues and fragmentation

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[1] Abbreviations: AGEs, advanced glycation end-products; AG, aminoguanidine; CML, N^{ϵ}-(carboxymethyl)lysine; DTPA, diethylenetriaminepentaacetic acid; fHSA, formylated human serum albumin; FL, N^{ϵ}-fructoselysine; GOPs, glycoxidation products; HFBA, heptafluorobutyric acid; HSA, human serum albumin; *o*-Tyr, *ortho*-tyrosine; P_i, phosphate buffer; PG-collagen, preglycated collagen; RP-HPLC, reversed-phase high-performance liquid chromatography; ROS, reactive oxygen species; SDS−PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SIM−GC/MS, selected ion monitoring−gas chromatography/mass spectrometry; N^{α}-*t*-Boc-Lys, N^{α}-*tert*-(butoxycarbonyl)lysine.

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of protein, and Cheng *et al.* (1991) also noted loss of histidine residues during metal-catalyzed oxidation of glycated albumin. Hunt *et al.* (1993) demonstrated that glycated albumin, in the presence of trace concentrations of transition metals, generated ROS capable of hydroxylating benzoate and caused protein fragmentation. Others have shown that Amadori compounds and glycated peptides also promote DNA damage (Komano *et al.*, 1986) and lipid peroxidation (Sakuri *et al.*, 1990) in *in vitro* systems. Overall, the formation of GOPs and the generation of ROS from Amadori compounds suggest a role for the Amadori adduct as both a source of GOPs and a propagator of oxidative damage during the Maillard reaction in biological systems.

Other evidence suggests that oxidation of free glucose also has an important role in the modification of proteins and formation of GOPs and ROS during the Maillard reaction. Wolff and Dean (1987) introduced the term *autoxidative glycosylation* to describe the modification of proteins by reactive carbonyl compounds formed during metal-catalyzed oxidation (autoxidation) of glucose. They noted that oxidation of glucose yielded α-dicarbonyl compounds and ROS, both in the presence and in the absence of protein (Wolff & Dean, 1987; Hunt *et al.*, 1988; Jiang *et al.*, 1990). Incubations of protein with glucose under oxidizing conditions yielded increased protein fragmentation and carbohydrate incorporation compared to incubations containing the transition metal chelator diethylenetriaminepentaacetic acid (DTPA). Similarly, incubations of low-density lipoprotein and phosphatidylcholine liposomes with glucose and copper produced higher levels of thiobarbituric acid-reactive species and hydroperoxides than incubations with glucose or copper alone (Hunt *et al.*, 1990). The kinetics of these reactions proceeded at a near-maximal rate from zero time, such that it was difficult to postulate that the Amadori adduct was an intermediate in the reaction. Chace *et al.* (1991) also found that incubation of glucose with copper, prior to addition of collagen, resulted in collagen cross-linking in excess of that found in collagen incubated with fresh glucose and copper under the same conditions. Recently, Wells-Knecht *et al.* (1995) identified glyoxal as the dicarbonyl sugar formed on autoxidation of glucose in the absence of protein and showed that glyoxal reacted with protein to form CML. Arabinose, which is known to be an efficient precursor of pentosidine (Sell & Monnier, 1989), was also formed on autoxidation of glucose (Wells-Knecht *et al.*, 1995). Thus, glucose autoxidation provides an additional mechanism, independent of Amadori adduct formation, to account for formation of GOPs and oxidative damage to protein during the Maillard reaction.

In this work, we confirm that Amadori adducts of glucose to protein are a source of GOPs and ROS that can lead to protein modification, as measured by specific markers of protein glycoxidation (CML and pentosidine) and oxidation (*ortho*-tyrosine, *o*-Tyr). We also show that both Amadori products and glucose contribute to formation of GOPs, and that the contribution from each source is dependent on the relative concentrations of Amadori product and glucose and on the phosphate buffer concentration. These results provide insight into the mechanisms of formation of GOPs and ROS from glucose in a model Maillard system and provide a foundation for understanding reaction pathways occurring *in vivo*.

Table 1: Comparison of Glycation, Glycoxidation, and Oxidation Products in Native and Preglycated Collagen

collagen	FL (mmol/mol of Lys)	CML (mmol/mol of Lys)	pentosidine (μmol/mol of Lys)	*o*-Tyr (μmol/mol of Phe)
native	1.0 ± 0.1	0.06 ± 0.03	<1	<1
preglycated[a,b]	104.6 ± 3.9	0.37 ± 0.08	2.8 ± 0.2	12.8 ± 3.8
preglycated[a,c]	78.2 ± 1.0	0.37 ± 0.09	2.4 ± 0.3	nd[d]

[a] Collagen was glycated under antioxidative conditions with 250 mM glucose for 5 weeks in 200 mM phosphate, pH 7.4, at 37 °C. [b] PG-collagen used in oxidative incubations in the absence of glucose. [c] PG-collagen used in oxidative incubations in the presence of [$^{13}C_6$]glucose. [d] nd = not determined.

EXPERIMENTAL PROCEDURES

Materials. Reagents were purchased from Sigma (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI) unless otherwise specified. CML, [$^2H_8$]-CML, and [$^{13}C_6$]-FL were prepared as described previously (Knecht *et al.*, 1991). [$^{13}C_6$]-Phe (99 atom %) was purchased from Cambridge Isotope Laboratories (Woburn, MA), and [$^{13}C_6$]-*o*-Tyr was synthesized by oxidation of [$^{13}C_6$]-Phe as described by Huggins *et al.* (1993) for [$^2H_4$]-*o*-Tyr. Rat tail tendon collagen was prepared by extraction with saline followed by washing with deionized water, as described previously (Fu *et al.*, 1992).

Preglycation of Collagen under Antioxidative Conditions. To obtain collagen rich in Amadori adducts but with minimal levels of GOPs, collagen was glycated under N_2 with 250 mM glucose in degassed and N_2-purged 200 mM phosphate buffer, pH 7.4, at 37 °C for 5 weeks. This preglycated collagen (PG-collagen) was washed thoroughly with deionized water prior to use in subsequent incubations. Preglycation yielded collagen with 78 and 105 mmol of FL/mol of Lys (two separate incubations) (Table 1) compared to approximately 5 mmol of FL/mol of Lys for nondiabetic skin collagen and 7−25 mmol of FL/mol of Lys in diabetic skin collagen (Dyer *et al.*, 1993).

Incubations of Preglycated Collagen under Oxidative Conditions. Incubations of PG-collagen (~5 mg wet weight/mL) were performed in 200 mM phosphate buffer, pH 7.4, at 37 °C under air in 20 mL glass scintillation vials in the dark; several drops of toluene were added to prevent microbial growth. At various times, separate reaction vials were removed and frozen at −20 °C until analyzed.

Analytical Methods. Selected ion monitoring−gas chromatography/mass spectrometry (SIM−GC/MS) analyses were performed on a Hewlett-Packard Model 5890 gas chromatograph/5970 mass selective detector system equipped with a high-energy dynode detector (Phrasor Scientific, Inc., Duarte, CA) and a 30 m Rtx-5 (95% methyl, 5% phenyl) capillary column (Restek, Bellefonte, PA). Reversed-phase high-performance liquid chromatography (RP-HPLC) analyses were performed on a Varian Model 5500 liquid chromatograph (Varian Instruments, Sunnyvale, CA) equipped with a Shimadzu RF-535 fluorescence detector (Shimadzu Corp., Tokyo, Japan) and a Zorbax SB-C18 column (MAC-MOD Analytical, Chadds Ford, PA). Hydrolysates were dried in a Savant Speed-Vac concentrator (Savant Instruments, Farmingdale, NY), and an N-Evap (Organomation, Berlin, MA) was used to remove derivatizing reagents under a stream of N_2.

Collagen (2−5 mg wet weight) was rinsed with deionized water to remove glucose and buffer in preparation for analyses described below. For SIM−GC/MS assays, peak areas of each analyte were normalized to their respective isotopically-labeled internal standards, and quantitation was based on calibration curves generated using increasing amounts of each analyte and a constant amount of internal standard. To normalize to the amount of protein analyzed, final values of glycation, glycoxidation, and oxidation products were expressed as a ratio to their precursor amino acids, lysine or phenylalanine.

Measurement of FL. Internal standards for FL (1 nmol of [$^{13}C_6$]-FL) and Lys (75 nmol of [$^2H_8$]-Lys) were added to collagen samples prior to hydrolysis in 7.8 N HCl under N_2 for 24 h at 110 °C (0.5−1 mg/mL protein concentration) (Erbersdobler, 1986). Dried hydrolysates were derivatized by adding 700 μL of trifluoroacetic anhydride and 500 μL of trifluoroethanol, followed by heating at 80 °C for 1 h to form the *N,O*-trifluoroacetyl trifluoroethyl ester derivatives (Gieseg *et al.*, 1993). Derivatizing reagents were removed under a stream of N_2, and the samples were dissolved in 100 μL of ethyl acetate for GC/MS analysis. FL was measured as the *N,O*-trifluoroacetyl trifluoroethyl ester derivative of its hydrolysis product furosine. FL, [$^{13}C_6$]-FL, Lys, and [$^2H_8$]-Lys were measured in the same sample by SIM−GC/MS using the 110, 116, 180, and 187 ions, respectively (Wells-Knecht, 1995). The following temperature program was used: 2 min at 150 °C, ramp at 6 °C/min to 180 °C, ramp at 15 °C/min to 300 °C, hold at 300 °C for 3 min. For samples incubated in the presence of [$^{13}C_6$]-glucose, the internal standard [$^2H_8$]-CML was used instead of [$^{13}C_6$]-FL since [$^{13}C_6$]-FL was formed in the collagen during the incubation.

Measurement of CML. Collagen was reduced with $NaBH_4$ (200 μL of 100 mg of $NaBH_4$/mL of 0.1 N NaOH) in 4 mL of 200 mM sodium borate buffer, pH 9.1, overnight at 4 °C; reduction was necessary to convert protein-bound FL to hexitollysine, thereby preventing artifactual formation of CML by oxidative cleavage of FL during acid hydrolysis (Knecht *et al.*, 1991). The reducing solution was decanted, and the collagen was rinsed with deionized water. Internal standards for CML (3 nmol of [$^2H_8$]CML) and Lys (75 nmol of [$^2H_8$]-Lys) were added, and the samples were hydrolyzed, derivatized, and analyzed as described for FL. CML, [$^2H_8$]-CML, Lys, and [$^2H_8$]-Lys were analyzed by monitoring the 460, 468, 180, and 187 ions, respectively (Wells-Knecht, 1995). [$^{13}C_2$]-CML was analyzed by monitoring the 462 ion and quantitated using the CML standard curve after correction for a 2.5% isotopic contribution from the 460 ion.

Measurement of o-Tyr. Internal standards for *o*-Tyr (0.05 nmol of [$^{13}C_6$]-*o*-Tyr) and Phe (130 nmol of [$^{13}C_6$]-Phe) were added to samples, and Phe and *o*-Tyr were measured in hydrolysates by SIM−GC/MS as their acetyl isopropyl ester derivatives, as described previously (Huggins *et al.*, 1993). The 265 and 271 ions were monitored for *o*-Tyr and [$^{13}C_6$]-*o*-Tyr, and the 249 and 255 ions for Phe and [$^{13}C_6$]-Phe, respectively.

Measurement of Pentosidine. Pentosidine was analyzed in reduced hydrolysates by RP-HPLC with fluorescence detection (E_x = 328 nm, E_m = 378 nm) (Dyer *et al.*, 1991). The buffer gradient was 28% B to 30% B from 0 to 45 min (buffer A = 0.1% HFBA in deionized water; buffer B = 0.1% HFBA in 50% acetonitrile/50% deionized water) at a flow rate of 1 mL/min. After each sample, the column was washed with 100% acetonitrile for 15 min and then equilibrated in 28% B. The hydroxyproline content of the sample was measured by the method of Stegemann and Stalder (1967). Pentosidine values were normalized to hydroxyproline to correct for differences in amounts of protein analyzed. Final pentosidine levels were expressed relative to the Lys content of the collagen based on the molar ratio of hydroxyproline to Lys in rat tail collagen, which was determined empirically to be 0.43 (Fu *et al.*, 1992).

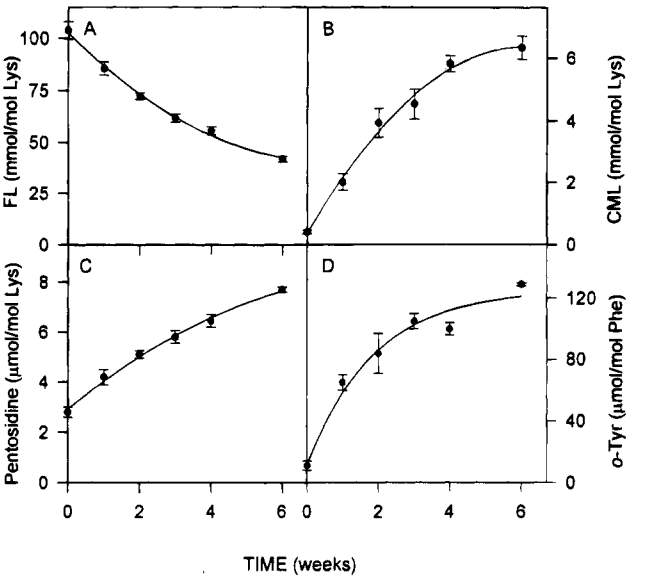

FIGURE 1: Kinetics of decomposition of FL and formation of CML, pentosidine, and *o*-Tyr during oxidative incubations of PG-collagen. PG-collagen was prepared under antioxidative conditions with 250 mM glucose and then incubated in the absence of glucose under oxidative conditions in 200 mM phosphate, pH 7.4 at 37 °C. Samples were analyzed at the time points indicated for (A) FL, (B) CML, (C) pentosidine, and (D) *o*-Tyr.

Data Analysis. Points in Figure 1 represent means ± 1 SD of three incubations for FL, CML, and *o*-Tyr and means ± ranges of two incubations for pentosidine. Results in tables are means ± 1 SD of triplicate analyses of the same incubation and are representative of results obtained for two or more incubations under each condition. Errors for calculations of percent contribution of [$^{13}C_2$]-CML to the total CML (Tables 2 and 4) were derived by propagation of errors (Taylor, 1982). Statistical significance was evaluated by the Student's *t*-test using SigmaStat (Jandel Scientific, San Rafael, CA).

RESULTS

Formation of Glycoxidation and Oxidation Products from Preglycated Collagen. PG-collagen, a protein rich in Amadori adducts but with only low levels of GOPs, was used for studies on formation of glycoxidation and oxidation products from Amadori adducts on protein. The FL, CML, pentosidine, and *o*-Tyr content of the native and PG-collagens is summarized in Table 1. PG-collagen was incubated for various times (0−6 weeks) under air in the absence of glucose. During this incubation, the Amadori adduct, FL, decreased in a first-order fashion to 40.4 ± 1.4 mmol of FL/mol of Lys at 6 weeks, representing a loss of 60% of the initial FL content of the protein, with a half-life of 4.6 ± 0.2 weeks (Figure 1A). At the same time, there was a time-

Table 2: Comparison of Glycoxidation and Oxidation Products in Preglycated Collagen Incubated in the Absence of Glucose and Native Collagen Incubated in the Presence of Glucose

collagen	glucose (mM)	CML (mmol/mol of Lys)	pentosidine (μmol/mol of Lys)	o-Tyr (μmol/mol of Phe)
preglycated[a]	0	6.3 ± 0.4	7.7 ± 0.1	129 ± 1
native[b]	250	34.6 ± 1.3	60.0 ± 6.5	1760 ± 96

[a] Collagen was glycated under antioxidative conditions with 250 mM glucose for 5 weeks and contained 104.6 ± 3.9 mmol of FL/mol of Lys and 0.37 ± 0.08 mmol of CML/mol of Lys (Table 1). It was then incubated under oxidative conditions for 6 weeks in 200 mM phosphate, pH 7.4, at 37 °C. [b] Native collagen was incubated under oxidative conditions for 5 weeks in 200 mM phosphate, pH 7.4, at 37 °C.

dependent increase in CML, pentosidine, and *o*-Tyr (Figure 1B–D), yielding approximately 6 mmol of CML/mol of Lys, 8 μmol of pentosidine/mol of Lys, and 120 μmol *of o*-Tyr/mol of Phe. The yields of CML and pentosidine accounted for 10% and 0.01% of the FL loss, respectively. These experiments establish that Amadori adducts on collagen are precursors of GOPs, both CML and pentosidine, and that oxidative degradation of Amadori adducts induces collateral oxidative damage to amino acids in the protein. Other products formed from degradation of Amadori adducts were not characterized, but are likely to include mannose, glucose, tetroses, and 3-deoxyglucosone, as described in similar systems by Zyzak *et al.* (1995).

As shown in Table 2, the oxidative incubation of PG-collagen for 6 weeks yielded only 20% of the CML, 13% of the pentosidine, and 7% of the *o*-Tyr produced in parallel oxidative incubations of native collagen with 250 mM glucose. At first glance, the higher yields of both GOPs and *o*-Tyr in the collagen incubated with glucose, compared to the oxidative incubation of PG-collagen, suggested that glucose, rather than Amadori adducts on protein, was the primary precursor of GOPs and ROS during the Maillard reaction. However, there were significant differences in oxidative stress in the two incubations, as indicated by the more than 10-fold difference in *o*-Tyr formation. It was, therefore, not possible to determine whether the lower production of GOPs during incubation of PG-collagen resulted from a dominant role of glucose in the formation of GOPs or from decreased oxidation of Amadori adducts because of decreased oxidative stress in the PG-collagen incubations.

Experimental Design for Assessing the Contribution of Amadori Adducts and Glucose to Formation of CML. To study the relative contributions of protein-bound Amadori adducts and free glucose to formation of CML at an equivalent level of oxidative stress, PG-collagen was incubated with [$^{13}C_6$]glucose under oxidative conditions. This experimental system permits differentiation between the two possible sources of CML because the Amadori adduct in PG-collagen, derived from natural glucose, would yield [$^{12}C_2$]-CML, while the [$^{13}C_6$]glucose would yield [$^{13}C_2$]-CML. This experimental design also eliminates problems resulting from differences in oxidative stress in parallel incubations. The formation of [$^{12}C_2$]-CML, [$^{13}C_2$]-CML, and [$^{13}C_6$]-FL and the decomposition of FL were compared as a function of both [$^{13}C_6$]glucose concentration (Tables 3 and 4) and phosphate concentration (Tables 5 and 6).

As shown in Table 3, at all concentrations of [$^{13}C_6$]glucose studied in 200 mM phosphate buffer, the majority of CML

Table 3: Effect of Glucose Concentration on CML Formation from FL and Glucose[a]

[$^{13}C_6$]glucose (mM)	[$^{12}C_2$]-CML[b] (mmol/mol of Lys)	[$^{13}C_2$]-CML (mmol/mol of Lys)	% total CML as [$^{13}C_2$]-CML[c]
0	6.38 ± 0.24	0.0	0.0
5	5.52 ± 0.11	10.60 ± 0.68	65.8 ± 1.5
25	4.42 ± 0.22	20.50 ± 1.13	82.3 ± 1.1
100	3.68 ± 0.08	29.07 ± 3.30	88.8 ± 1.2
250	3.40 ± 0.29	38.97 ± 0.66	91.9 ± 0.6

[a] Collagen was glycated under antioxidative conditions with 250 mM glucose (PG-collagen) and contained 78.2 ± 1.0 mmol of FL/mol of Lys and 0.37 ± 0.09 mmol of CML/mol of Lys. PG-collagen was then incubated under oxidative conditions for 5 weeks in 200 mM phosphate, pH 7.4, at 37 °C in the presence of [$^{13}C_6$]glucose at the concentrations indicated. [b] [$^{12}C_2$]-CML = CML derived from FL on PG-collagen. [c] % total CML as [$^{13}C_2$]-CML = [$^{13}C_2$]-CML/([$^{13}C_2$]-CML + [$^{12}C_2$]-CML) × 100.

Table 4: Effect of Glucose Concentration on FL Decomposition and Conversion to CML[a]

[$^{13}C_6$] glucose (mM)	FL[b] (mmol/mol of Lys)	[$^{13}C_6$]-FL[b] (mmol/mol of Lys)	% FL loss[c]	% FL to CML[d]
0	33.4 ± 5.5	0.0	57.3 ± 7.1	8.2 ± 0.3
5	31.8 ± 4.8	0.8 ± 0.2	59.4 ± 6.8	7.1 ± 0.1
25	34.6 ± 3.6	5.4 ± 0.5	55.7 ± 4.6	5.6 ± 0.3
100	32.1 ± 6.7	19.1 ± 3.9	58.9 ± 8.5	4.7 ± 0.2
250	33.7 ± 5.3	46.8 ± 6.9	57.0 ± 6.8	4.4 ± 0.4

[a] PG-collagen, containing 78.2 mmol of [$^{12}C_6$]-FL/mol of Lys (Table 1), was incubated under oxidative conditions for 5 weeks in 200 mM phosphate, pH 7.4, at 37 °C in the presence of [$^{13}C_6$]glucose at the concentrations indicated. [b] FL and [$^{13}C_6$]-FL values are the amounts after 5 weeks incubation with [$^{13}C_6$]glucose. [c] % FL loss was calculated using the initial amount of FL in the PG-collagen. [d] % FL to CML was calculated using the initial amount of FL in the PG-collagen and the amount of CML formed after 5 weeks (Table 3).

was derived from [$^{13}C_6$]glucose, rather than from FL on PG-collagen. It is notable that the PG-collagen was prepared in 250 mM glucose and was heavily glycated, but even in the presence of 5 mM [$^{13}C_6$]glucose which caused minimal protein glycation (0.8 ± 0.2 mmol of [$^{13}C_6$]-FL/mol of Lys at 5 weeks, Table 4), the majority of CML was derived from [$^{13}C_6$]glucose. Both the amount of [$^{13}C_2$]-CML formed (Table 3, column 3) and the relative contribution of [$^{13}C_6$]-glucose to total CML formation (Table 3, column 4) were affected by the [$^{13}C_6$]glucose concentration. The percent contribution of [$^{13}C_2$]-CML to the total yield of CML ([$^{13}C_2$]-CML + [$^{12}C_2$]-CML) ranged from 66% at 5 mM [$^{13}C_6$]-glucose to 92% at 250 mM [$^{13}C_6$]glucose. These estimates of the fraction of total CML derived from [$^{13}C_6$]glucose in solution are upper limits since the calculation ignores the contribution of the Amadori adduct, [$^{13}C_6$]-FL, formed *in situ* from [$^{13}C_6$]glucose to the formation of [$^{13}C_2$]-CML. This error would be only a small fraction of the total [$^{13}C_2$]-CML formed. For example, in the experiment with 250 mM [$^{13}C_6$]-glucose, only 3.4 mmol of CML/mol of Lys is formed from FL on PG-collagen containing 78 mmol of FL/mol of Lys (Table 3), representing 4.4% conversion of FL to CML (Table 4), while 39 mmol of [$^{13}C_2$]-CML is formed from [$^{13}C_6$]glucose (Table 3). During this experiment, the mean concentration of FL present on PG-collagen prepared in 250 mM glucose would be comparable to the amount of [$^{13}C_6$]-FL formed in 250 mM [$^{13}C_6$]glucose, so that the yield of [$^{13}C_2$]-CML from [$^{13}C_6$]-FL should be approximately 3 mmol/

Table 5: Effect of Phosphate Concentration on CML Formation from FL and Glucose[a]

P_i[b] (mM)	[$^{13}C_6$]glucose (mM)	[$^{12}C_2$]-CML[c] (mmol/mol of Lys)	[$^{13}C_2$]-CML (mmol/mol of Lys)	% total CML as [$^{13}C_2$]-CML
10	0	1.13 ± 0.07	0.0	0.0
50	0	2.47 ± 0.03	0.0	0.0
200	0	6.29 ± 0.10	0.0	0.0
10	100	1.14 ± 0.15	0.92 ± 0.05	44.7 ± 3.5
50	100	2.16 ± 0.14	6.09 ± 0.50	73.8 ± 2.0
200	100	3.58 ± 0.14	27.90 ± 0.45	88.6 ± 0.4

[a] PG-collagen, containing 78.2 ± 1.0 mmol of FL/mol of Lys and 0.37 ± 0.09 mmol of CML/mol of Lys (Table 1), was incubated under oxidative conditions for 5 weeks in the presence of 0 or 100 mM [$^{13}C_6$]glucose and various concentrations of phosphate buffer, pH 7.4, at 37 °C. [b] P_i = phosphate buffer. [c] [$^{12}C_2$]-CML = CML derived from FL in PG-collagen. [d] % total CML as [$^{13}C_2$]-CML = [$^{13}C_2$]-CML/([$^{13}C_2$]-CML + [$^{12}C_2$]-CML) × 100.

mol of Lys, *i.e.*, less than 10% of the 39 mmol of [$^{13}C_2$]-CML/mol of Lys observed. In the experiment performed in 5 mM [$^{13}C_6$]glucose, the contribution of [$^{13}C_6$]-FL to formation of [$^{13}C_2$]-CML would be even smaller because of the small amount of [$^{13}C_6$]-FL formed (Table 4, column 3).

As also shown in Table 3 (column 2) and Table 4 (column 5), oxidation of the Amadori adduct to form [$^{12}C_2$-CML] was inhibited by [$^{13}C_6$]glucose, even at a [$^{13}C_6$]glucose concentration of 5 mM. This inhibition increased from 13 to 47% between 5 and 250 mM [$^{13}C_6$]glucose, although the extent of decomposition of FL was not affected by the presence of [$^{13}C_6$]glucose (Table 4, column 4). While approximately 60% of the initial FL on the collagen was lost during the 5 week incubation under air (Table 4, column 4), the amount of [$^{12}C_2$]-CML formed (Table 4, column 5) was a small fraction of the initial FL content of the protein, from 4% for PG-collagen incubated with 250 mM [$^{13}C_6$]glucose to 8% for PG-collagen incubated in the absence of glucose. These results indicate that although glucose enhances the overall production of ROS, it inhibits the oxidation of FL to CML without affecting the overall rate of decomposition of FL. This inhibition by glucose might result from competition with the Amadori product for available free transition metal ions or ROS. Alternatively, if similar reactive intermediates, such as glyoxal, are involved in CML formation from both glucose and FL autoxidation, these intermediates may compete for the amino groups of Lys residues.

Because phosphate buffer catalyzes the rates of both glycation and oxidation reactions (Fu *et al.*, 1992), we studied the possible role of phosphate buffer concentration as a determinant of the relative rates of oxidation of glucose and Amadori adducts on collagen. As shown in Table 5, phosphate buffer concentration had a significant effect on both the yield and distribution of [$^{13}C_2$]-CML and [$^{12}C_2$]-CML formed during incubation of PG-collagen with [$^{13}C_6$]-glucose. The yield of both forms of CML increased in response to increased phosphate concentrations, with the production of [$^{13}C_2$]-CML from [$^{13}C_6$]glucose being most markedly affected. Thus, conversion of FL to CML increased by a factor of 3 when phosphate was increased from 10 to 200 mM (column 3), while the yield of [$^{13}C_2$]-CML from [$^{13}C_6$]glucose increased by a factor of 30 (column 4). For comparison, Ahmed *et al.* (1986) reported a 7-fold increase in CML formation from the model Amadori compound N^{α}-formyl-N^{ϵ}-fructoselysine over the same range of phosphate concentrations, while Thornalley *et al.* (1984) observed a 9-fold increase in the rate of glyceraldehyde autoxidation when phosphate was raised from 10 to 125 mM.

As observed in 200 mM phosphate buffer, [$^{13}C_2$]-CML was the primary form of CML produced in 50 mM phosphate (Table 5, column 5); however, the yield of [$^{13}C_2$]-CML from the Amadori adduct on PG-collagen slightly exceeded that of [$^{13}C_2$]-CML in 10 mM phosphate. The inhibitory effect of [$^{13}C_6$]glucose on the conversion of FL to CML was also eliminated at 10 mM phosphate (Table 5, column 3, 0 mM compared to 100 mM [$^{13}C_6$]glucose), while at 50 and 200 mM phosphate, conversion of FL to CML was inhibited approximately 10% and 40%, respectively, by [$^{13}C_6$]glucose (Table 5, column 3, 0 mM compared to 100 mM [$^{13}C_6$]-glucose). The extent of decomposition of FL did not differ significantly between 10 and 50 mM phosphate buffer, but was increased in 200 mM phosphate (Table 6, columns 3 and 5), both in the presence and in the absence of [$^{13}C_6$]-glucose. Formation of [$^{13}C_6$]-FL was not affected by phosphate concentration (Table 6, column 4), contrasting with the catalytic effect of phosphate shown previously in glycation of native collagen (Fu *et al.*, 1992); this discrepancy may result from differences in susceptibility to glycation of the unmodified Lys residues remaining in the PG-collagen, compared to the original Lys residues in native collagen. In summary, the above experiments demonstrate that both glucose and Amadori adducts can serve as precursors to CML on collagen and that their conversion to CML is affected by both the glucose and phosphate concentrations in the incubation. At high phosphate concentrations, glucose is clearly the primary source of CML (Tables 3 and 5), but at low phosphate concentration, characteristic of physiological systems, the contribution of the Amadori compound to formation of CML becomes more significant.

Contribution of Amadori Adducts and Glucose to Formation of Pentosidine. Since pentosidine and [$^{13}C_5$]pentosidine could not be differentiated by RP-HPLC with fluorescence detection, pentosidine levels were compared in PG-collagen incubated with and without 250 mM glucose and in native collagen incubated with 250 mM glucose under oxidative conditions (Table 7). PG-collagen incubated in the absence of glucose yielded pentosidine levels approximately 10% of the levels formed in native or PG-collagen incubated in the presence of glucose. There was no statistically significant difference in the amounts of pentosidine in PG-collagen and native collagen incubated with 250 mM glucose under oxidative conditions. If pentosidine were formed to a significant extent from Amadori adducts on the protein, the yield of pentosidine would have been significantly higher in PG-collagen incubated with glucose, compared to native collagen incubated with glucose. While not as conclusive as the results for CML, these experiments suggest that Amadori adducts are only minor precursors to pentosidine in incubations of protein in high glucose and phosphate buffer concentrations.

DISCUSSION

Understanding the mechanism of protein modification during the Maillard reaction is important for the design of therapeutic approaches for limiting chemical damage to protein during aging and diabetes and for assessing the role of the Maillard reaction in the pathogenesis of diabetic

Table 6: Effect of Phosphate Concentration on FL Decomposition and Conversion to CML[a]

P_i[b] (mM)	[$^{13}C_6$]glucose (mM)	FL[c] (mmol/mol of Lys)	[$^{13}C_6$]-FL[c] (mmol/mol of Lys)	% FL loss[d]	% FL to CML[e]
10	0	49.6 ± 0.8	0.0	36.6 ± 1.0	1.4 ± 0.1
50	0	47.6 ± 1.6	0.0	39.5 ± 2.0	3.2 ± 0.1
200	0	37.1 ± 0.6	0.0	52.5 ± 0.8	8.0 ± 0.1
10	100	52.4 ± 3.8	17.4 ± 1.0	33.0 ± 4.9	1.5 ± 0.2
50	100	52.3 ± 7.3	17.3 ± 1.4	33.2 ± 9.4	2.8 ± 0.2
200	100	32.8 ± 2.2	15.2 ± 2.8	58.1 ± 2.9	4.6 ± 0.2

[a] PG-collagen, containing 78.2 mmol of [$^{12}C_6$]-FL/mol of Lys (Table 1), was incubated under oxidative conditions for 5 weeks in the presence of 0 or 100 mM [$^{13}C_6$]glucose and various concentrations of phosphate buffer, pH 7.4, at 37 °C. [b] P_i = phosphate buffer. [c] FL and [$^{13}C_6$]-FL values are the amounts after 5 weeks incubation with [$^{13}C_6$]glucose. [d] % FL loss was calculated using the initial amount of FL in the PG-collagen. [e] % FL to CML was calculated using the initial amount of FL in the PG-collagen and the amount of CML formed after 5 weeks (Table 5).

Table 7: Comparison of Pentosidine in Preglycated Collagen Incubated in the Absence or Presence of Glucose and Native Collagen Incubated in the Presence of Glucose[a]

collagen	glucose (mM)	pentosidine (μmol/mol of Lys)
preglycated	0	7.6 ± 0.6
preglycated	250	62.4 ± 8.2
native	250	60.0 ± 6.5

[a] PG-collagen, described in Table 1, contained 2.4 ± 0.3 μmol of pentosidine/mol of Lys. Native collagen and PG-collagen were incubated in the presence or absence of 250 mM glucose under aerobic conditions for 5 weeks in 200 mM phosphate, pH 7.4, at 37 °C.

complications. Amadori adducts are known to be precursors of CML and pentosidine (Ahmed *et al.*, 1986, 1988; Dyer *et al.*, 1991; Smith & Thornalley, 1992; Glomb & Monnier, 1995), while Wells-Knecht *et al.* (1995) showed recently that GOPs can also be formed by autoxidative glycosylation, involving glyoxal and arabinose as intermediates in the formation of CML and pentosidine. The goal of the present study was to determine the relative contributions of glucose and protein-bound Amadori adducts to the formation of GOPs in a defined model system. Our studies indicate that glucose and Amadori adducts serve simultaneously as precursors of GOP formation on collagen *in vitro*, but that the relative role of these two sources of GOPs varies with the relative concentrations of Amadori adducts and glucose and with the phosphate buffer concentration. Increasing glucose concentration both favored the formation of CML from glucose and inhibited the formation of CML from Amadori adducts on protein. At high phosphate concentrations (50−200 mM), glucose was the primary source of CML, even at low glucose, while at lower phosphate concentrations (10 mM) oxidation of the Amadori adduct was favored. It is not clear whether this effect of phosphate *in vitro* is due to the variation in the concentration of phosphate or of transition metal ion catalysts present in the buffer, or to a combination of both effects. The resolution of this matter *in vivo* is even more complicated by the debate regarding the nature, availability, and role of metal ions for catalysis of nonenzymatic oxidation reactions in biological systems (Baynes, 1994). Regardless of the precise mechanism, this phosphate buffer effect is a significant observation because it establishes that in experiments conducted at high phosphate concentration *in vitro*, glucose, rather than the Amadori adduct, is the primary source of GOPs. In contrast, in physiological systems the relative roles of glucose and Amadori adducts may be reversed. Thus, inhibitors which prevent the oxidation of glucose or trap glucose oxidation products *in vitro* may not be as potent in inhibiting modification of protein derived from the Amadori compound at glucose and phosphate concentrations and metal ion availability found *in vivo*.

In contrast to our observations, Hunt and colleagues (Hunt *et al.*, 1993) concluded that oxidation of Amadori adducts to protein was a more important source of oxidative damage to protein than glucose autoxidation. Their conclusions were based on experiments in 100 mM phosphate with added copper ions, showing that formylated human serum albumin (fHSA), which was resistant to glycation, was also protected from glucose-mediated oxidation in the presence of copper ions, while native HSA was prone to oxidative damage. Thus, glucose apparently needed to be attached to the protein for the damage to occur. As shown in Figure 1 of their study, however, glycation-resistant fHSA underwent significant glucose-mediated fragmentation, as assessed by SDS−PAGE. In addition, as shown in Figure 2 of their study, although fHSA underwent less fragmentation than HSA in the presence of glucose and copper, it was also less susceptible than HSA to copper-generated fragmentation in the absence of glucose. Thus, their data do not satisfactorily demonstrate that the mechanism of protection of fHSA from glucose-mediated damage is the result of the inability of fHSA to be glycated. A more quantitative interpretation of their studies is hindered by the lack of specific indices of oxidation of glucose and Amadori adducts and of protein oxidation by these precursors.

Based on inhibition studies using aminoguanidine (AG) and borate, Glomb and Monnier (1995) concluded recently that Amadori products were responsible for over 50 % of CML formation in incubations of glucose (42.2 mM) and N^{α}-(*tert*-butoxycarbonyl)lysine (N^{α}-*t*-Boc-Lys) (42.2 mM) in 200 mM phosphate buffer, pH 7.4. AG (5 mM) caused 15% inhibition of CML formation in incubations of the Amadori product, N^{α}-*t*-Boc-N^{ϵ}-fructoselysine, and 50% inhibition of CML formation in incubations of glucose with N^{α}-*t*-Boc-Lys at approximately 1 week. However, the effects of various concentrations of AG were not explored to determine whether maximum inhibition was achieved. In other studies, borate (420 mM) completely blocked CML formation from the Amadori product, but only inhibited CML formation by 37% in incubations of glucose with N^{α}-*t*-Boc-Lys, suggesting that CML was partially derived from the Amadori adduct formed in incubations of glucose with N^{α}-*t*-Boc-Lys. However, the high borate concentration used, over twice the concentration of the phosphate buffer, could influence the mechanism of formation of CML beyond complexation with the Amadori adduct, e.g., by interfering with phosphate ion interactions with various components of the reaction system. Our results indicate that at the concentrations of glucose (42.2 mM) and phosphate buffer (200 mM) used in their experi-

ments, the percent contribution of the Amadori adduct to CML formation would be ≤10% rather than their estimate of 50%. Because of differences between our model systems and uncertainties about the relationship of both model systems to physiological systems, it is difficult to make more definitive conclusions regarding the relative importance of glucose and Amadori adducts *in vivo*.

The mechanism of formation of CML from glucose, independent of the Amadori adduct, is also a question of interest. Glomb and Monnier (1995) propose that Schiff base (or carbinolamine) adducts of glucose to protein, which are in rapid equilibrium with free glucose, are the major precursor(s) of glyoxal and CML via the Namiki pathway (Hayashi & Namiki, 1986) and that glucose autoxidation is not an important source of glyoxal or CML. While our work does not permit us to discriminate between formation of ROS and GOPs from free glucose or Schiff bases, it should be noted that the conclusions of Glomb and Monnier were based on detection of lower amounts of 3-amino-1,2,4-triazine, a product of reaction of glyoxal and AG, in oxidative incubations of glucose compared to oxidative incubations of glucose and N^{α}-*t*-Boc-Lys with AG. The presence of AG, however, may alter product formation in these systems by trapping glyoxal precursors or by catalyzing formation of products not formed in the absence of AG. Further studies are needed to determine if the mechanism of formation of ROS and GOPs from glucose involves autoxidation of the sugar in solution or requires reversible association of glucose with amino groups on protein prior to oxidation.

In conclusion, the studies described in this paper bring together earlier evidence that both glucose and glycated proteins are sources of ROS and GOPs. At high glucose and phosphate buffer concentrations commonly used for *in vitro* studies of the Maillard reaction, the major contributor to oxidation and glycoxidation of collagen is glucose, or rapidly formed carbinolamine or Schiff base adducts of glucose to protein, but not Amadori adducts. At lower glucose and phosphate concentrations, closer to conditions found *in vivo*, our studies suggest an increased role for the Amadori adduct as a source of CML and possibly other GOPs (Tables 3−6). However, an increase from 5 to 25 mM glucose concentration in 200 mM phosphate decreased the fractional conversion of FL to CML (Table 3), suggesting that hyperglycemia *in vivo* may both decrease the conversion of preformed Amadori adducts to CML and stimulate the formation of CML from precursors of Amadori adducts or by autoxidative glycosylation. Similarly, at 10 mM, compared to higher phosphate concentrations, the Amadori adduct accounts for about half of CML formation (Table 5, row 4), suggesting comparable roles for free glucose and Amadori adducts at physiological buffer concentrations. The physiological relevance of these observations is bolstered by the fact that the PG-collagen contains 5 times the level of Amadori adducts found in human skin collagen in diabetes [78 mmol of FL/mol of Lys in PG-collagen (Table 1), compared to 15 ± 5 mmol of FL/mol Lys in diabetic human skin collagen (Dyer *et al*, 1993)], while the 100 mM [$^{13}C_6$]-glucose concentration is also 5 times the glucose concentration in blood in diabetes. Thus, we conclude that both glucose and Amadori adducts are potential sources of GOPs *in vivo* and that their relative contributions to GOP formation *in vivo* depend not only on their relative concentrations but also on the local oxidative environment. In regions of high oxidative stress, such as areas of local inflammation or monocyte/macrophage activation, the shower of ROS during oxidative bursts may be more characteristic of glycation reactions carried out *in vitro* in high phosphate concentration, so that glucose and/or precursors of Amadori adducts may be more important sources of GOPs. In contrast, if antioxidant defenses are strong, the situation may be more comparable to experiments conducted at low phosphate concentration which favors the oxidation of more readily oxidized substrates, such as Amadori adducts. It is possible that a shift from low to high levels of oxidative stress in diabetes may serve as a switch for enhancing the contribution of free glucose to formation of GOPs, exacerbating the development of diabetic complications. These considerations must be balanced against the fact that some glycoxidation reactions *in vivo* are likely to be site-specific, so that the contribution of glucose and Amadori adducts to protein modification may be dictated by the proximity of each substrate to catalysts of oxidation reactions. Other reactive carbohydrates, such as ascorbate, pentoses, and deoxyglycosones, may also have a significant role in the formation of glycoxidation products *in vivo*. Since the same products may be formed by separate mechanisms and at different rates depending on glucose, phosphate, metal ion, and substrate concentrations, our results suggest caution in the interpretation of *in vitro* studies designed to elucidate mechanisms and intermediates or to evaluate the effectiveness of inhibitors of the Maillard reaction in biological systems.

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