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Age-Dependent Accumulation of N^{ϵ}-(Carboxymethyl)lysine and N^{ϵ}-(Carboxymethyl)hydroxylysine in Human Skin Collagen†

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ABSTRACT: N^{ϵ}-(Carboxymethyl)lysine (CML) is formed on oxidative cleavage of carbohydrate adducts to lysine residues in glycated proteins in vitro [Ahmed et al. (1988) *J. Biol. Chem. 263*, 8816–8821; Dunn et al. (1990) *Biochemistry 29*, 10964–10970]. We have shown that, in human lens proteins in vivo, the concentration of fructose–lysine (FL), the Amadori adduct of glucose to lysine, is constant with age, while the concentration of the oxidation product, CML, increases significantly with age [Dunn et al. (1989) *Biochemistry 28*, 9464–9468]. In this work we extend our studies to the analysis of human skin collagen. The extent of glycation of insoluble skin collagen was greater than that of lens proteins (4–6 mmol of FL/mol of lysine in collagen versus 1–2 mmol of FL/mol of lysine in lens proteins), consistent with the lower concentration of glucose in lens, compared to plasma. In contrast to lens, there was a slight but significant age-dependent increase in glycation of skin collagen, 33% between ages 20 and 80. As in lens protein, CML, present at only trace levels in neonatal collagen, increased significantly with age, although the amount of CML in collagen at 80 years of age, ~1.5 mmol of CML/mol of lysine, was less than that found in lens protein, ~7 mmol of CML/mol of lysine. The concentration of N^{ϵ}-(carboxymethyl)hydroxylysine (CMhL), the product of oxidation of glycated hydroxylysine, also increased with age in collagen, in parallel with the increase in CML, from trace levels at infancy to ~5 mmol of CMhL/mol of hydroxylysine at age 80. Thus, accumulation of *N*-(carboxymethyl) amino acids appears to be a general feature of the aging of long-lived proteins by glycation and oxidation reactions.

Glycation (nonenzymatic glycosylation) is a posttranslational modification of proteins resulting from reaction of glucose with amino groups in protein (Baynes et al., 1989). The ϵ-amino group of lysine residues is the primary site of modification of most proteins, resulting in the formation of the Amadori compound fructose–lysine (FL)[1] (Figure 1). We reported previously that FL may be cleaved oxidatively to form N^{ϵ}-(carboxymethyl)lysine (CML) residues in proteins in vitro (Ahmed et al., 1986, 1988) and showed recently (Dunn et al.,

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[1] Abbreviations: CMhL, N^{ϵ}-(carboxymethyl)hydroxylysine; CML, N^{ϵ}-(carboxymethyl)lysine; GC/MS, gas chromatography–mass spectrometry; FL, N^{ϵ}-(1-deoxyfructos-1-yl)-L-lysine (fructose–lysine); Hyl, hydroxylysine; SIM, selected-ion monitoring; TFAME, trifluoroacetyl methyl ester.

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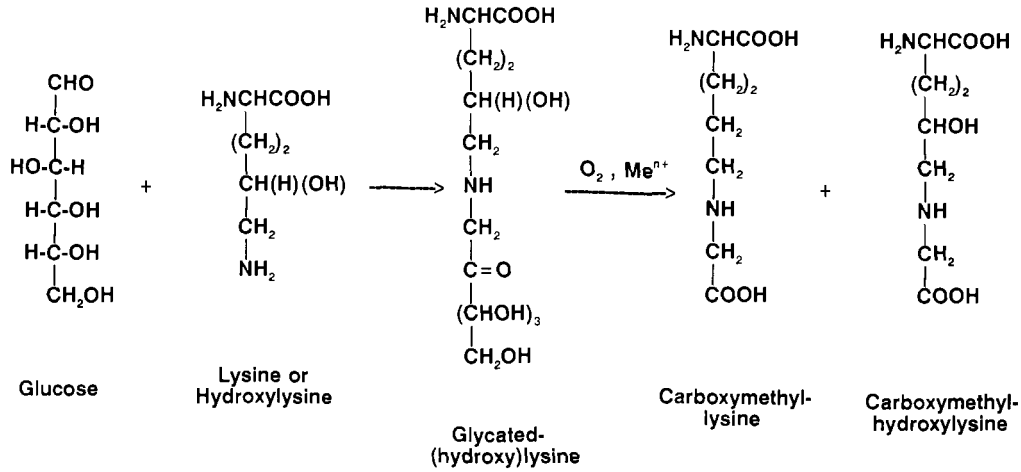

FIGURE 1: Reaction scheme for glycation and oxidation of lysine and hydroxylysine residues in collagen. CML has been identified as the oxidation product of fructose–lysine (Ahmed et al., 1986, 1988), while CMhL is the analogous product derived from oxidation of glycated hydroxylysine residues in collagen.

1990) that CML may also be formed on reaction of ascorbate and other sugars with protein under autoxidative conditions. In other work we studied the relationship between age and concentrations of FL and CML in lens proteins, which are among the longest lived proteins in the body and serve as a useful model for studies on age-dependent chemical modifications of protein (Zigler & Goosey, 1981). We observed (Dunn et al., 1989) that while the concentration of FL was constant, the concentration of CML increased linearly with age, so that after age 10, the concentration of CML exceeded that of FL in human lens proteins. We interpreted these results to indicate that the concentration of Amadori products, such as FL, reaches a steady-state concentration with respect to lens glucose concentration, while the accumulation of oxidation products, such as CML, is the result of cumulative exposure of lens proteins to oxidative stress (Dunn et al., 1989).

To determine whether the accumulation of CML is unique to lens proteins or characteristic of the aging of long-lived proteins, in general, we have extended our studies to the analysis of another major class of long-lived proteins in the body, the insoluble collagens in skin. Since collagen contains hydroxylysine (Hyl), in addition to lysine, and both hydroxylysine and lysine residues are known to be glycated in collagen (Garlick et al., 1988), we have also analyzed for the collagen content of N^{ϵ}-(carboxymethyl)hydroxylysine (CMhL), the expected product of oxidation of glycated hydroxylysine residues in collagen. The results of these studies, described below, suggest that the accumulation of *N*-(carboxymethyl) amino acids is a general feature of the aging of tissue proteins by glycation and oxidation reactions.

Experimental Procedures

Materials. Unless otherwise indicated, reagents were purchased from Sigma Chemical Co., St. Louis, MO. N^{α}-Formyl-N^{ϵ}-(1-deoxyfructos-1-yl)lysine (the standard for measurement of FL) and CML were prepared as described previously (Ahmed et al., 1986, 1988). CMhL was prepared by reacting N^{α}-formylhydroxylysine (prepared according to Hofmann et al., 1960) and glyoxylic acid, each at 0.4 M in 0.2 M phosphate buffer, pH 8, overnight at room temperature with a 4-fold molar excess of $NaBH_3CN$. The CMhL, obtained in 65% yield, was then purified by sequential anion- and cation-exchange chromatography, as described previously for the purification of CML (Ahmed et al., 1986, 1988). The identity of CMhL was confirmed by mass spectrometry (see Figure 2) and its concentration determined by amino acid analysis using the calibration factor for CML.

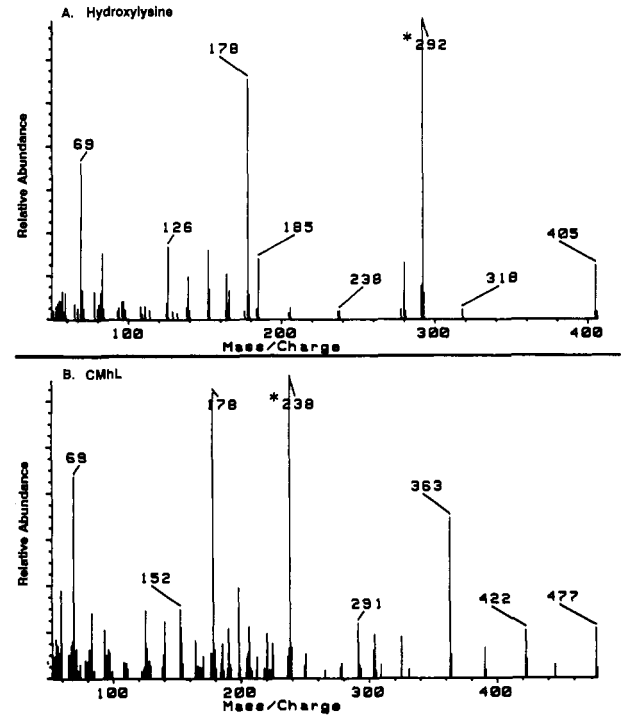

FIGURE 2: Full-scan mass spectra of the TFAME derivatives of (A) hydroxylysine (Hyl) and (B) N^{ϵ}-(carboxymethyl)hydroxylysine (CMhL). The ions used for quantitation of these compounds by SIM-GC/MS are indicated by asterisks, i.e., the m/z = 292 and 238 ions for Hyl and CMhL, respectively.

Skin Samples. Skin samples (n = 52) were obtained from healthy, nondiabetic donors or at autopsy at the Royal Victoria Hospital in Belfast, Northern Ireland. Autopsy samples were taken within 6 h of death from patients who had died of acute illness, e.g., stillbirth, severe congenital heart disease, trauma, or myocardial infarction. These patients had no history of diabetes and, where possible, had documented normal plasma glucose levels in their terminal illness. An elliptical full thickness skin section (approximately 0.5 × 1.0 cm) was taken from the medial aspect of the buttock (under 2% lidocaine local anesthesia in live donors). The study was approved by the Ethical Committee of the Royal Victoria Hospital and the Human Subjects Review Board at the University of South Carolina.

Sample Preparation for Gas Chromatography–Mass Spectrometry (GC/MS) Analysis. Skin samples were stored

at −70 °C before preparation for assay. The sample preparation was as described previously (Lyons & Kennedy, 1985). Briefly, after thawing, the tissue was scraped vigorously with a scalpel to remove adherent fat and vascular tissue and then extracted sequentially for 24 h at 4 °C with 1 M NaCl, chloroform–methanol (2:1), and 0.5 N acetic acid in order to remove lipids and soluble proteins; 50 volumes (w/v) of each extractant was used, with replacement of the solution once at 8 h. After extraction, the samples were rinsed in distilled water, dried by lyophilization, and stored frozen at −70 °C. Approximately 20–30 mg of protein was obtained from each sample by this procedure. Analyses of the extracts from representative skin samples indicated that less than 5% of the hydroxyproline content, measured by the procedure of Stegemann and Stalder (1967), as modified by Maekawa et al. (1970), was removed during the extraction procedures. On the basis of hydroxyproline content, the samples contained approximately 80% collagen by weight, assuming 14% hydroxyproline in skin collagen (Hamlin & Kohn, 1971). The ratio of Hyl/Lys in these samples (methodology discussed below) was constant with age, 0.19 ± 0.02 mol of Hyl/mol of Lys, consistent with previous analyses of human skin [ratio = 0.20–0.21, calculated from data of Bornstein and Piez (1964) and Rojkind (1973)].

For analysis of FL, samples of skin collagen (3 mg in 3 mL of 7.8 N HCl) were hydrolyzed for 24 h at 110 °C under nitrogen in 13 × 100 mm screw-top test tubes (Teflon-lined caps). These hydrolysis conditions were selected to maximize the percent conversion of FL to furosine (Erbersdobler, 1986). Because of partial (3–4%) conversion of FL to CML (and possibly of glycated Hyl to CMhL) during the hydrolysis step, CML and CMhL were measured separately in 3-mg samples reduced in 500 μL of 0.1 M $NaBH_4$ in 1 M NaOH for 12 h at room temperature. Reduction of FL was judged complete, on the basis of the failure to detect furosine in chromatograms of reduced samples. Excess $NaBH_4$ was discharged by addition of 6 N HCl and the sample dried by centrifugal evaporation. Prior to acid hydrolysis, borate was removed as the methyl ester by two additions of 1 mL of glacial acetic acid in methanol (1:10), followed by incubation at 65 °C for 30 s and drying under a stream of nitrogen. Acid hydrolysates were dried in vacuo by using a Savant Speed-Vac concentrator and converted to their trifluoroacetyl methyl ester (TFAME) derivatives for analysis. For preparation of the methyl esters, the hydrolysate was dissolved in 1.5 mL of 1 N methanolic HCl and heated for 0.5 h at 65 °C. Solvent was evaporated at room temperature under a stream of nitrogen by using an N-Evap Analytical Evaporator (Organomation, South Berlin, MA), and the product was redissolved in 0.5 mL of dry methylene chloride. Trifluoroacetic anhydride (1 mL) was then added and the mixture incubated at room temperature for 1 h to obtain the trifluoroacetyl derivatives. After removing the solvent under a stream of nitrogen, the sample was dissolved in 150 μL of methylene chloride and 2 μL of this solution was injected for GC/MS analysis.

GC/MS Analyses. GC/MS analyses were performed on a Hewlett-Packard Model 5890 Gas Chromatograph equipped with a Model 7673A Autosampler and Model 5970 Mass Selective Detector, using a 30-m DB-5 capillary column (J & W Scientific, Folsom, CA). The temperature program was as follows: 2 min at 70 °C; ramp to 260 °C at 5 deg/min and then to 290 °C at 15 deg/min; hold for 4 min at 290 °C. The lysine, hydroxylysine, FL, CML, and CMhL content of the collagen was determined by selected-ion monitoring GC/MS (SIM-GC/MS). FL was measured as the TFAME derivative

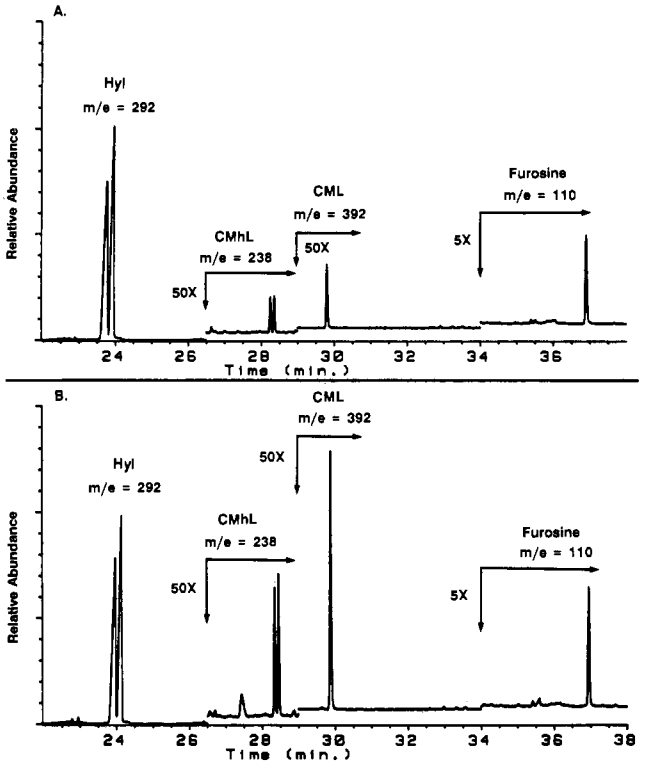

FIGURE 3: Typical SIM-GC/MS chromatograms of skin collagen samples from donors aged 19 and 71. For comparative purposes two chromatograms were chosen that had similar lysine peak areas. The lysine peaks (m/e = 180) are not shown in the chromatograms because of coelution of lysine with hydroxylysine. Note the significant increase in the CML and CMhL peaks, the small increase in FL (as furosine), and no change in the Hyl content of the collagen between ages 19 and 71. The scales are expanded 50× at 26.5 min and then reduced to 5× at 34 min; i.e., the expansions are not multiplicative.

of furosine, which was formed in ~40% yield from FL during the acid hydrolysis (Erbersdobler, 1986). Lysine, hydroxylysine, CML, and CMhL were measured directly as their TFAME derivatives. The mass spectra of the TFAME derivatives of hydroxylysine and CMhL are shown in Figure 2; the spectra of the lysine, furosine, and CML derivatives have been reported previously (Dunn et al., 1989). The 320, 110, 392, 292, and 238 ions were used for detection and quantitation of lysine, furosine, CML, hydroxylysine, and CMhL, respectively, by SIM-GC/MS. Detector response (peak area) was linear for these ions within the range of concentrations reported. The amounts of FL, CML, and CMhL were normalized to the lysine or hydroxylysine content of the collagen by external standardization (Kennedy, 1984), using calibration curves generated from solutions with constant lysine or hydroxylysine content and variable concentration of the other analytes. The calibration curves were linear and covered the range of analyte concentrations in all samples assayed. Samples were analyzed in batches to minimize interassay variation. The coefficients of variation for the assays of FL/Lys, CML/Lys, and CMhL/Hyl were 7, 5, and 6%, respectively.

Results

GC/MS Analyses of Skin Collagen. Figure 3 shows typical ion chromatograms obtained on analysis of young and old human skin collagen by SIM-GC/MS. The identification of the various compounds was confirmed by both the retention time of the selected ion and the presence of other fragment ions in the mass spectrum that were characteristic of the authentic compounds. CML, hydroxylysine, and CMhL could be quantified directly by this technique, while FL was measured as the acid hydrolysis product, furosine (Erbersdobler,

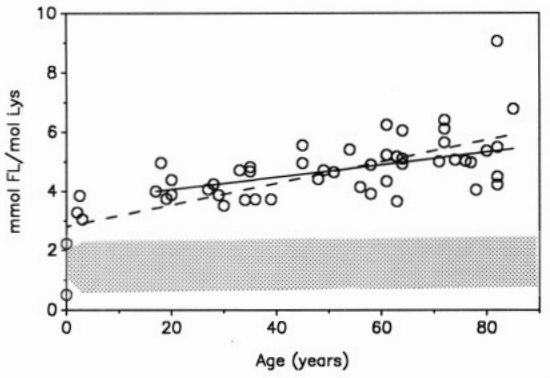

FIGURE 4: Age-dependent changes in the concentration of FL in human skin collagen. Two linear least-squares fits to the data are shown, the dashed line, which includes all data points, and the solid line, which includes the data between ages 15 and 85, excluding the outlier at age 82. The equation for the solid line is mmol of FL/mol Lys = $2.1 \times 10^{-2} \times$ age + 3.6 ($r = 0.55$; $P < 0.001$). The shaded area at the bottom includes the 95% confidence limits for similar analyses of normal lens proteins (Dunn et al., 1989).

1986). The extent of glycation of hydroxylysine could not be determined simultaneously because the major fragment ions from glycated hydroxylysine exceeded the 800 amu mass range of our laboratory mass spectrometer and adequate sensitivity and specificity were not obtainable with smaller ions. We did determine, however, that glycated hydroxylysine does not yield furosine on acid hydrolysis, so that its presence did not interfere with the measurement of FL.

Measurement of the FL Content of Skin Collagen. Figure 4 shows the results of analyses of the FL content of collagen samples spanning the range between neonatal and 85-year-old donors. The concentration of FL ranges from trace levels in the neonate to ~5 mmol of FL/mol of Lys at age 80, i.e., 0.5% of the lysine residues in the protein. The concentration of FL appears to be significantly lower in the neonatal compared to 2-year-old samples. However, after 20 years of age the slope of the line, 0.02 mmol of FL·(mol of Lys)$^{-1}$·year^{-1} (Figure 4), indicates only a slight, but significant ($r = 0.55$, $P < 0.001$), rate of increase in the FL content of collagen with age. This amounts to a 33% increase in glycation of lysine residues in collagen between ages 20 and 80. The shaded area at the bottom of the graph in Figure 4 represents the 95% confidence limits for similar analyses of human lens proteins (Dunn et al., 1989). It is apparent that the concentration of FL, relative to lysine, is 3–4 times higher in skin collagen than in lens proteins.

Measurement of the CML Content of Skin Collagen. Figure 5A shows that the concentration of CML in collagen increases significantly with age, from trace levels at infancy to ~1.6 mmol of CML/mol of Lys at age 80, i.e., ~0.16% of the lysine residues in the protein. The shaded area on this graph represents the 95% confidence limits for similar analyses of lens proteins (Dunn et al., 1989). Thus, despite the lower concentration of FL in lens proteins (Figure 4), the concentration of CML in the lens exceeds that in skin collagen by about 4-fold at all ages. In Figure 5B (lower frame), the concentration of CML is normalized to the FL concentration in the collagen, showing that CML, if it is derived exclusively from FL in vivo (see under Discussion), accounts for only 5–30% of the total known products of glycation of lysine residues (FL + CML) detectable in human skin collagen. In contrast, the ratio of CML to FL in the lens (Figure 5B, upper frame; shaded area = 95% confidence limits) increases to 7 at age 80, so that CML represents ~80% of the total known products of glycation of lysine (FL + CML) in lens protein.

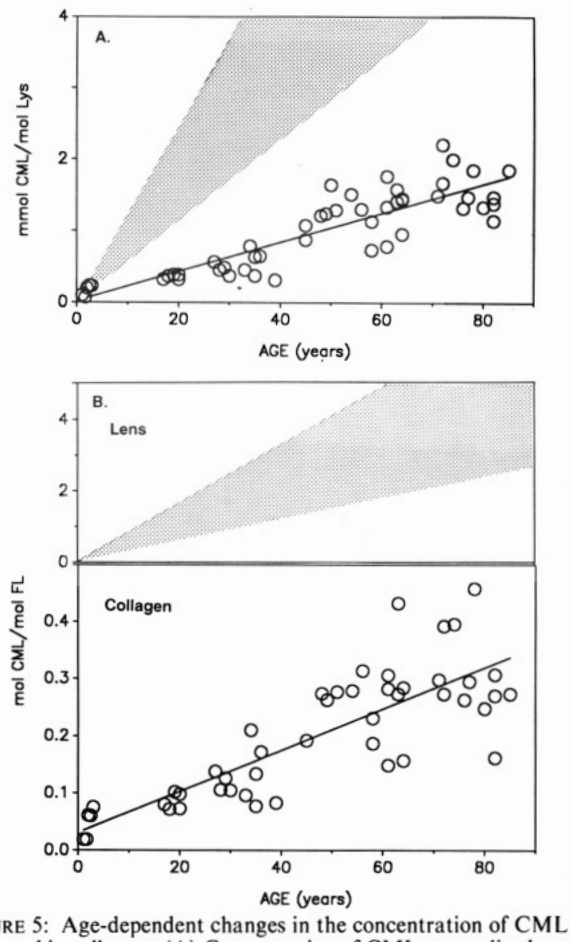

FIGURE 5: Age-dependent changes in the concentration of CML in human skin collagen. (A) Concentration of CML, normalized to the lysine content of the collagen. The shaded area represents the 95% confidence limits for similar analyses of normal lens proteins (Dunn et al., 1989). (B) Concentration of CML, normalized to the FL content of the collagen. The shaded area at the top is the 95% confidence limits for similar analyses of normal lens proteins. The upper scale has been compressed 20-fold, compared to the lower, for purposes of presentation; i.e., the slope for the lens data versus age is approximately 20 times that observed for skin collagen.

It is apparent from Figure 5 that, while the CML content of skin collagen is significantly lower than that of lens protein, whether normalized to the lysine or FL content of the protein, in both cases there is a 3–4-fold increase in the CML content of the protein between ages 20 and 80.

Measurement of the CMhL Content of Skin Collagen. Although our analytical method did not permit us to measure the glycated hydroxylysine content of collagen, the concentration of CMhL, the putative product of oxidation of glycated hydroxylysine, could be quantified simultaneously with that of CML. As shown in Figure 6A, the concentration of CMhL, like that of CML, increased linearly with age in human skin collagen, with a 3–4-fold increase in CMhL between ages 20 and 80. Figure 6B shows that the ratios of CML/Lys (Figure 5A) and CMhL/Hyl (Figure 6A) are strongly correlated with one another, illustrating the constant relationship between the CML and CMhL concentrations in skin collagen with age. Thus, when the samples are divided into four cohorts, on the basis of age (plotted on the graph by using four different symbols), the cohorts are distributed into age-dependent clusters along the slope axis. Clearly, the correlation between levels of CML and CMhL in collagen (Figure 6B) is much stronger than the correlations between age and levels of either CML or CMhL (Figures 5 and 6A). The slope of the line in Figure 6B, ~3 (mmol of CMhL/mol of Hyl)/(mmol of

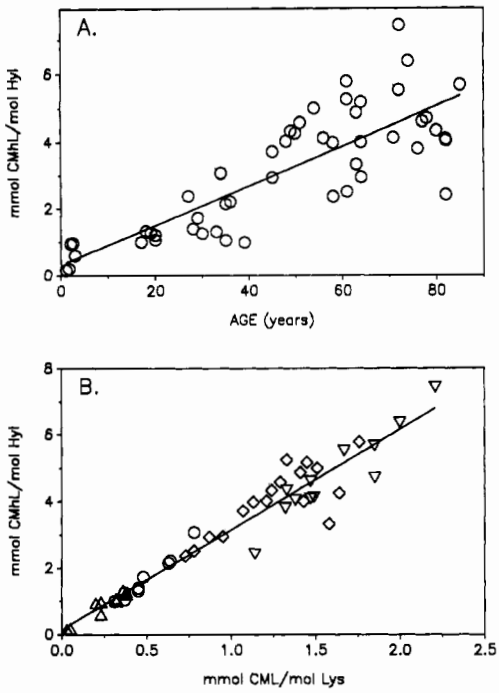

FIGURE 6: Age-dependent changes in the concentration of CMhL in human skin collagen. (A) Concentration of CMhL, normalized to the hydroxylysine content of the collagen. (B) Comparison of the CML/Lys and CMhL/Hyl ratios in the collagen samples. While it is not apparent on a two-dimensional graph, there is an age gradient in the data. To illustrate this point, symbols have been assigned to arbitrary groups in the ranges 0–22 years (△), 23–44 years (○), 45–66 years (◇), and 67–85 years (▽).

CML/mol of Lys), indicates that a greater fraction of hydroxylysine residues in collagen exists as CMhL than lysine residues as CML.

Discussion

Comparative Glycation of Lens Proteins and Skin Collagen. In earlier studies on the relationship between aging and glycation of proteins we observed that glycation of human lens proteins was relatively constant throughout adult life (Dunn et al., 1989). We concluded that glycation was not an age-dependent chemical modification of proteins and that the extent of glycation of lens proteins was probably in an equilibrium or steady-state relationship with lens glucose concentration. In the present study we observed that glycation of insoluble skin collagen was also relatively insensitive to age in adult humans. An age-dependent increase in glycation of collagen was observed but was modest, only a 33% increase between ages 20 and 80. Schleicher and Wieland (1986), using an HPLC assay for furosine, also reported only a slight age-dependent increase in glycation of human tendon collagen, comparable to that which we observed for skin collagen. In essential agreement with our work and that of Schleicher and Wieland (1986), Vishwanath et al. (1986) found no change in glycation of human skin collagen with age, based on measurement of [^{3}H]hexitol–lysines in [^{3}H]$NaBH_4$-reduced collagen. Earlier reports of significant age-dependent increases in glycation of human skin (Schnider & Kohn, 1981) and glomerular basement membrane (Cohen & Yu-Wu, 1983) collagens employed the relatively nonspecific thiobarbituric acid assay (Schleicher & Wieland, 1981; Baynes et al., 1989) for measurement of protein glycation. In general, the more rigorous chemical analyses indicate that there is, at best, only a slight age-dependent increase in glycation of human collagens. Quantitative comparisons of the results of the above studies with our own are difficult because of differences in methodology and standardization of the assays. However, our estimate of ~5 mmol of FL/mol of Lys in skin collagen is consistent with results of Garlick et al. (1988) who measured [^{3}H]hexitol–lysine in [^{3}H]$NaBH_4$-reduced human glomerular basement membrane collagen and found an average value of 7.6 mmol of FL/mol of Lys and also no increase in glycation with age.

The lower extent of glycation of lysine residues in lens [~1.4 mmol of FL/mol of Lys (Dunn et al., 1989)] compared to skin or glomerular basement collagens (5–7.6 mmol of FL/mol of Lys) is consistent with the lower, 1–2 mM, glucose concentration in ocular fluids (de Berardinis et al., 1965) and the lens (Harding & Crabbe, 1984; Cotlier, 1987), compared to the ~5 mM glucose concentration in plasma and extravascular fluid. These data also support the argument that the extent of glycation of long-lived proteins is in an equilibrium or steady-state relationship to ambient glucose concentration.

Comparative Carboxymethylation of Lysine Residues in Lens Proteins and Skin Collagen. Figure 5B shows that the concentration of CML in skin collagen is significantly lower than that in lens protein, when compared to either the lysine or FL content of the protein. This may indicate that the collagen is exposed to less oxidative stress than is the lens, perhaps because of lower exposure of skin collagen (particularly from the buttock area) to ionizing radiations or because of lower concentrations of oxygen or free redox metal catalysts in the collagenous matrix of skin. In the latter case, the metal ion catalysts might be inactivated by chelation by acidic glycosaminoglycans in the extracellular matrix. The increase in CML (and CMhL) with age might result from constant oxidative stress, combined with a negligible or gradually decreasing rate of turnover of insoluble skin collagen with age (Prockop et al., 1979). Alternatively, the average age of skin collagen may be increasing with age as the result of a constant rate of deposition of collagen into the insoluble collagen pool (Molnar et al., 1986, 1988). The possibility that insoluble skin collagen is turning over at a constant rate but that the level of oxidative stress is increasing with age is not consistent with other work from our laboratory (Knecht et al., 1990) showing that the concentration of CML in human urine is constant with age, therefore, arguing against increased oxidative stress with age. Ames and colleagues (Saul et al., 1987; Ames, 1988) have also concluded that oxidative stress is constant with age, based on the constant rate of urinary excretion of thymine and thymidine glycols, which are products of oxidation of DNA.

Comparative Carboxymethylation of Lysine and Hydroxylysine Residues in Collagen. The data in Figure 6B illustrate that the aging of collagen is accompanied by proportional increases in carboxymethylation of both the lysine and hydroxylysine residues in the protein. However, the slope of the line indicates that there is an approximate 3-fold greater carboxymethylation of hydroxylysine compared to lysine residues in collagen. The reason for this difference is unknown but might result from either preferential glycation of hydroxylysine, compared to lysine, or preferential oxidation of glycated hydroxylysine. In this respect, Perejda et al. (1984) have reported a 2-fold increased rate of glycation of hydroxylysine versus lysine residues in type I (chick embryo tendon) collagen in vitro. Thus, some fraction of the greater CMhL/Hyl, compared to CML/Lys, ratio in human skin collagen could result from increased glycation of hydroxylysine versus lysine in vivo. As noted earlier, because of methodological and instrument limitations we were unable to measure the extent of glycation of hydroxylysine in our samples. However, the answer to this question may be important for

understanding the role of glycation and oxidation reactions in the development of complications in diabetes. Oxidation of Amadori compounds yields superoxide (Jones et al., 1987; Sakurai & Tsuchiya, 1988) and may initiate oxidative damage to neighboring molecules in the extracellular matrix (Hicks et al., 1988). Thus, collagens richer in hydroxylysine (and CMhL) may be inherently more susceptible to damage from glycation and/or oxidation reactions.

Origin of CML and CMhL in Collagen. In our original studies (Ahmed et al., 1986, 1988) we had observed that CML was formed spontaneously on autoxidation of FL in glycated proteins in vitro. The detection of CML in tissue proteins and urine then led us to conclude that CML was also a product of oxidation of glycated proteins in vivo. In more recent work (Dunn et al., 1990) we have learned that CML is also formed on reaction of ascorbate with proteins under autoxidative conditions, i.e., in the presence of air and traces of metal ions. The pathway for formation of CML from ascorbate involves threose as an intermediate, and the chemistry for formation of CML from threose is similar to that for its formation from glucose, i.e., oxidative cleavage of an Amadori adduct between C-2 and C-3 of the carbohydrate chain to form CML. These results suggest that in vivo, in addition to its possible formation from glucose, CML may also be derived from oxidation of ascorbate or other aldose or aldose phosphate adducts to proteins. At the present time, despite uncertainty about the exact origin of CML in proteins, it does seem that its formation in proteins in the lens and extracellular matrix is indicative of posttranslational oxidative reaction and that glucose, and possibly ascorbate and other sugars, are likely precursors. Regardless of its origin, however, it is clear that CML accumulates with age in both lens proteins and collagen and that the analogous compound, CMhL, accumulates in parallel in collagen. These compounds, individually or together, may constitute a useful chemical marker or biomarker of age-dependent chemical modification of long-lived proteins by nonenzymatic glycation and oxidation reactions.

Registry No. CML, 5746-04-3; CMNL, 130985-18-1; FL, 21291-40-7.

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