Conversion of Arginine into Ornithine by Advanced Glycation in Senescent Human Collagen and Lens Crystallins*

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Long lived proteins undergo age-related postsynthetic modifications that destabilize them by altering their conformation, charge, and helicity, thereby enhancing their resistance toward proteolysis and propensity to aggregate. The unexpected finding of substantial amounts of ornithine, the nonprotein amino acid, and decarbamidation product of arginine in acid hydrolysates of lens crystallins and skin collagen led us to investigate its source and mechanism of formation. In order to exclude ornithine formation as an artifact of acid hydrolysis, proteins were reductively alkylated with formaldehyde to convert ornithine to dimethyl-ornithine. The proteins were assayed for carboxymethyl-ornithine and glycated ornithine (“furornithine”) by liquid chromatography coupled to electrospray ionization mass spectrometry. Ornithine in acid hydrolysates of human lens and skin proteins increased from 1 to 15 nmol/mg protein from ages 10 to 90 years, whereas dimethyl-ornithine increased from 0.5 to 15 and from 0 to 5 nmol/mg protein, respectively. Carboxymethyl-ornithine and furornithine increased with age in lens and skin from ∼0 to 60 and 0 to 180 pmol/mg protein, respectively. In collagen, ornithine was elevated above levels of nondiabetic controls only when both diabetes and end stage renal disease were present. The age-related increase of these modifications provides evidence for substantial in vivo formation of ornithine in aging human tissue proteins. The mechanism of ornithine formation is not known, but data suggest that arginine-derived advanced glycation end products might serve as precursors for the in vivo conversion of ornithine from arginine.

Reducing sugars react with long lived proteins in vivo, resulting in molecular adducts and cross-links collectively referred to as advanced glycation end products (AGEs),¹ which are thought to be important in diabetic complications and the aging phenomenon (1–6). Glycation begins with the reducing sugar reacting nonenzymatically with the free amino group of the protein to form an Amadori product (1). The latter can undergo a series of complex reactions involving both oxidative and nonoxidative chemistry resulting in AGE formation (7). Although the e-amino group of lysine bears primary importance in its reactivity with reducing sugars in forming Amadori products and AGEs, evidence suggests that the guanidino group of arginine is also actively involved in these reactions. Arginine can react with the oxoaldehydes methylglyoxal (MG), glyoxal (G), and 3-deoxyglucosone (3DG), to produce the hydroimidazolones MG-H₁, G-H₁, and 3DG-H₁, respectively (Fig. 1) (8, 9). Likewise, MG can also react directly with arginine to form argpyrimidine (Fig. 1) (10). Additionally, ornithine is also able to undergo reaction with the lysine-derived Amadori products of pentoses and glucose to form the cross-links pentosidine (11) and glucosepane (12). Similarly, MG, G, and 3DG can react with lysine and arginine to form the imidazoline cross-links MODIC, GODIC, and DOGDIC, respectively (Fig. 1) (12–14).

In the present research, the unexpected and consistent finding of ornithine in our chromatographic profiles of acid-hydrolyzed insoluble human lens and collagen in association with chronological age led us to investigate its source and mechanism of formation. In vivo, ornithine is formed in the liver by the urea cycle via enzymatic cleavage of arginine into ornithine and urea by arginase (15, 16). Similar cleavage occurs in extrahepatic tissues, where ornithine is a precursor for the biosynthesis of proline, spermidine, spermine, and nitric oxide (17). However, ornithine, to our knowledge, has never been described in the primary structure of proteins, since there is neither any codon nor any aminoacyl-tRNA and amino acid synthetase specific for ornithine for transcription, translation, and protein synthesis (16). Thus, ornithine is not found in native proteins (16). Because of these issues, a number of interesting questions arose concerning the origin of ornithine in our biological specimens (i.e. as to whether it is truly present in proteins in vivo or whether it is merely an artifact of acid hydrolysis of proteins rich in hydroimidazolones as suggested by Paul et al. (18)).

Thus, the purpose of the present research is 2-fold: (i) to investigate whether ornithine truly exists in the primary structure of long lived proteins and (ii) to investigate its biological significance as to its age-related increase and mechanism of glyoxal-derived hydroimidazolone; GODIC, glyoxal-derived imidazoline cross-link; HPLC, high pressure (performance) liquid chromatography; LC, liquid chromatography; NCE, normalized collision energy; MS, mass spectrometry; MG, methylglyoxal; MG-H₁, methylglyoxal-derived hydroimidazolone; MODIC, methylglyoxal-derived imidazoline cross-link; RT, retention time; SRM, selected reaction monitoring; TDFHA, tridecafluoro-oroheptanoic acid; ESI, electrospray ionization.
formation in insoluble skin collagen and lens proteins. The major outcome of this study is that it provides the first and unequivocal demonstration that ornithine forms in substantial quantities during aging of collagen and lens crystallins and that it itself becomes glycated and glycoxidized with age.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Except as noted below, most chemicals and solvents were from Aldrich, Sigma, or Fisher and were of the highest grade available. Methyloxime was from Fluka (Milwaukee, WI) and was purified by distillation according to McLellan and Thorndyke (19). Standards of MG-H1 and G-H1 were generously provided by Dr. Paul Thornalley (University of Essex, Colchester, Essex, UK); glucosamine, MODIC, GODIC, and DOGDIC were from Dr. Klaus Biebel (University of Hohenheim, Stuttgart, GR); argpyrimidine was from Dr. Marcus Glomb (Technical University of Berlin, Berlin, GR); and N-{

Carboxymethyllysine-4,4,5,5-}
d4-ornithine (d4-ornithine) was purchased from Cambridge Isotope Laboratories (Andover, MA). N\n\nCarboxymethylornithine (CMO), N\n\n[2-furoylmethyl]-l-ornithine ("furornithine"), and N\n\n,N\n\n-dimethylornithine (DMO) were synthesized as detailed elsewhere (see Supplemental Material). For derivatization, aliquots of acid-hydrolyzed collagen, the equivalent of 50 \mu\g of hydroxyproline (~357 \mu\g of collagen) were pipetted into borosilicate tubes together with the following deuteriated internal standards: 7 \nmol of d4-ornithine and 98 \nmol of d3-CML. The content of each tube was dried by a SpeedVac evaporator (Thermo Savant, Holbrook, NY) followed by conversion of amino acids into their isobutyl esters by heating the tubes in a 1:5 in the presence of diisobutyl ketone and with an added solution of 0.4 \mM thionyl chloride. The latter reagents were removed by the Speed-Vac set to a high drying rate. The dried residue was reconstituted with 25 \mu\l of an injection solvent consisting of 11.5 \mM formic acid and 2.75 \mM tridecafluorooctanoic acid (TDFHA) and transferred to a microjection vial.

In cases where samples were injected in the undervitized form, and acid-hydrolyzed collagen or lens hydrolysates were injected directly into the HPLC using the autosampler as described below.

**Ornithine Determination in Enzymatic Digests of Insoluble Lens Crystallins**—In order to determine the effects of acid hydrolysis on ornithine in human lenses, the enzymatic lens digest was analyzed as described below. The recovered pellet was freeze-dried and acid-hydrolyzed according to the procedure of Jentoft and Dearborn (25). Totals of 3 \mg of insoluble lens pellet or 6–7 \mg of insoluble skin collagen were acid-hydrolyzed followed by esterification with 2-propanol for HPLC ESI-MS analysis. Ornithine concentration was determined by its parent mass at m/z 175 with correction for the response of the internal standard, d3-ornithine, at m/z 181. Additionally, enzymatic digests of lens crystallins were prepared without acid hydrolysis for ornithine assay by HPLC ESI-MS analysis. The sample digest was spiked with internal standards and derivatized with 2-butanol.

**Reducive Alkylation of Insoluble Collagen and Lens Crystallins**—In order to prove that ornithine is present in proteins prior to any manipulation, samples were reductively alkylated in order to generate acid-stable DMO using the procedure of Jentoft and Dearborn (25). Totals of 3 \mg of insoluble lens pellet or 6–7 \mg of insoluble skin collagen were reductively alkylated by continuous rotation overnight at 4 °C. Tubes containing 83 \mM each of sodium cyanoborohydride and formeldehyde in 0.1 \mM sodium phosphate buffer, pH 7 (see Supplemental Material). The recovered pellet was freeze-dried and acid-hydrolyzed according to previously published procedures (26). Additionally, in order to study the effect of protein unfolding on the yield of DMO, some samples were reductively alkylated in the presence of urea. The urea was removed simultaneously with that described for reductive alkylation, except that 7 \mM urea was added to the buffer prior to adjusting the pH to 7.

**Reduction of Collagen with Borohydride and Cyanoborohydride—**Collagen was reduced according to procedures stated above for reductive alkylation, except formeldehyde was used in the buffer. The purpose of this experiment was to investigate whether precursors of ornithine exist that may be composed of acid-labile reducible chemical bonds. Collagen was prepared by weighing insoluble skin samples from n = 11 different donors of age range 15–90 years. Each sample was weighed into three portions consisting of 6 \mg each followed by treating each portion in one of three ways as follows: (i) control, no reduction; (ii) reduction with 0.1 \mM sodium borohydride; or (iii) reduction with 0.1 \mM sodium cyanoborohydride.

**Quantitative Analyses of Ornithine, CMO, Furornithine, and DMO by HPLC ESI-MS/MS SRM—**Liquid chromatography was carried out using a Michrom Magic 2002 HPLC (Michrom BioResources, Auburn, CA). The pump flow at 120 \mu\l/min was split 1:12 (column/waste) by the Magic Precolumn Variable Splitter (Michrom), which resulted in a column flow at 10 \mu\l/min. Samples of 1–2 \mg were injected into a 15 \mM 0.32 mm, 5 \muM pore size, 50 \mM phosphate buffer (pH 7) by a SpeedVac evaporator (Savant, Thermo Electron, San Jose, CA) as detailed elsewhere (see Supplemental Material).

Solvents used for HPLC were as follows: solvent A, water; solvent B, 70% acetonitrile in water (Burdick & Jackson Certified Solvents, Honeywell, Inc., Muskegon, MI). For ornithine, CMO, and DMO assays,
Preparation of AGE-BSA from the Incubation of BSA with Reducing Sugar—Bovine serum albumin (BSA, Fraction V, heat shock, fatty acid-free; Roche Applied Science) was incubated at 15 mg/ml in 0.05 M sodium phosphate buffer, pH 7.4, for 7 days at 37 °C in 10-ml volumes with 20 mM each of the following sugars: D-ribose, DL-glyceraldehyde, glyoxal, and methylglyoxal. In addition, BSA was incubated similarly with 200 mM D-glucose for either 3 or 82 days (see Supplemental Material).

Reductive Alkylation, Acid Hydrolysis, and Assay for Ornithine and DMO in AGE-BSA—AGE-BSA preparations were reductively alkylated as described above with minor modifications. Samples of ~7 mg each of AGE-BSA preparation, but also unmodified BSA as a control, were reductively alkylated in duplicates. Further procedures for acid hydrolysis and assays of these preparations are detailed in the Supplemental Material.

Acid Hydrolysis of Arginine and a Series of AGE Standards for Screening of Precursors for Ornithine Formation—A series of experiments were designed to determine whether ornithine could be generated from arginine and arginine-derived AGEs shown in Fig. 1. In the first experiment, aliquots of l-arginine (SigmaUltra) consisting of 0.25, 0.5, 1.0, and 1.5 nmol were each acid-hydrolyzed in a total of 300 μl of 6 N HCl (see Supplemental Material). In a second experiment, aliquots of standards (Table I) were each evaporated and acid-hydrolyzed in 300 μl of 6 N HCl at concentrations as follows: (i) 0.1–0.60 pmol of pentosidine; (ii) 0.25–1.5 nmol each of argpyrimidine, G-H1, and MG-H1; (iii) 112–1050 nmol each of 70 mM glucosepane, MODIC, GODIC, and DOG-DIC. Procedures for assays of these products are detailed in the Supplemental Material.

Statistical Methods—Statistical analyses were done using procedures previously described by us (20, 26, 28) using SPSS software (Graduate Pack version 11.5, SPSS Inc., Chicago, IL). In these analyses, race, gender, diabetes, and renal failure were coded with indicator variables as follows: race, 1 = Caucasian, 2 = African-American; gender, 1 = male, 2 = female; diabetes, 0 = absence of diabetes, 1 = type 1, 2 = type 2; renal failure, 0 = absence of chronic renal disease or end stage renal disease (ESRD), 1 = chronic renal disease, 2 = ESRD, as previously defined (20). For skin, there were n = 68 Caucasians and n = 45 African-Americans. All lens samples were from Caucasians.

RESULTS

The products assayed in this study and their presumed chemical relationships are summarized in Fig. 2, whereby any of the arginine-AGEs in Fig. 1 is regarded as a potential precursor for ornithine formation in vivo or during acid hydrolysis. Ornithine, CMO, and Furornithine Levels in Acid-hydrolyzed Human Insoluble Skin Collagen—Ornithine, glycated ornithine (furornithine), and CMO were measured in acid hydrolysates of insoluble skin collagen by HPLC ESI-MS/MS SRM.

Amino acids were converted into their isobutyl ester derivatives, as detailed under “Experimental Procedures,” whereupon ornithine eluted at ~25–26 min in the chromatogram (Fig. 3). Skin levels followed as a function of age, diabetes, and renal failure are shown in Fig. 4A. Ornithine increased linearly (p < 0.0001) with age in nondiabetic subjects without renal failure and reached ~4 nmol/mg collagen at age 90 years (Fig. 4A). Stepwise multivariate linear regression analyses revealed that age (p < 0.0001) and renal failure (p = 0.035) were selected as predictors for ornithine formation in skin with the equation, ornithine = 1.4 + 0.03(age) + 0.458(renal failure), whereby renal failure included both chronic renal disease and ESRD as defined under “Statistical Methods.”
For further investigation of the effects of renal failure on ornithine levels in skin collagen, levels were age-adjusted to 50 years and categorized into nine different groups according to the presence or absence of diabetes and renal failure as previously published (20; i.e. type 1 versus type 2 diabetes; chronic renal failure versus end stage renal disease (ESRD) (data not shown). Statistical comparisons using the Mann-Whitney test showed that ornithine was elevated above levels for nondiabetic control subjects without renal failure, but only when both diabetes and ESRD were present (p = 0.022).

Since ornithine is reportedly not found in native proteins (16), further evidence for its presence in the primary structure of collagen was sought. Based on the structural homology between lysine and ornithine, we hypothesized that if ornithine was actually present in intact protein and not an artifact of acid hydrolysis, then the Amadori product, 8-fructosyl-ornithine, and the AGE, CMO, should be present in senescent proteins. In analogy to the conversion of fructosyl-lysine to furosine and CML, we expected to detect “furornithine” and CMO (Fig. 2) in the acid hydrolysate, but in lower quantities compared with furosine and CML, since furornithine and CMO are secondary modifications. Thus, quantitative analysis of these ornithine-derived AGE modifications, furornithine and CMO (Fig. 2), would be considerably more challenging and require assays of higher sensitivity than furosine and CML. On the other hand, the finding that ornithine is a major age-related modification with levels up to 7 nmol/mg collagen in some skin samples (Fig. 4) suggested that even at an expected yield at 1% of total ornithine, sufficient sensitivity for the detection of ornithine modifications would be achieved.

The isobutyl ester of CMO (RT $\sim$ 30.8 min) eluted just before $d_4$-CML (RT $\sim$ 31.1 min) (Fig. 3). CMO levels in the acid hydrolysate of collagen samples (Fig. 4) showed more variation compared with ornithine (Fig. 4A). Levels increased with age (p < 0.0001), reaching about 60 pmol/mg collagen at age 90 years (Fig. 4B). However, the effects of diabetes (p = 0.27) and renal failure (p = 0.58) were not significant, as confirmed by stepwise regression analysis. In addition, CMO significantly (p < 0.0001) correlated (r = 0.70) with ornithine levels within the same individuals (data not shown).

Furornithine (i.e. the acid-hydrolyzed product of glycated ornithine) (Fig. 2) was measured in the acid hydrolysate of insoluble skin collagen samples by HPLC ESI-MS/MS SRM without prior derivatization. Furornithine eluted at $\sim$ 21.9 min (Fig. 5) (i.e. after ornithine (RT $\sim$ 19.5 min) but before furosine (RT $\sim$ 22.5 min)). Results showed that levels significantly (p < 0.0001) increased from 0 to 175 pmol/mg collagen between the ages of 8 and 90 years (Fig. 4C). Levels were significantly (p = 0.022) elevated by diabetes but not renal failure (p = 0.46). These three factors, including gender and race, were further examined by stepwise multivariate linear regression analyses. Both age (p < 0.0001) and diabetes (p = 0.013) were selected as predicting factors for furornithine formation in skin with the equation, ornithine = 15 + 0.8(age) + 11(diabetes). Levels were age-adjusted to 50 years, and the effect of diabetes was further investigated by analysis of variance and the Student-Newman-Keuls post hoc test. The results showed that diabetes was still a significant (p = 0.007) factor even after adjustment for age. Levels were significantly (p < 0.05) elevated for individuals with type 2 diabetes versus nondiabetic controls by the Student-Newman-Keuls test (data not shown).

Ornithine, CMO, and Furornithine Levels in Acid-hydrolyzed Insoluble Human Lens Crystallins—Ornithine significantly (p = 0.0012) increased with age in acid-hydrolyzed lens crystallins from nondiabetic subjects without renal disease (Fig. 6A). Univariate regression analysis showed significant effects due to age (p = 0.013) and diabetes (p = 0.044), but not gender (p = 0.92) and Pirie grade (p = 0.11). Multivariate analysis consisting of ornithine levels versus age and diabetes showed that the bivariate model was significant (p = 0.025), but each
contributing factor within this model was not (i.e., age, $p = 0.068$; diabetes, $p = 0.26$). Furthermore, stepwise regression analysis selected age ($p = 0.013$), but not diabetes ($p = 0.82$), Pirie grade ($p = 0.63$), and gender ($p = 0.99$). Last and surprisingly, levels were positively ($r = 0.63$) and significantly ($p = 0.004$) correlated with age for Pirie grade I (i.e., the least pigmented lenses) (Fig. 6A). No such correlation was found for Pirie grades $>1$ ($r = 0.21$, $p = 0.31$), possibly due to a larger variability in these groups.

CMO in lens crystallins (Fig. 6B) was not associated with any factor. There was a nonsignificant ($p = 0.091$) trend for levels to increase with age in nondiabetic subjects, as shown in
which showed that Pirie grade is strongly associated with age ($r = 0.63, p < 0.0001$).

Overall, these results suggest that more samples would be needed to increase the statistical power of the tests as reflected by the borderline significance of diabetes and the lack of association of ornithine with lens samples for Pirie grade $> 1$.

**Ornithine Levels in Enzymatic Digests of Insoluble Human Lens Crystallins**—Similar studies (data not shown) were also carried out with insoluble human lens crystallins that were sequentially digested by four different enzymes. This digestion protocol resulted in an average of 65% of the lens protein being digested into amino acids as determined by ninhydrin. Ornithine measurements in these digests showed levels nonsignificantly ($p = 0.17$) increased with age by univariate regression analysis. All other factors, including diabetes ($p = 0.69$), Pirie grade ($p = 0.37$), and gender ($p = 0.24$), were nonsignificant. However, since it was noted that more variation occurred in ornithine levels for lens samples of Pirie grade I, these data ($n = 19$) were subsequently removed from the analysis. Re-analysis of the remaining levels (i.e. Pirie grade $> 1$, $n = 27$) showed that ornithine significantly ($p = 0.003$) increased with age ($r = 0.55$) (data not shown). These results suggest incomplete cleavage of ornithine from proteins in digests of insoluble lens crystallins and perhaps more resistance to enzymatic digestion, particularly in the diabetic lens samples. Surprisingly, more ornithine was recovered from these digests (i.e. up to 30 nmol/mg protein) in comparison with acid hydrolysis (i.e. about 15 nmol/mg protein), suggesting that the digestion procedure partly converted arginine or arginine-AGEs into ornithine.

**The Effect of Chemical Reduction by Borohydride on Measured Ornithine Levels in Collagen**—The experiments so far have unequivocally demonstrated the presence of fructosyl ornithine and CMO in collagen and lens crystallins. However, uncertainty persisted as to how much ornithine was being artifically generated either by enzymatic or acid hydrolysis. In particular, one question was whether a borohydride- or cyanoborohydride-reducible labile precursor served as ornithine source during processing. To investigate this question, insoluble skin collagen samples were reduced with either sodium borohydride or cyanoborohydride. Ornithine levels in the acid hydrolysates were compared with those from unreduced samples ($n = 11$ donors/group). The results showed that ornithine levels increased significantly ($p = 0.008$) with age in both reduced and nonreduced samples. However, the effect of reduction by either agent was insignificant ($p = 0.76$), suggesting that the ornithine formation pathway does not involve reducible chemical bonds (data not shown).

**The Effect of Reductive Alkylation of Collagen and Lens Crystallins on Levels of DMO**—In all studies so far, regression lines for ornithine did not go through the zero point at age “zero,” in contrast to those for furornithine and CMO (Figs. 4 and 6), again suggesting that some ornithine was being generated during processing of the protein. In order to unequivocally address the question of whether and how much ornithine was actually present in lens crystallins and collagen prior to any treatment, the intact protein was reductively alkylated with formaldehyde in order to convert the ornithine ε-amino group into the acid-stable dimethyl adduct (i.e. DMO). An HPLC ESI-MS/MS SRM assay was developed for detection of DMO derivatized as isobutyl esters. As shown in Fig. 7, DMO eluted as a single peak at $-0.4$ min after ornithine (RT $25.1–25.4$ min) in skin collagen samples. The peak area of DMO assayed in a reductively alkylated skin sample from an 89-year-old donor is larger than that from a 15-year-old. DMO was not detected in protein samples without reductive alkylation at any

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**Fig. 4.** The effect of age, diabetes, and renal failure on formation levels of ornithine and its modifications in acid-hydrolyzed insoluble human skin collagen. Regression line and 95% confidence intervals computed for nondiabetic subjects without renal failure are shown. Nondiabetics and diabetics are represented by open and closed symbols, respectively. A, ornithine, \( y = 1.4 + 0.031x, n = 59, r = 0.75, p < 0.0001 \); B, CMO, \( y = 2.3 + 0.35x, n = 59, r = 0.60, p < 0.0001 \); C, furornithine, \( y = 1.6 + 0.25x, n = 59, r = 0.52, p < 0.0001 \). D, nondiabetic; ◊, nondiabetic with renal failure; ▲, type I diabetic with and without renal failure; ▼, type 2 diabetic with and without renal failure.

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Fig. 6B. Univariate analyses of all data (i.e. both diabetic and nondiabetic) showed no effects of age ($p = 0.31$) and gender ($0.34$) and nonsignificant trends for CMO to be associated with diabetes ($p = 0.082$) and Pirie grade ($p = 0.13$).

Furornithine in lens crystallins increased significantly ($p = 0.04$) with age in nondiabetic subjects (Fig. 6C). When all data were collectively analyzed, there were significant effects due to age ($p = 0.029$) and Pirie grade ($p = 0.028$) but not gender ($p = 0.67$). The effect of diabetes approached statistical significance ($p = 0.066$). Stepwise regression analysis failed to select Pirie grade as a significant ($p = 0.77$) factor, suggesting that Pirie and age were intercorrelated. This latter association is well known and was confirmed by Spearman’s correlation analysis,
time during these analyses. DMO levels were modeled as linear functions for both collagen (Fig. 8A) and lens (Fig. 8B). Levels significantly increased in both insoluble human skin collagen \((p < 0.0001)\) and lens crystallins \((p = 0.004)\), reaching up to \(\sim 5\) and \(\sim 8\) nmol/mg protein, respectively. In addition, both lines intercepted approximately at the point of origin as evidenced by the \(y\) intercept at age 0: collagen (controls), \(-0.16 \pm 0.31\); lens, \(0.042 \pm 1.7\) (Fig. 8).

Finally, arguing that the reductive alkylation reaction may not have hit all existing ornithine residues due to protein folding and hiding of residues, selected samples of collagen were denatured using \(7 \text{ M urea}\) prior to the reaction. A small but significant \((p = 0.037)\) increase of about 10% in recovery of DMO was noted in urea-treated skin samples \textit{versus} untreated controls (Fig. 8A).

The overall conclusion from the comparison of the data above (Fig. 8) with that of Figs. 4 and 6 is that acid hydrolysis and protein manipulation apparently contributed little to total ornithine levels.

\textbf{Mechanism of Ornithine Formation: The Role of Spontaneous versus Acid-catalyzed Conversion of AGEs into Ornithine—Further studies were undertaken to address the role of arginine-derived AGEs in ornithine formation. Various AGE-protein preparations were prepared by incubation of BSA at 37 °C for 7 days with 20 mM each of ribose, glyceraldehyde, glyoxal, and methylglyoxal. In addition, the effects of both short and long}
term glycation on ornithine formation were investigated by incubating BSA with 200 mM glucose for either 3 or 82 days. In order to verify ornithine formation in these preparations, aliquots were reductively alkylated with formaldehyde before acid hydrolysis to convert putative ornithine into DMO. Subsequently, the preparations were acid-hydrolyzed, and DMO levels were measured as isobutyl esters.

Fig. 9A indeed shows that ornithine is present in substantial quantities in acid hydrolysates of AGES, whereby methylglyoxal was the most active precursor (p < 0.0001 versus control) followed by glyceraldehyde (p < 0.001) > glyoxal (p < 0.001) > ribose (p < 0.05) > 82-day glucose (p < 0.05) > 3-day glucose (p > 0.05, not significant).

Most interestingly, however, a strong indication of ornithine’s presence in these AGE-BSA samples prior to acid hydrolysis was found (Fig. 9B). The highest amount of DMO was measured in 82-day glucose (p < 0.0001 versus control), where levels reached ~2 nmol/mg protein followed by glyoxal (p < 0.0001) > ribose (p < 0.001) > glyceraldehyde = methylglyoxal (p < 0.01) > 3-day glucose (p > 0.05, not significant). Thus, these results clearly indicate that ornithine can form spontaneously during glycation reactions but that acid hydrolysis of AGES can generate substantial amounts of ornithine as judged by the 10-fold difference in scale between Fig. 9, A and B.

Finally, several experiments were designed to determine the possible origin of ornithine formation during acid hydrolysis. The results showed that the commercial source of ultrapure arginine was contaminated with ornithine. However, after correction for this factor, there was no net conversion of arginine into ornithine over concentrations examined (data not shown). We then sought to address the question of which AGE contributes most ornithine in an acid hydrolysate. Each of the arginine-based AGES listed in Fig. 1 was acid-hydrolyzed, and recovery analysis was performed. As shown in Table I, pentosidine, argpyrimidine, MG-H1, and GH-1 generated the most ornithine, whereby we estimated based on the data from Ahmed et al. (29) that MG-H1 and G-H1 would generate most of the artifactual ornithine in a hydrolysate from a 90-year-old lens. Since MG-H1 and G-H1 levels in collagen are not known, this estimation cannot be made. Most interestingly, however, the sum of artifactual ornithine formed in hydrolysate from the lens is still less than 1 nmol/mg (i.e. almost 8 times lower than total ornithine or ornithine that can be measured as DMO).

From these data, it can be concluded that a rough estimate of ornithine in biological proteins with about 15% error can be made from acid hydrolysates without prior derivatization into DMO. The other surprise was that 40 and 75% of MG-H1 and GH-1, respectively, can be recovered from a 6 N HCl hydrolysate after 16 h at 110 °C. This result, however, did not hold true for glucosepane, which was completely destroyed (Table I).

DISCUSSION

This study provides, to our knowledge, the first evidence for the presence of ornithine and its glycation products fructosyl-ornithine and CMO in biological proteins. The data strongly suggest that ornithine formation in senescent proteins is the result of chemical conversion of arginine into an AGE product that subsequently spontaneously degrades into ornithine. As a lysine analogue, ornithine is then available to undergo the same modifications as those that affect lysine residues. Amid these conclusions, a number of observations need to be taken into consideration.

The first and most important question was to precisely understand the contribution of acid hydrolysis on ornithine formation. Paul et al. (18) reported the presence of ornithine in the amino acid analysis chromatograms of acid hydrolysates of MG-modified proteins. They attributed this observation to the breakdown by acid hydrolysis of the hydroimidazolones (e.g. MG-H1 and G-H1) (Fig. 1), characterized as the major AGE adducts in the in vitro reaction between ribose and collagen (18). This proposition led us to perform extensive studies on the origin of ornithine in our analytical samples.

Several data unequivocally support the notion that most ornithine measured forms in situ during protein aging and is not simply an artifact of acid hydrolysis. First, frornithine, the acid-catalyzed rearrangement product of glycated ornithine (Fig. 2), significantly increased with age in both insoluble collagen (p < 0.0001) and lens (p = 0.029), whereas CMO signif-
Significantly (p < 0.0001) increased in insoluble collagen, suggesting that ornithine must be present in intact protein before acid hydrolysis. Second, the derivatization of the intact proteins into DMO prior to hydrolysis revealed that the latter significantly increased with age in both collagen (p < 0.0001) and lens (p < 0.005). Third, DMO was present in reductively alkylated AGE-modified BSA samples, implying that incubation of the proteins under mild conditions with glycating agents suffices to convert substantial quantities of arginine into ornithine. Expressed as a percentage of arginine residues, ornithine level in Figs. 4A and 6A represents an estimated 1.1% modification (mol/mol) in collagen and 1.5% modification in lens crystallins in a 90-year-old person. Finally, ornithine was also detected in enzymatic digests of insoluble lens crystallins and significantly increased with age (p = 0.009) for all data excluding those of nondiabetic Pirie I (not shown).

Thus, whereas our data both unequivocally demonstrate that ornithine is present in senescent proteins and can form during acid hydrolysis of AGEs, the next question was what percentage of ornithine in the acid hydrolysates of biological samples was generated during the procedure. A strong significant (p < 0.0001) correlation between ornithine and DMO levels within

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**Fig. 7. Chromatograms for assay of DMO in insoluble human skin collagen.** DMO was assayed by HPLC ESI-MS/MS SRM using $d_6$-ornithine (Orn) as the internal standard (see Fig. 3). DMO was converted into isobutyl esters by derivatization with 2-butanol. The parent ion of DMO at m/z 217 was fragmented and monitored for product (daughter) ions at m/z = 172 and m/z = 116. Volumes consisting of 2 μl containing ~28.6 μg of collagen were injected as described in Fig. 3.
Throughout the study, reductive alkylation was used to modify proteins before acid hydrolysis. For lens, more variation was noted in this relationship (Fig. 10), which approached significance (p = 0.093). DMO recovery was 87 ± 32% (S.D.) of that for ornithine.

The following points are elaborated in the text:

- **Fig. 8.** DMO levels as a function of age in insoluble human skin collagen and lens crystallins with reductive alkylation before acid hydrolysis. DMO was assayed by HPLC ESI-MS/MS SRM described in Fig. 7. The regression line and 95% confidence intervals of prediction are indicated in both graphs. A, insoluble human skin collagen (all data used in regression line computations as shown): $y = 0.042x - 0.08$, $n = 44$, $r = 0.84$, $p < 0.0001$; reductive alkylation in the presence of 7 M urea, $y = 0.042x - 0.15$, $n = 24$, $r = 0.85$, $p < 0.0001$; reductive alkylation without urea (controls), $y = 0.039x - 0.16$, $r = 0.85$, $n = 20$, $p < 0.0001$. B, insoluble human lens crystallins, $y = 0.042 + 0.091x$, $r = 0.68$, $n = 16$, $p = 0.004$. The two outliers shown above the confidence interval for lens were not used in the analysis. ⊗, reductive alkylation; ⊕, reductive alkylation in 7 M urea.

- **Fig. 9.** The effect of acid hydrolysis on ornithine and DMO levels in AGE-BSA preparations with and without reductive alkylation. AGE-BSA preparations were made by the incubation of BSA with various reducing sugars as listed. Mean levels ± S.D. are stated as nmol/mg BSA. Error bars that do not have the same letter superscript in common with each other are significantly (p < 0.05) different by the Student-Newman-Keuls test.

The text elaborates on the importance of ornithine in biological samples, including skin and lens crystallins. It is noted that ornithine is present in skin donors >50 years of age, more than 90 ± 20% (S.D.) of measured ornithine levels is present before acid hydrolysis. For lens, more variation was noted in this relationship, which approached significance (p = 0.093). DMO recovery was 87 ± 32% (S.D.) of that for ornithine.

Several hypothetical mechanisms may explain the origin of ornithine in biological samples, whereby decreased protein turnover with age (32) undoubtedly plays a major role in its age-related accumulation. The first and most attractive hypothesis involves enzymatic cleavage of the arginine moiety of specific AGEs. This would represent a physiological repair mechanism to maintain homeostasis by regeneration of the positive charge loss during AGE formation, thus retaining both protein structure and function. This hypothesis was proposed by Sletten et al. (30) as a possible explanation of ornithine in the urate-binding $\alpha_1\alpha_2$ globulin as previously discussed. Sletten et al. (30) hypothesized the splitting off of urea from arginine residues in this protein by catalysis involving a specific arginase. Indeed, widespread expression of arginase I in mouse tissues including skin has been reported (17). However, in skin, localization was to the hair follicle, not the ECM (17).

A second mechanism would involve the covalent attachment of free ornithine to glycated proteins. This hypothesis is supported by studies that show that free lysine and soluble proteins like albumin can covalently attach to glycated calf skin collagen (33). The strongest argument against covalent incorporation of ornithine as an explanation for our findings is that ornithine would have to be chemically bound via an N-α-amino group in order to leave the δ-amino group free for derivatization into DMO and yet allow intact release upon acid hydrolysis. Whereas sugar amide bonds have been described (23), this type of linkage is yet unheard of.

The third and most likely mechanism involves in situ decomposition of arginine-derived AGEs via a nonenzymatic, spontaneous mechanism, similar to the age-dependent deamidation of asparagine and glutamine residues in proteins (34). Some of the AGEs known to accumulate with age in collagen and lens crystallins are shown in Fig. 1. Interestingly, a recent study revealed large amounts of glyoxal and methylglyoxal hydroimi-
diazolones in human lens crystallins (29) that could serve as precursors for ornithine (Table I). However, it has recently been reported that the hydroimidazolones are short lived under physiological conditions (29, 35). Whether the same phenome-

Table I

| AGE | Total amount of acid hydrolyzed in 300 µl of 6 n HCl | Samples (n) | Generated ornithine (percentage of total AH) | Recovery after AH (percentage of total AH) | Estimated contribution in vivo* |
|-----|--------------------------------------------------|-------------|---------------------------------------------|------------------------------------------|-------------------------------|
| Adducts | | | | | |
| Argpyrimidine | 250–1500 pmol | 3 | 13.2 ± 4.4 | 27 | 22 | 90 |
| GH-1 | 250–1500 pmol | 4 | 5.5 ± 3.2 | 75 | 7 | 215 |
| MG-H1 | 250–1500 pmol | 4 | 5.7 ± 4.4 | 40 | 399 |
| Cross-links | | | | | |
| Pentosidine | 100–600 pmol | 3 | 15.8 ± 7.3 | 62 | 9 | 0.95 |
| Glucosepane | 7–1551 nmol | 3 | 11.4 ± 0.42 | 16 | 3 | |
| MODIC | 176–1055 nmol | 4 | 0.22 ± 0.02 | 0 | 0 | |
| GODIC | 219–1315 nmol | 4 | 0.34 ± 0.06 | 0 | 0.04 | |
| DOGDIC | 112–448 nmol | 3 | 0.51 ± 0.12 | 0 | 0.02 | |

***Based upon the percentage of generated ornithine level from this table multiplied by level of the specific AGE estimated from the literature for a 90-year-old nondiabetic individual as follows. For skin, these values are 167, 57, 1400, 17, 10, and 4 pmol/mg protein for argpyrimidine, pentosidine, glucosepane, MODIC, GODIC, and DOGDIC, respectively (D. Sell, V. K. Biemel, O. Reihl, M. Lederer, and V. Monnier, unpublished results and Sell et al. (20)). For lens, these values are 300, 3966, 7000, 6, 250, and 3 for argpyrimidine, GH-1, pentosidine, glucosepane, and GODIC, respectively (Biemel et al. (12), Ahmed et al. (29), Tessier et al. (43), and Wilker et al. (44)).

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