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Oxidative Degradation of Glucose Adducts to Protein

FORMATION OF 3-(N'-LYSINO)-LACTIC ACID FROM MODEL COMPOUNDS AND GLYCATED PROTEINS*

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The chemistry of Maillard or browning reactions of glycated proteins is being studied in model systems in vitro in order to characterize potential reaction pathways and products in biological systems. In previous work with the Amadori rearrangement product N'-formyl-N'-fructoselysine (fFL), an analog of glycated lysine residues in proteins, we showed that fFL was oxidatively cleaved between C-2 and C-3 of the carbohydrate chain to yield N'-carboxymethyllysine (CML) and d-erythronic acid. We then detected CML in proteins glycated in vitro, as well as in human lens proteins and collagen in vivo (Ahmed, M. U., Thorpe, S. R., and Baynes, J. W. (1986) J. Biol. Chem. 261, 4889–4894). This work provided an explanation for the origination of CML in human urine and evidence for non-browning pathways of the Maillard reaction in vivo. In this report we describe the identification of a second set of products resulting from oxidative cleavage of fFL between C-3 and C-4 of the sugar chain, i.e., 3-(N'-lysino)-lactic acid (LL) and d-glyceric acid. The formation of LL from fFL was increased at slightly acid pH, representing about 30% of the yield of CML at pH 6.4, compared with 4% at pH 7.4 in phosphate buffer. By gas chromatography-mass spectroscopy, LL was detected in proteins glycated in vitro and then identified as a natural product in human lens proteins and urine. Our results indicate that oxidative degradation of Amadori adducts to proteins occurs in vivo, leading to formation and excretion of CML and LL. These non-browning pathways for reaction of Amadori compounds may be physiologically relevant mechanisms for averting potentially damaging consequences of the Maillard reaction.

The Maillard or browning reaction is a complex series of reactions which occur between reducing sugars and amines, leading to the formation of poorly characterized, polymeric, brown, and fluorescent compounds (1–4). Maillard reactions between sugars and proteins under physiological conditions in vitro (pH 7.4, 37 °C) may also lead to denaturation, cross-linking, and precipitation of the protein, as well as the formation of protein-bound pigments with a characteristic brown color and fluorescence. The first step in this reaction is the formation of a Schiff base between a reducing sugar and an amino group on the protein, followed by an Amadori rearrangement to yield a relatively stable ketoamine adduct. The early stages of the Maillard reaction, resulting in the formation of the Amadori adduct, are described as glycation or nonenzymatic glycosylation of proteins and are known to occur between glucose and proteins in vivo (5–9). The level of glycation of numerous body proteins, such as hemoglobin, albumin, and collagen, also corresponds closely with the mean circulating blood glucose concentration, and glycation of protein is increased in proportion to the degree of hyperglycemia in diabetes. It is widely believed that subsequent browning reactions of glycated proteins in vivo may contribute to the denaturation, cross-linking, and insolubilization of tissue proteins and the gradual development of the chronic pathophysiology of diabetes (5–9). In addition, because of the decline in glucose tolerance and decreased rates of protein turnover with age, there is reason to believe that Maillard reactions may also be one mechanism involved in the age-dependent chemical modification of proteins (9).

One of the goals of research in our laboratory has been to study the intermediates and mechanisms of protein browning and cross-linking during the later stages of the Maillard reaction. We recently showed that the compound N'-carboxymethyllysine (CML) is formed on oxidation of the Amadori compound N'-formyl-N'-fructoselysine (fFL), an analog of glycated lysine residues in proteins (Fig. 1, top) (10, 11). We also observed that CML was formed following glycation of proteins in vitro and was detectable in human lens protein and collagen. Our observations suggested that CML, which had been detected previously in human urine (12), is derived from the oxidative degradation of glycated proteins. Both CML and the split product erythronic acid are inactive in the Maillard reaction, since the carboxylic acid functional groups of CML and erythronic acid are relatively inert compared to the carbonyl functionality in the Amadori adduct. We have now identified the hitherto unknown compound 3-(N'-lysino)-lactic acid (LL) as a second product of oxidation of fFL in vitro (Fig. 1, bottom). In this report we describe the characterization of LL and some factors affecting its formation in vitro, and we also report the presence of this compound in human lens proteins and as the free amino acid in human urine.

EXPERIMENTAL PROCEDURES

General Methods—Diethylenetriaminepentacetic acid (DTPA), Tiron (4,5-di hydroxy-1,3-benzendisulfonic acid), poly-L-lysine-HBr

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**RESULTS**

**Identification of 3-(N'-Lysino)-lactic acid**—In previous work we showed that oxidative degradation of Amadori compounds occurs spontaneously in phosphate buffer under air in the presence of trace amounts of iron salts (11). CML was identified as the major product of oxidation of fFL at pH 7.4 (11). However, we also observed a minor product which eluted at 42 min on the amino acid analyzer, about 3 min later than CML (Fig. 2A). The yield of this compound was about 5% of the yield of CML at pH 7.4 (Fig. 2A) but increased to >20% of CML at pH 6.4 (Fig. 2B). As with CML, this product was formed in only trace amounts in the absence of air (see Table II, discussed below), and was also formed during glycation of poly-L-lysine (Fig. 2, C and D). Its elution time and yield were not affected by reduction with NaBH₄ (Fig. 2, C versus D), nor could it be labeled by reduction with [³H]NaBH₄ (data not shown). The formation of this product during glycation of proteins in vitro could not be confirmed by HPLC amino acid analysis because of its coelution with leucine or isoleucine in several gradient systems. The compound was isolated by pooling peak fractions from several semi-preparative runs of fFL reaction mixtures on the amino acid analyzer. Following derivatization by methyl esterification and acetylation, it was tentatively identified as LL, based on its mass spectrum obtained by direct probe analysis (Fig. 3). This mass spectrum was identical to that of authentic LL prepared by reaction of bromopyruvic acid with N'-formyllysine, followed by reduction in situ with NaBH₄, as described under “Experimental Procedures.”

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*Fig. 1. Pathways for oxidative degradation of fructoselysine in glycated proteins. The upper pathway, leading to formation of CML, was characterized in Ref. 11. Evidence for the lower pathway, leading to formation of LL, is presented in this paper.*
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Although LL could be characterized as its acetylated methyl ester derivative using direct probe MS after its purification from a reaction mixture, it was not detectable in reaction mixtures by GC/MS, either because of its poor volatility or adsorption to the GC column. As an alternative approach to derivatization, the fFL reaction mixture was reduced with NaBH₄, (converting fFL to the hexitollysines, glucitol- and mannitollysine), desalted on Dowex 1-X8 acetate, deformedylated, and derivatized directly by trimethylsilylation. The total ion chromatogram (Fig. 4) and mass spectra revealed the presence of both LL and its putative split product, glyceric acid (Fig. 1), and also of CML and erythronic acid. In addition, mannose and glucose (as mannitol and glucitol) and lysine, which are formed on reversal of the Amadori rearrangement, were among the major products detected in the reaction mixture. The hecitollysines, glucitol- and mannitollysine, formed on reduction of the remaining fFL, were also detected in the chromatogram. In other studies (data not shown), the reaction mixture was dried by rotary evaporation and then trimethylsilylated directly without prior reduction, desalting, and deformingylation. Although the trimethylsilyl derivative of fFL was not detectable in the ion chromatograms, erythronic and glyceric acids were observed in amounts comparable to those obtained following the reduction and deformingylation procedure. There were also prominent peaks at retention times and ion intensities relative to lysine and with mass spectra expected for the trimethylsilyl derivative of N⁴-formyl-CML and N⁴-formyl-LL. Thus, CML and LL appear to be formed in the reaction mixture in situ, rather than by degradation of labile intermediates during the work-up for analysis.

Characteristics of the Reaction—Having established that LL is a product of oxidative cleavage of fFL, we compared the kinetics of formation of LL and CML and the effects of various reaction conditions on their yield. As shown in Fig. 5, the kinetics of formation of LL closely paralleled those for formation of CML. The data in Table I indicate that, within the range of pH 5.4-8.4, formation of LL, relative to CML, was favored at more acidic pH, while the absolute yield of LL was maximal at pH 6.4. The experiments described in Table II indicate that LL, like CML, is formed by a mechanism involving oxygen free radicals. These reactions require both oxygen and traces of redox-active metal ions, such as copper or iron, and are inhibited by strong chelators and oxygen radical scavengers (16, 17). Thus, only traces of CML or LL were formed under nitrogen or in the presence of the chelator DTPA. Partial inhibition was observed with cyanide, probably as a result of precipitation or formation of complexes with metal ions. Good inhibition was also obtained with mannitol, a hydroxyl radical scavenger, and with Tiron which may act as either a chelator (18) or a superoxide radical scavenger (19, 20). The differential effect of Tiron on formation of CML and LL was consistently observed but is not fully understood. Overall, however, these data clearly support a free radical mechanism for the formation of both CML and LL.

Detection of LL in Biological Samples—Having detected LL in model reaction systems in vitro we next tested for the presence of this compound in biological samples. The trimethylsilyl derivatization procedure proved unsatisfactory for this purpose because of incomplete and variable efficiency of derivatization of amino acids in protein hydrolysates and concentrated urine samples. However, preparation of trifluoroacetyl methyl esters yielded reproducible results with lysine, CML, and LL, although FL and the hexitollysines were not detectable by this procedure. The total ion chromatogram of a typical fFL reaction mixture, prepared by trifluoroacetyl methyl ester derivatization, is shown in Fig. 6A, along with the mass spectrum of the compounds identified as CML and LL (Fig. 6, B and C, respectively). Using the trifluoroacetyl methyl ester derivatives, both CML and LL were also detected in hydrolysates of ribonuclease and lysozyme glycated under air in vitro (data not shown) but were not detectable in the native proteins. As shown in the selected ion chromatograms in Fig. 7, CML and LL were also detected in hydrolysates of human lens proteins and as the free amino acids in human urine. The peaks identified as CML and LL co-eluted with authentic standards in mixing experiments and had total ion chromatograms characteristic of CML and LL, respectively. The possibility that these compounds were formed from en-
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Fig. 3. Direct probe electron impact mass spectrum of 3-(N'-lysino)-lactic acid. LL was isolated by HPLC from an fFL reaction mixture incubated at pH 6.4 for 15 days at 37 °C, derivatized by methyl esterification and N- and O-acetylation, and analyzed on a Finnigan Model 4521C mass spectrometer.

Fig. 4. Total ion chromatogram of an fFL reaction mixture after incubation for 7 days at pH 6.4. The products were reduced with NaBH₄, deformylated, and then derivatized by trimethylsilylation. GA, glyceric acid; EA, erythronic acid; EAL, erythronic acid lactone; GlcOH and ManOH, glucitol and mannitol; ML and GL, mannitol- and glucitol-lysine. Peaks marked with an asterisk, Lys*, ML*, and GL*, contain one less trimethylsilyl group than the major peak. CML' contains 5 trimethylsilyl groups versus 4 in CML.

dogenous fL during the hydrolysis or derivatization procedures was excluded since similar results were obtained following prior reduction of the samples with NaBH₄. Overall, the trifluoroacetyl methyl ester derivatization procedure not only permitted the detection of LL in biological samples but also provided significantly higher sensitivity for detection of CML than was obtained previously using the N-acetyl methyl ester derivative (11). Preliminary experiments indicate that CML and LL are present at 3.5 ± 0.9 and 0.2 ± 0.6 mmol/mol lysine in adult human lens proteins (n = 4, donor age = 45–60) and 1.6 ± 0.8 and 0.35 ± 0.3 mg/g creatinine in fasting adult human urine (n = 5, age = 20–50).

DISCUSSION

The studies reported here extend our previous observations (10, 11) on the oxidative degradation of Amadori adducts to
by the activity of numerous endogenous chemical and enzymatic inhibitors of free radical reactions. The yield of browning products from fFL incubation would be oxidized, and CML and LL would accumulate in human urine is comparable to amounts of FL in lens protein, as reported from other laboratories, i.e. 1–2 nmol/mg lens protein (21, 22). Although LL has not been described previously, our estimate of the free CML concentration in human lens protein is 10–20-fold lower than that previously reported, i.e. 27 ± 17 mg/g creatinine (calculated from data in Table I of Wadman et al. (12)). The difference may result from differences in the populations under study, i.e. pediatric (12) versus adult donors, or from dietary sources of CML in the pediatric population (12). Our estimate of daily urinary excretion of free CML and LL (1–2 μmol/kg body weight/day) is about 10% of the estimated daily excretion of total (free plus peptide bound) glycated amino acids (2.7 μmol/kg/day, estimated from data of Brownlee et al. (23)). Although we have not yet obtained an estimate of total urinary CML and LL, the amounts detected are reasonable since only a fraction of glucose adducts would be oxidized, and CML and LL would accumulate primarily on longer-lived proteins which turn over more slowly. Even on these proteins, the rates of formation of CML and LL would be limited by the concentration of active oxygen and metal ion species in tissues and body fluids and inhibited by the activity of numerous endogenous chemical and enzymatic inhibitors of free radical reactions.

Since the products of oxidation of FL, such as CML, LL, erythronic and glyceric acid, are colorless, the reactions leading to their formation may be described as non-browning pathways of the Maillard reaction. These oxidation products are chemically inert compared with FL, and thus do not contribute to the further chemical modification and cross-linking of protein via the Maillard reaction. Indeed, the oxidation reactions appear to compete with the browning reactions since the yield of browning products from FL incubation mixtures in vitro is significantly depressed under air compared with nitrogen (11). The fact that the non-browning reactions occur in vivo suggests that they may function as a physiological mechanism for limiting extended damage to long-lived glycated proteins via the Maillard reaction. Thus, if Maillard reactions of glycated proteins have any role in the development of pathophysiology in diabetes and aging (5–9), differences in the relative rates of browning versus non-browning pathways among individuals may be important in determining their relative susceptibility to tissue damage from these reactions.
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Fig. 7. Simultaneous detection of CML and LL in human lens and urine samples by GC/MS using selected ion monitoring. Chromatographic conditions were as described in Fig. 6A, except for focus on the M-30 ions, m/z = 392 and 518 for CML and LL, respectively. A, analysis of hydrolysate of human lens protein. Samples were prepared as described under "Experimental Procedures." The amount of sample injected in 1 µl of methylene chloride corresponded to approximately 80 µg of lens protein, estimated by the Biuret assay prior to hydrolysis using a standard of bovine serum albumin. B, analysis of human urine. The urine sample was enriched for amino acids by sequential anion- and cation-exchange chromatography, as described under "Experimental Procedures." The quantity of sample injected in 1 µl of methylene chloride corresponded to approximately 25 µl of the original urine sample. Differences in retention times between chromatograms result from differences in sample load.

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