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Reaction of Ascorbate with Lysine and Protein under Autoxidizing Conditions: Formation of N^{ϵ}-(Carboxymethyl)lysine by Reaction between Lysine and Products of Autoxidation of Ascorbate†

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ABSTRACT: N^{ϵ}-(Carboxymethyl)lysine (CML) has been identified as a product of oxidation of glucose adducts to protein in vitro and has been detected in human tissue proteins and urine [Ahmed, M. U., Thorpe, S. R., & Baynes, J. W. (1986) *J. Biol. Chem. 261*, 4889-4894; Dunn, J. A., Patrick, J. S., Thorpe, S. R., & Baynes, J. W. (1989) *Biochemistry 28*, 9464-9468]. In the present study we show that CML is also formed in reactions between ascorbate and lysine residues in model compounds and protein in vitro. The formation of CML from ascorbate and lysine proceeds spontaneously at physiological pH and temperature under air. Kinetic studies indicate that oxidation of ascorbic acid to dehydroascorbate is required. Threose and N^{ϵ}-threuloselysine, the Amadori adduct of threose to lysine, were identified in the ascorbate reaction mixtures, suggesting that CML was formed by oxidative cleavage of N^{ϵ}-threuloselysine. Support for this mechanism was obtained by identifying CML as a product of reaction between threose and lysine and by analysis of the relative rates of formation of threuloselysine and CML in reactions of ascorbate or threose with lysine. The detection of CML as a product of reaction of ascorbate and threose with lysine suggests that other sugars, in addition to glucose, may be sources of CML in proteins in vivo. The proposed mechanism for formation of CML from ascorbate is an example of autoxidative glycosylation of protein and suggests that CML may also be an indicator of autoxidative glycosylation of proteins in vivo.

The Maillard or browning reaction between reducing sugars and amines is a complex series of reactions that leads eventually to the formation of brown and fluorescent polymeric products, known as melanoidins (Hodge, 1953; Feather & Waller, 1983; Fujimaki et al., 1986; Baynes & Monnier, 1989). The early products of the reaction are Schiff base and ketoamine (Amadori) adducts formed between sugars and amines, while the brown products, which are formed during later stages of the reaction, are not well characterized. Maillard reactions between glucose and amino groups of proteins are thought to contribute to the browning, fluorescence, and cross-linking of protein in vivo, suggesting a mechanism for the development of pathophysiology in diabetes and aging (Fujimaki et al., 1986; Brownlee et al., 1988a,b; Kennedy & Lyons, 1989; Baynes & Monnier, 1989). However, evidence in support of this hypothesis is limited because of the lack of information on the structure of browning products formed by reactions of proteins with glucose either in vitro or in vivo.

In recent studies on the Maillard reaction we have begun to characterize products formed from the model Amadori compound N^{α}-formyl-N^{ϵ}-(1-deoxy-D-fructos-1-yl)-L-lysine

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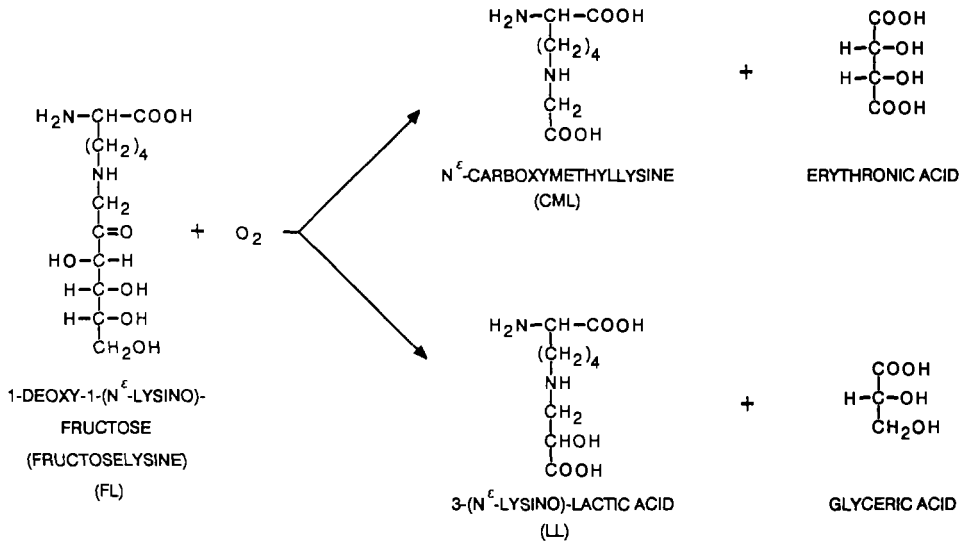

FIGURE 1: Reaction scheme for formation of CML and LL from FL in glycated proteins. The Amadori adduct, FL, in glycated proteins may be oxidatively cleaved between C-2 and C-3 or C-3 and C-4 of the carbohydrate chain to yield CML or LL, respectively. Split products that have been identified include erythronic and glyceric acids (Ahmed et al., 1986, 1988).

(formylfructoselysine, fFL),[1] an analogue of glycated lysine residues in protein. In these studies we identified two products, N^{ϵ}-(carboxymethyl)lysine (CML) (Ahmed et al., 1986; Baynes et al., 1986) and 3-(N^{ϵ}-lysino)lactic acid (LL) (Ahmed et al., 1988), which are colorless, inert compounds formed by oxidative cleavage of fructoselysine (FL) (Figure 1). CML and LL were also detected in human lens proteins, skin collagen, and urine (Wadman et al., 1975; Ahmed et al., 1986, 1988; Dunn et al., 1989). Their formation in tissue proteins has suggested that oxidation of Amadori adducts to CML and LL may be a physiological mechanism for limiting the potentially damaging consequences of browning reactions in vivo (Ahmed et al., 1986, 1988; Kennedy & Lyons, 1989).

Both CML and LL were shown to be formed in vitro from FL or glycated proteins by oxygen radical reactions. Since ascorbic acid (AA) is a potent reducing agent and radical scavenger in biological systems (Bielski, 1982; Bendich et al., 1986) and alterations in both ascorbate[2] concentration and the equilibrium between AA and dehydroascorbic acid (DHA) are known to occur in diabetes (Chatterjee & Banerjee, 1979; Som et al., 1981; Jennings et al., 1987; McLennan et al., 1988), we set out to study the effects of AA on the oxidative degradation of FL and the Maillard reaction, in general. We had hoped that this study would yield insight into possible effects of altered ascorbate metabolism on the oxidation of glycated proteins in diabetes. However, we observed unexpectedly that both CML and LL were formed in control reactions between ascorbate and lysine, even in the absence of glucose. The resulted of our studies on the reactions of AA and DHA with lysine are described here, along with discussion of their significance with respect to chemical, modification of proteins by ascorbate in vitro and the source and mechanism of formation of tissue and urinary CML and LL in vivo.

Experimental Procedures

Unless otherwise indicated, reagents were purchased from Sigma Chemical Co., St. Louis, MO. fFL was prepared from N^{α}-formyllysine (fL) and glucose, as described previously (Ahmed et al., 1986). AA, DHA, and threose solutions were prepared freshly by diluting weighed amounts of the carbohydrates into 0.2 M phosphate buffer, pH 7.4; the solutions were filter sterilized (Gelman 0.2 μm Acrodisc filters) and reaction mixtures incubated at 37 °C under air. The concentration of AA in reaction mixtures was determined by gas chromatography of the trimethylsilyl (TMS) derivative, as described by Vecchi and Kaiser (1967). In some experiments the reaction mixtures were reduced by addition of $NaBH_4$ (25-fold molar excess over ascorbate or threose, from a solution of 1 M $NaBH_4$ in 0.1 N NaOH), followed by incubation for 1 h at room temperature. Excess $NaBH_4$ was destroyed by addition of 6 N HCl.

Reaction products were characterized by high-performance liquid chromatography (HPLC) or gas chromatography–mass spectrometry (GC/MS). Prior to analysis, the products were deformylated by acid hydrolysis (2 N HCl for 1 h at 95 °C), and the HCl removed by rotary evaporation. The samples were redissolved in 1 mL of H_2O and then applied to a 1-mL C-18 Sep-Pak column (Supelco); the column was eluted with 4 mL of distilled water and the eluate dried by rotary evaporation. Amino acids were separated by ion-exchange chromatography on a Waters amino acid analyzer system and quantified by postcolumn reaction with *o*-phthaldialdehyde, using procedures described previously (Watkins et al., 1985; Ahmed et al., 1986, 1988). For GC/MS analysis amino acids were converted to their *N,O*-diacetyl or *N,O*-bis(trifluoroacetyl) methyl ester (TFAME) derivatives. Briefly, amino acids were esterified by heating in 1 N methanolic HCl for 0.5 h at 65 °C, dried under a stream of nitrogen, and then acylated with either acetone–triethylamine–acetic anhydride (5:2:1) for 5 min at 65 °C (Adams, 1974) or trifluoroacetic anhydride–methylene chloride (2:1) for 1 h at room temperature. The derivatized samples were evaporated under nitrogen and redissolved in CH_2Cl_2 for GC/MS analysis. Trimethylsilylation was carried out with 1 mL of 1% trimethylchlorosilane in bis(trimethylsilyl)trifluoroacetamide (Pierce Chemicals) for 10 min at 95 °C. GC/MS was performed on a Hewlett-Packard Model 5890A GC, interfaced to a Model 5970 mass selective detector, using a 30-m DB-5

[1] Abbreviations: AA, ascorbic acid; CML, N^{ϵ}-(carboxymethyl)lysine; DHA, dehydroascorbic acid; GC/MS, gas chromatography–mass spectrometry; fL, N^{α}-formyllysine; FL, N^{ϵ}-(1-deoxyfructos-1-yl)-L-lysine (fructoselysine); fFL, N^{α}-formyl-N^{ϵ}-(1-deoxyfructos-1-yl)-L-lysine; HPLC, high-performance liquid chromatography; LL, 3-(N^{ϵ}-lysino)-DL-lactic acid; TFAME, *N,O*-bis(trifluoroacetyl) methyl ester; TL, tetritol lysine(s); TMS, trimethylsilyl.

[2] The term ascorbate is used instead of the abbreviations AA and DHA to refer in a general sense to both AA and DHA, e.g., "ascorbate metabolism".

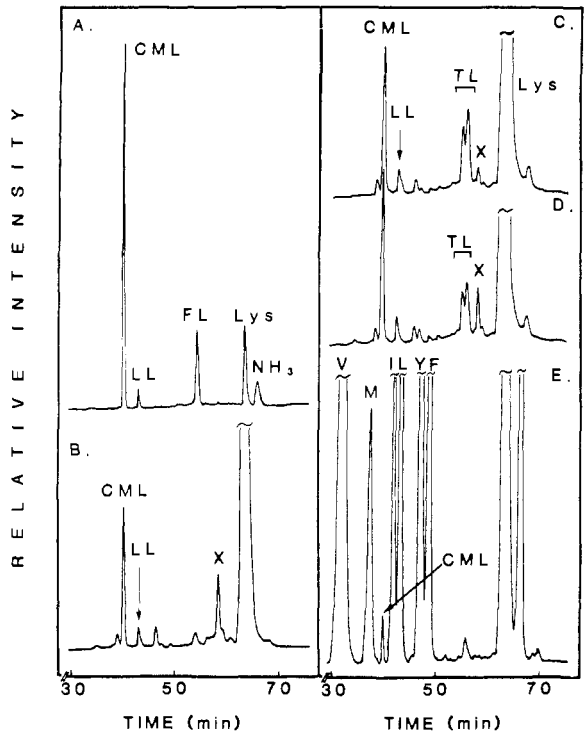

FIGURE 2: Detection of CML and LL in sugar–amine condensation reactions by HPLC. (A) Analysis of an fFL solution (15 mM in 0.2 M phosphate buffer, pH 7.4) after incubation for 7 days at 37 °C under air. After mild acid hydrolysis to remove the N^{α}-formyl group, the products were identified by amino acid analysis, as described under Experimental Procedures. The reaction products, CML, LL, and Lys, and their characterization were described previously (Ahmed et al., 1986, 1988). The yield of CML from fFL in this incubation was 41%. About 10% of the original fFL (identified as FL on the chromatogram) remained at the end of 7 days. Free lysine, formed in part by reversal of the Amadori rearrangement (Ahmed et al., 1986) is also a major product, accounting for ~30% of the original fFL. (B) Analysis of a reaction mixture containing AA and fL (15 mM each) incubated and analyzed as described in (A). The yield of CML, based on AA or fL, varied from 1.5 to 4% in different incubations conducted over a 1-year period. An unidentified product eluting at ~60 min is labeled X (see text). (C) Reaction mixture identical with that described in (B), except that the products were reduced by addition of $NaBH_4$ prior to workup for amino acid analysis. Two new products eluting at 55–60 min, labeled TL, were eventually identified as tetritollysine epimers (see text). (D) Reaction mixture identical with that described in (B), except using DHA instead of AA. The workup included reduction of the reaction mixture with $NaBH_4$, as in (C). (E) Amino acid analysis of RNase (5 mg/mL) after incubation with AA (50 mM), as described above. The protein was dialyzed and then hydrolyzed in 6 N HCl for 24 h at 110 °C. The peak identified as CML is absent from the native protein but accounts for ~1% of the total lysine content of the modified protein. LL cannot be detected by this HPLC method because of coelution with isoleucine.

capillary column (J&W Scientific). The temperature program was as follows: injector temperature, 275 °C; column temperature, 70 °C, hold for 2 min; ramp to 245 °C at 5 °C/min and then to 280 °C at 15 °C/min.

Results

The original intent of this study was to evaluate the effects of AA on both the glycation of lysine to form FL and the oxidation of FL to form CML or LL (Figures 1 and 2A). However, we observed that compounds with HPLC retention times equivalent to those of CML and LL were also formed in reactions of AA with fL (Figure 2B,C), even in the absence of glucose. The formation of CML and LL in these reactions was confirmed by GC/MS, where the retention times and mass spectra of reaction products were identical with those previously reported for CML (Ahmed et al., 1986) and LL (Ahmed et al., 1988) (data not shown). Other unidentified products were also detected in the AA reaction mixture (Figure 2B), with a major peak, labeled X, eluting at about 60 min. Two additional peaks (labeled TL because of their eventual identification as tetritollysines) were also detected in the same region of the chromatogram (~55 min) when the reaction mixture was reduced with $NaBH_4$ prior to deformylation and derivatization (Figure 2C). The pair of TL peaks in the chromatograms of the reduced reaction mixtures suggested that they were derived from a carbonyl adduct to lysine, reducible to the acid-stable epimeric alditol adducts, i.e., compounds analogous to N^{ϵ}-mannitollysine and N^{ϵ}-glucitollysine, which are formed on reduction of FL. Comparison of parts C and D of Figure 2 shows that these products were also produced by reaction of DHA with fL, suggesting that their formation from AA was preceded by oxidation of AA to DHA. The yield of X was unaffected by $NaBH_4$ reduction and ranged from 20 to 100% of the total TL area in different $NaBH_4$-reduced reaction mixtures (compare parts C and D of Figure 2). Compound X appears to be an inert, acid-stable end product, but its structure has not yet been determined and it will not be discussed further. As shown in Figure 2E, CML was also detected in reactions of AA with bovine pancreatic ribonuclease (RNase), indicating the susceptibility of lysine residues in proteins to modification by ascorbate. The identity of the CML peak and the presence of LL in the RNase were also confirmed by GC/MS analysis of the protein hydrolysate.

These results suggested initially that formation of CML from glucose adducts to protein might proceed through intermediates structurally related to adducts formed between AA or DHA and lysine. Parallels between the ascorbate and

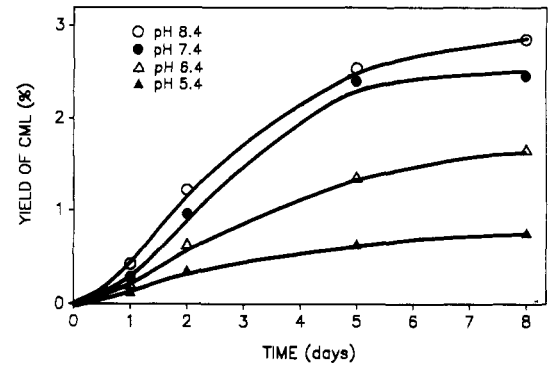

FIGURE 3: Effect of pH on the kinetics of formation and yield of CML from AA and fL. Reaction mixtures were prepared and incubated as described in Figure 2B. Freshly prepared solutions of AA were diluted into phosphate buffer at the appropriate pH and then mixed with a solution of fL at the same pH.

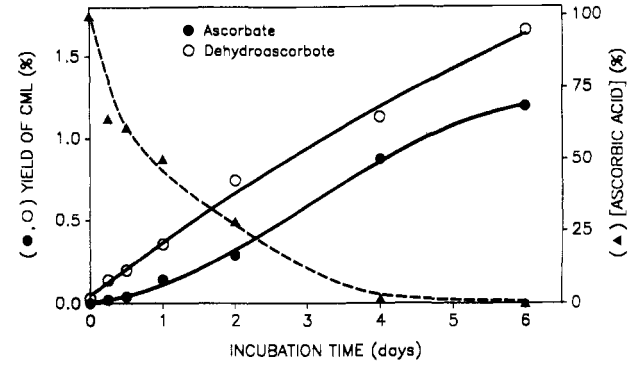

FIGURE 4: Relative rates of formation of CML from AA and DHA. Reaction mixtures were prepared and analyzed as described in Figure 2B. The kinetics of disappearance of AA from the AA reaction mixture is shown by the dotted line.

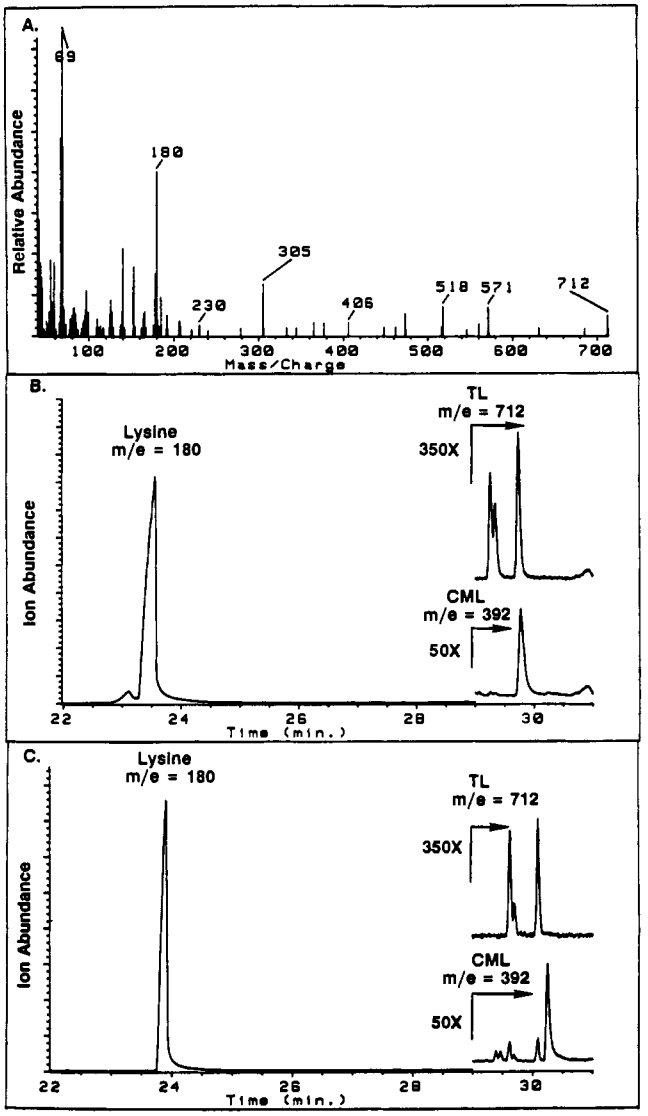

FIGURE 5: Comparative GC/MS analysis of AA and threose reaction mixtures. (A) Mass spectrum of the TFAME derivative of TL epimers isolated from $NaBH_4$-reduced reaction of AA with fL, as described in Figure 2C. TL peaks in the chromatogram shown in Figure 2C were isolated by multiple runs on the HPLC system, then desalted, and converted to their TFAME derivatives. These products yielded identical mass spectra that were consistent with their identification as TL epimers. (B) SIM-GC/MS ion chromatograms of products formed in reaction between AA and fL. Incubation conditions were as described in Figure 2C; a sample was removed at 3 days and worked up by $NaBH_4$ reduction and TFAME derivatization. The elution of lysine was monitored by the m/e = 180 ion; the insets show the partial ion chromatograms for the m/e = 392 (CML) and m/e = 712 (TL) ions. The peaks identified as TL had the same elution times and major fragment ions as the TL products shown in (A), above. (C) SIM-GC/MS ion chromatograms of $NaBH_4$-reduced products of reaction of L-threose with fL. Threose and fL, each at 15 mM, were incubated in 0.2 M phosphate buffer, pH 7.4 at 37 °C; a sample was removed at 12 h and worked up as described in (B).

glucose reaction systems were also suggested by the fact that, with either glucose or ascorbate as reactant, LL was a minor product, compared to CML (compare part A to parts B–D of Figure 2). In addition, as shown in Figure 3, the overall reaction rate and yield of CML in AA reaction mixtures was increased at neutral and slightly alkaline pH, in agreement with results reported previously on the effects of pH on the yield of CML from fFL (Ahmed et al., 1988). With AA, as with glucose, the relative yield of LL, compared to that of CML, was also increased at acidic compared to neutral pH (data not shown).

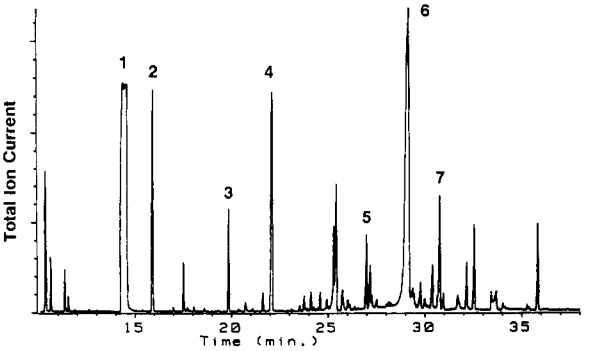

FIGURE 6: Characterization of products formed during the reaction of AA with fL. An aliquot of a reaction mixture, as described in Figure 2C, was removed at 3 days, dried under a stream of nitrogen, and then derivatized by trimethylsilylation (without hydrolysis for deformylation). Product identification is based on elution time and/or mass spectra compared to authentic compounds. Peak identification: 1, phosphate; 2, glyceric acid; 3, threose; 4, threonic acid; 5, pentonic (ribonic and/or arabinonic) acid; 6, fL; 7, ascorbic acid. N^{α}-Formyl-CML was also detectable in this incubation mixture by SIM-GC/MS.

Studies on the kinetics of formation of CML (Figure 4) revealed that there was a lag phase in the formation of CML from AA and lysine but not from DHA and lysine. As also shown in Figure 4, AA was rapidly depleted from the reaction mixture, with a half-life of ~1 day, while the yield of CML continued to increase thereafter. These experiments support the hypothesis that DHA, formed in situ by oxidation of AA under air, is the reactive species involved in the formation of CML and LL. The results are also in agreement with recent work by Ortwerth et al. (1988) which shows that the reaction of ascorbate with proteins is inhibited by glutathione, which maintains ascorbate in the reduced state (Winkler, 1987).

In kinetic studies we had observed that the compounds labeled TL in the HPLC chromatograms in Figure 2C,D were transient products, which disappeared as the reaction progressed. This, together with the fact that $NaBH_4$ reduction yielded a pair of peaks, suggested the reducible carbonylamine compounds formed in the reaction mixture might be the immediate precursors of CML. Thus, the TL compounds were isolated by semipreparative ion-exchange chromatography on the amino acid analyzer, desalted on Dowex-50 (Ahmed et al., 1986), and then converted to their TFAME derivatives. The mass spectra of the TFAME derivatives of the two compounds were identical (Figure 5A) and consistent with their identification as the C-2 epimers of $NaBH_4$-reduced N^{ϵ}-(1-deoxytetrulose-1-yl)-L-lysine, i.e., the TL epimers, L-threitollysine and L-erythritollysine. This assignment was confirmed by chemical ionization mass spectrometry of the TFAME derivative, which yielded the expected molecular ion with m/e = 744. GC/MS analysis also revealed the presence of TL in $NaBH_4$-reduced AA reaction mixtures (Figure 5B), as well as in reactions of L-threose with fL (Figure 5C). Three TL peaks were observed in the GC/MS chromatograms (Figure 5B and 5C), apparently resulting from epimerization at C-3 of the Amadori adduct of tetroses to lysine. These epimers were not resolved in the lower resolution HPLC analyses shown in Figure 2, and the expected fourth epimer of TL was also apparently not resolved in the GC/MS analysis. CML was detected in both the AA and threose reaction mixtures (Figure 5B,C), and threose itself was detected as a prominent product in AA reaction mixtures (Figure 6). The similarity in products obtained in the AA and threose reaction mixtures (Figure 5B,C) argued that an Amadori adduct between threose and lysine was the precursor to CML and LL in the ascorbate reactions (see the reaction scheme in Figure 9).

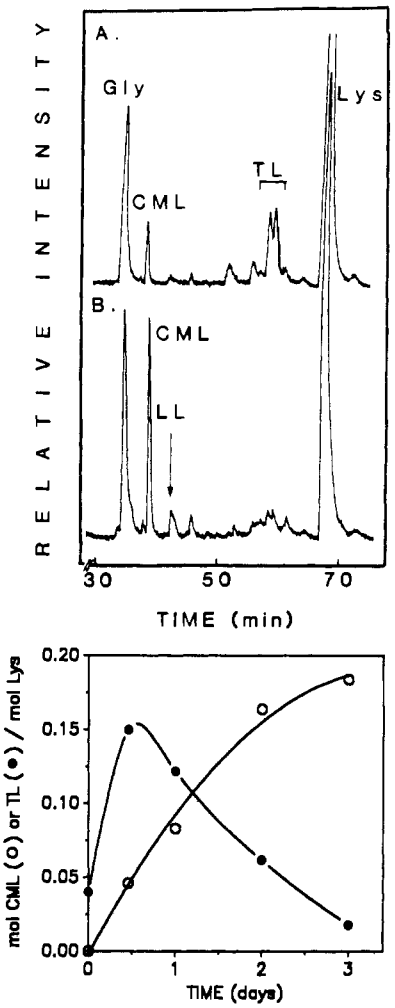

FIGURE 7: Kinetics and products of reaction between L-threose and fL. (A) L-threose and fL, each at 15 mM, were incubated in 0.2 M phosphate buffer, pH 7.4 at 37 °C; *N*-formylglycine, 1 mM, was included as an internal standard. An aliquot was withdrawn at 12 h, reduced with $NaBH_4$, and deformylated by mild acid hydrolysis and then analyzed by HPLC amino acid analysis. The presence of CML, LL, and TL was confirmed by GC/MS. Note the similarity between this chromatogram and those in Figure 2C,D. (B) Same reaction as in (A), but after incubation for 3 days. Note the disappearance of the TL peaks and the corresponding increase in CML and LL. (C, lower panel) Kinetics of formation of TL (sum of TL peaks) and CML in the incubation mixture described in (A) and (B). The analysis and quantitation were done by HPLC, assuming a color factor for TL equivalent to that for CML.

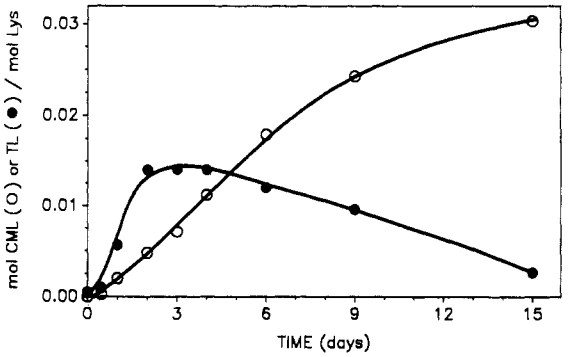

FIGURE 8: Kinetics of formation of TL and CML in reactions of AA with fL. Analysis and quantitation were as described in Figure 7.

As a further test of the role of threose as an intermediate in these reactions, we conducted kinetic studies on the formation of CML in reactions of AA or threose with fL. Figure 7A shows that TL is readily detected in the reaction between L-(+)-threose and fL but disappears during the course of the reaction, concurrent with an increase in CML (Figure 7B). The kinetics of formation of threuloselysine (as TL) and CML in these reactions, shown in the lower panel of Figure 7, are consistent with a precursor–product relationship between the two compounds. Similar results were obtained in reactions between AA and fL (Figure 8), supporting the role of tetruloselysines as intermediates in the formation of CML from ascorbate and lysine.

Discussion

Glycation of Protein and Source of CML in Vivo. The scheme in Figure 9 summarizes our hypothesized route for formation of CML and LL from AA and lysine. The role of DHA and threose as intermediates in this pathway is supported both by similarities in lysine adducts formed in reactions of AA, DHA, and threose with fL (Figures 2 and 5) and by studies on the relative rates of formation of tetruloselysine (as TL) and CML in these reaction mixtures (Figures 7 and 8). To assess the physiological significance of this reaction pathway, we attempted to detect tetrose adducts to lens proteins. However, even though the lens has a relatively high concentration of AA (1–2 mM; Heath, 1962), we were unable to detect TL in $NaBH_4$-reduced human lens proteins. (Control experiments established that TL was stable to conditions used for acid hydrolysis of the protein.) It is likely that, despite the high concentration of AA, the concentration of the reactive species, DHA, is much lower and that, in addition, the high reactivity of tetruloselysine may preclude the accumulation of detectable amounts of tetrose adducts to lens proteins. Thus, while CML is readily detected in human proteins and urine, there is no evidence at this point that it is derived from ascorbate, as opposed to oxidation of proteins glycated by glucose. We have recently detected CML in guinea pig lens and rodent tissues, and future studies on the effects of low and high ascorbate diets on levels of AA and CML in tissues may yield insight into the role of ascorbate as a source of CML in tissue protein. A role for ascorbate autoxidation products in chemical modification of protein might also be indicated if increased carboxymethylation of protein is observed in oxidatively stressed tissues. Thus, despite a decrease in total ascorbate, the DHA concentrations in sera and synovial fluid are increased 2–3-fold in rheumatoid arthritis (Lunec & Blake, 1985). Under these circumstances proteins in the rheumatoid joint, and perhaps throughout the body, may be subject to increased chemical modification by DHA, threose, and other autoxidation products. Increases in the absolute concentration of DHA (Chatterjee & Banerjee, 1979; Som et al., 1981) or the ratio of DHA to AA (Jennings et al., 1987) have also been reported in diabetics. In this case, however, it may be difficult to distinguish between carboxymethylation of protein resulting from autoxidation of FL (derived from glucose) and that resulting from autoxidation of ascorbate.

Autoxidative Glycosylation of Proteins. Autoxidative glycosylation is defined as the chemical modification of proteins by products formed on spontaneous oxidation of sugars by oxygen. Wolff and colleagues (Wolff, 1987; Wolff & Dean, 1987; Hunt et al., 1988) have suggested that autoxidative glycosylation may enhance the damage to tissue proteins by glucose in diabetes. Although the significance of autoxidative glycosylation in vivo is controversial (Harding & Beswick, 1988; Wolff & Dean, 1988), the chemical modification of RNase by ascorbate to yield CML (Figure 2E) is, to our knowledge, the first example of autoxidative glycosylation in which a specific product has been identified. Thus, threose

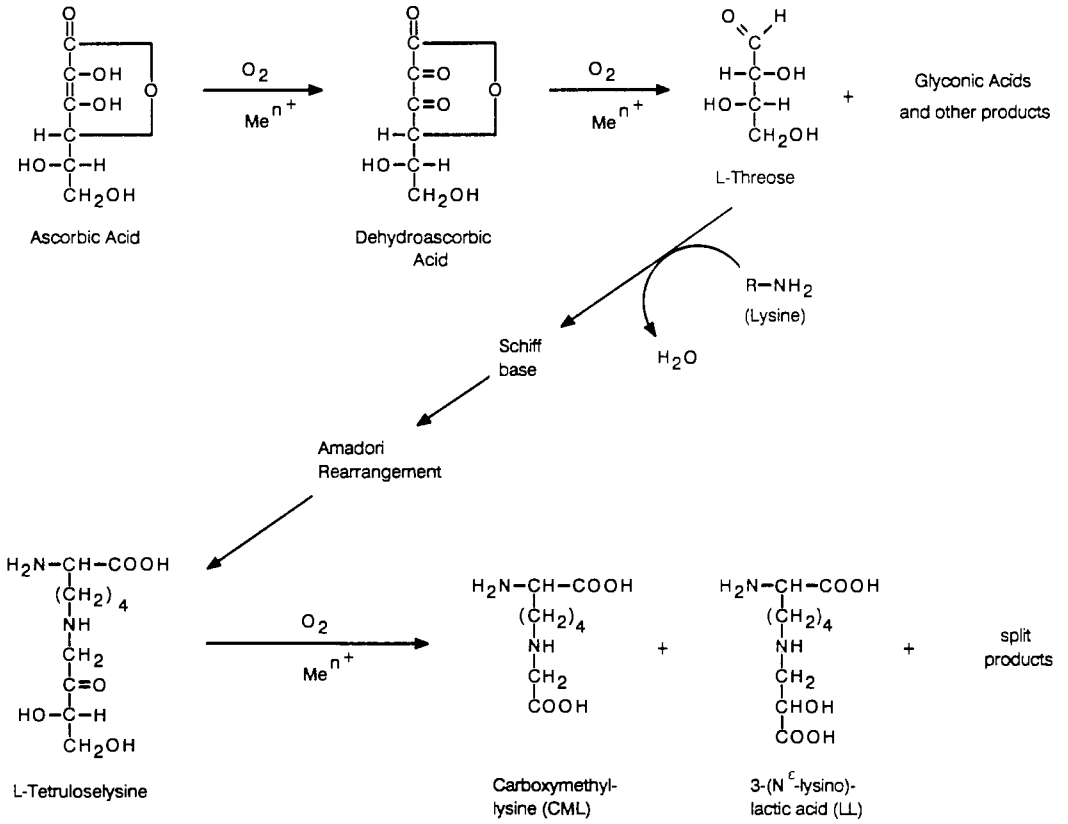

FIGURE 9: Proposed scheme for formation of CML in reactions of AA with lysine. AA is oxidized to DHA, followed by fragmentation to yield L-threose. L-Threose reacts with lysine to form a Schiff base adduct, followed by an Amadori rearrangement to form N^{ϵ}-(1-deoxy-L-tetrulos-1-yl)lysine. The ketoamine is oxidatively cleaved between C-2 and C-3 to form CML or between C-3 and C-4 to form LL.

is an identifiable product of autoxidation of the sugar ascorbate, which goes on to glycate protein and eventually to yield a stable chemical modification of the protein, i.e., CML. The oxidation of FL to CML was originally proposed as a mechanism for limiting damage consequent to glycation of proteins (Ahmed et al., 1986, 1988). However, now that ascorbate has been identified as a possible source of CML, it could also be an indicator of the extent of modification of proteins by autoxidative glycosylation. In either case the product, CML, is an oxidation product and is relatively inert, compared to the precursor carbonyl compounds. While the biological significance of autoxidative glycosylation by ascorbate may still be uncertain, the detection of significant amounts of CML in food products rich in AA suggests that AA may be a source of dietary CML. Thus, Büser and Erbersdobler (1986) have shown that in foods, such as wurst or canned stews, that are stabilized with AA as an antioxidant, the concentration of CML may be equal to or exceed that of Amadori adducts of glucose or lactose to lysine residues in protein.

In addition to DHA and threose, numerous other carbonyl compounds are formed on autoxidation and fragmentation of ascorbate (Mikova & Davidek, 1974). Like threose, many of these compounds are reactive with nucleophilic groups in protein and may also contribute to the chemical modification, cross-linking, and browning of proteins by ascorbate under autoxidizing conditions (Bensch et al., 1985; Ortwerth & Olesen, 1988; Slight et al., 1990). The complexity of the chemistry and the possible variations in reaction conditions, catalytic species, and their concentrations make it difficult to assess the quantitative role of threose in reactions of protein with ascorbate in biological systems. It is equally difficult to determine the role of ascorbate, compared to glucose, other sugars, or fatty acid oxidation products in the browning of proteins in vivo. The answer to this question will require the structural characterization of other compounds that, unlike CML, are uniquely derived from reaction of protein with products of autoxidation of ascorbate.

Chemical Modification of Proteins by Ascorbate in Metal-Catalyzed Oxidation Systems in Vitro. AA is frequently used with Fe(III) ion and O_2 for inducing oxidative modification of proteins [reviewed by Stadtman (1988)]. In this system the AA functions to recycle the iron catalyst between the ferric and ferrous states. Arginine, histidine, lysine, and proline are among the amino acids in protein that are most sensitive to modification in the iron–AA systems (Amici et al., 1989; Gordillo et al., 1989). While some of the amino acid oxidation products have been identified, the structure of the oxidized lysine residue is unknown (Amici et al., 1989). Since lysine is among the amino acids most rapidly modified in the presence of ascorbate, reaction of lysine residues in protein with autoxidation products of AA, such as DHA, threose, and numerous other possible products (Mikova & Davidek, 1974), rather than oxidative degradation of the lysine residue, may be the primary reaction occurring in these oxidation systems. Thus, presumed oxidation of lysine residues, on the basis of loss of primary amino groups in protein or loss of lysine by amino acid analysis, could be the result of formation of ascorbate-derived adducts to the amino acid, rather than oxidation of the lysine side chain. The tetrose and possibly DHA and other adducts to lysine may also react with carbonyl reagents [[^{3}H]$NaBH_4$ or (2,4-dinitrophenyl)hydrazine] under the assay conditions used, creating, in addition, the false impression that the lysine residues have been oxidatively modified to carbonyl compounds. On the basis of the loss of lysine residues reactive with trinitrobenzenesulfonic acid, Gordillo et al. (1989) have recently concluded, for example, that lysine residues in proteins are more sensitive to oxidative damage than histidine. While the conclusion may be correct, it is possible

that, in the experimental system described, the loss of lysine is attributable to reactions of lysine with ascorbate-derived compounds rather than to oxidation of the lysine residues in the protein. Similarly, vicinal dicarbonyl sugars, or reductones (Hodge, 1953), formed from ascorbate may also react with arginine residues in protein. The loss in arginine content of the protein (by amino acid analysis) during oxidation in iron–AA systems may then be the result of formation of dicarbonyl adducts, as well as the oxidation of the arginine side chain.

Most studies on protein oxidation using the iron–AA system are conducted for shorter times than the reactions between ascorbate and lysine described in the present work. However, only trace modifications of amino acids in proteins are generally reported and trace modification of the protein by ascorbate-derived oxidation products has not been rigorously excluded. In addition, in metal-catalyzed oxidation reactions, the molar ratio of ascorbate to lysine residues in protein is generally higher than the equimolar ascorbate:lysine used in the present work, so that more extensive modification of the protein by ascorbate might occur. The higher concentration of iron salts used for metal-catalyzed oxidation reactions may also stimulate the autoxidative degradation of ascorbate (Thornalley, 1985), thus accelerating the formation of AA-derived adducts to proteins, even in short-term incubations. While many of the observations made with proteins oxidized in iron–AA systems have been confirmed independently by using other metal-catalyzed oxidation systems (Stadtman, 1988), the results of the studies reported here suggest some caution in the interpretation of experiments with ascorbate. On the other hand, the chemical characterization of ascorbate adducts to protein in vitro and in vivo may provide insight into a possible role for ascorbate in the chemical aging and the regulation of the aging of proteins in biological systems.

Registry No. CML, 5746-04-3; LL, 116448-40-9; fL, 19729-28-3; dehydroascorbic acid, 490-83-5; L-threose, 95-44-3; L-lysine, 56-87-1; L-threitollysine, 130031-91-3; L-erythritollysine, 130143-26-9; ascorbic acid, 50-81-7; *N*-threuloselysine, 130031-92-4.

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