N^{ϵ}-(Carboxymethyl)lysine Is a Dominant Advanced Glycation End Product (AGE) Antigen in Tissue Proteins†

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ABSTRACT: Advanced glycation end products (AGEs) and glycoxidation products are formed during Maillard or browning reactions between sugars and proteins and are implicated in the pathophysiology of aging and the complications of diabetes. To determine the structure of AGEs, antibodies were prepared to protein browned by incubation with glucose and used in ELISA assays to measure AGEs formed in model reactions between bovine serum albumin (BSA) or N^{α}-acetyllysine and glucose, fructose, or glyoxal. AGEs were formed from glucose and fructose only under oxidative conditions, but from glyoxal under both oxidative and antioxidative conditions. Gel permeation chromatographic analysis indicated that a similar AGE was formed in reactions of N^{α}-acetyllysine with glucose, fructose, and glyoxal and that this AGE co-eluted with authentic N^{α}-acetyl-N^{ϵ}-(carboxymethyl)lysine. Amino acid analysis of AGE proteins revealed a significant content of N^{ϵ}-(carboxymethyl)lysine (CML). In ELISA assays using polyclonal antibodies against AGE proteins, CML−BSA (~25 mol of CML/mol of BSA), prepared by chemical modification of BSA, was a potent inhibitor of the recognition of AGE proteins and of AGEs in human lens proteins. We conclude that AGEs are largely glycoxidation products and that CML is a major AGE recognized in tissue proteins by polyclonal antibodies to AGE proteins.

The term advanced glycosylation (glycation) end product (AGE)[1] was introduced by Vlassara *et al.* (1984) to describe the brown, fluorescent, and cross-linked structures formed during late stages of the Maillard reaction between sugars and proteins *in vivo*. Age-dependent structural and functional alterations in proteins resulting from accumulation of AGEs are now implicated in the development of pathophysiology of normal aging and in the pathogenesis of long-term complications of diabetes (Brownlee, 1994; Vlassara *et al.*, 1994). Sell and Monnier (Sell & Monnier, 1989) chemically characterized the first AGE, pentosidine, a fluorescent lysine−arginine cross-link which accumulated with age in tissue collagens (Sell & Monnier, 1989; Dyer *et al.*, 1993) and lens proteins (Dyer *et al.*, 1991) and was increased in collagen in diabetes (Dyer *et al.*, 1991b; Sell *et al.*, 1992). The term AGE is now applied to a broad range of advanced products of the Maillard reaction which have been detected in tissue proteins, including N^{ϵ}-(carboxymethyl)lysine (CML) (Dunn *et al.*, 1989, 1991b; Dyer *et al.*, 1993), N^{ϵ}-(carboxymethyl)hydroxylysine (CMhL) (Dunn *et al.*, 1991), and pyrraline (Hayase *et al.*, 1989), compounds which do not have color or fluorescence; neither are they cross-links in protein. However, like pentosidine, CML and CMhL accumulate with age in collagen (Dunn *et al.*, 1991; Sell *et al.*, 1992; Dyer *et al.*, 1993), and age-adjusted levels of CML and pentosidine in skin collagen are increased in diabetes (Dyer *et al.*, 1993), particularly in patients with complications (Sell *et al.*, 1992; Beisswenger *et al.*, 1993; McCance *et al.*, 1993). Both CML and pentosidine are now known to be formed from a wide range of carbohydrates, including glucose, ascorbate, pentoses, and tetroses, suggesting common intermediates in their formation from carbohydrate precursors (Dunn *et al.*, 1990; Dyer *et al.*, 1991b; Grandhee & Monnier, 1991). Pyrraline is not known to accumulate in proteins with age, but the pyrraline content of plasma proteins is increased in diabetes (Hayase *et al.*, 1989). Nakamura *et al.* (1993a) have recently described another candidate AGE structure, an epimeric pair of glucose-derived lysine−lysine cross-links, known as crosslines. Thus far, crosslines have been isolated from model reactions *in vitro*, and they have been detected immunohistochemically in renal collagen in rats where their concentration increases in diabetes.

Baynes (1991) noted that all AGEs known to accumulate in tissue proteins (pentosidine, CML, and CMhL) required autoxidative conditions (molecular oxygen and metal ion catalysis) for their formation from glucose in model systems. In subsequent work, Fu *et al.* (1992, 1994) showed that the formation of these compounds was inhibited under anaerobic conditions and, under aerobic conditions, by a number of chelators, reducing agents, and oxygen radical scavengers. Because of the requirement for both glycation and oxidation in their formation, CML, CMhL, and pentosidine were designated "glycoxidation" products (Baynes, 1991), and because glycoxidation products accumulated irreversibly in proteins during the Maillard reaction, oxygen was described as a fixative of Maillard reaction damage to proteins (Dyer *et al.*, 1991a). At this time glycoxidation products are still

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[1] Abbreviations: AcCML, N^{α}-acetyl-N^{ϵ}-(carboxymethyl)lysine; AGE, advanced glycation end product; BSA, bovine serum albumin; CMhL, N^{ϵ}-(carboxymethyl)hydroxylysine; CML, N^{ϵ}-(carboxymethyl)lysine; KLH, keyhole limpet hemocyanin; NAL, N^{α}-acetyllysine; PBS, phosphate-buffered saline; RbSA, rabbit serum albumin.

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the only structurally characterized AGEs known to accumulate in proteins with age and at an accelerated rate in diabetes, suggesting a role for oxidation in the formation of AGEs and in the irreversible, glucose-dependent, oxidative chemical modification of proteins in aging and disease. During glycation of collagen under antioxidative conditions, not only is the formation of glycoxidation products inhibited, but the browning, cross-linking, and development of fluorescence are also markedly suppressed (Chace *et al.*, 1991; Fu *et al.*, 1992, 1994), supporting a strong relationship between oxidation reactions of glucose and the formation of AGEs.

Although only a few AGEs have been structurally characterized, several laboratories have developed antibodies to proteins browned by glucose and have used these antibodies to detect AGEs in tissue proteins by immunohistochemical methods (Nakamura *et al.*, 1993b) and to measure the AGE content of tissue proteins by ELISA assays. In agreement with the results of analyses of the CML and pentosidine content of tissue proteins during age and in diabetes, these ELISA assays have also detected increases in AGEs in human lens proteins with age (Araki *et al.*, 1992), in human serum (Makita *et al.*, 1992a) and red cell (Makita *et al.*, 1992b) proteins in diabetes, and in aortic (Makita *et al.*, 1992a) and renal (Mitsuhashi *et al.*, 1993) collagen in diabetic rats. Thus far, the anti-AGE antibodies have not led to the isolation or structural charactization of the AGE antigens. However, Horiuchi *et al.* (1991) concluded, on the basis of cross-competition in ELISA assays, that a common AGE structure (or structures) was formed in browning reactions from a variety of sugar and amine precursors *in vitro*. Nakayama *et al.* (1991) also observed that AGEs produced by reaction of glucose with N^{α}-acetyllysine (NAL) yielded a single, apparently low molecular weight product peak during gel permeation chromatography on Bio-Gel P-2. In this paper we show that oxidation is required for the formation of AGEs during browning reactions involving glucose and fructose and that CML, which chromatographs similarly to the AGE product described by Nakayama *et al.* (1991), is a major AGE antigen formed in browning reactions. We conclude that AGE antigens measured in ELISA assays using polyclonal antibody to AGE protein are, in fact, glycoxidation products and that, although anti-AGE antibodies are generally assumed to measure brown, fluorescent, and cross-linking products in tissue proteins, CML is a major, if not the primary, AGE antigen detected in tissue proteins by polyclonal anti-AGE antibodies.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise indicated, biochemical and immunological reagents were obtained from Sigma Chemical Company (St. Louis, MO). Bio-Gel P-2 (extra fine) resin was from Bio-Rad Laboratories (Hercules, CA). CML and N^{α}-acetyl-N^{ϵ}-(carboxymethyl)lysine (AcCML) were synthesized using NAL, glyoxylic acid, and $NaBH_3CN$, as described previously (Dunn *et al.*, 1991). *N*-(Carboxymethyl)bovine serum albumin (CML−BSA) was prepared by incubating BSA (fraction V, 175 mg) in 1 mL of 0.2 M phosphate buffer, pH 7.8 (0.15 M lysine equivalents), containing glyoxylic acid (0.15 M) and $NaBH_3CN$ (0.45 M) for 24 h at 37 °C. Human lenses were obtained from the South Carolina Lions Eye Bank, Columbia, SC, and the Medical College of Georgia Eye Bank, Augusta, GA. Lens homogenates and extracts were prepared as described by Araki *et al.* (1992).

Kinetic Studies. For comparative studies on the kinetics of formation of AGEs, NAL or BSA was reacted with glucose, fructose, or glyoxal in 0.2 M phosphate buffer, pH 7.5, at 45 °C. Reactions were conducted under either oxidative (aerobic) or antioxidative conditions (nitrogen atmosphere with 1 mM diethylenetriaminepentaacetic acid and 1 mM phytic acid) (Fu *et al.*, 1992, 1994). Aliquots (100 μL) were removed at various times and frozen at −70 °C.

Analytical Procedures. Protein concentration was determined by the Lowry assay (Lowry *et al.*, 1951) using BSA as standard. The CML content of CML−BSA and AGE proteins was measured by amino acid analysis following hydrolysis of the protein in 6 N HCl for 24 h at 110 °C. Amino acid analyses were performed on a Waters ion-exchange HPLC amino acid analyzer system with fluorescence detection using *o*-phthaldialdehye as the postcolumn reagent (Ahmed *et al.*, 1986).

Immunological Techniques. Two different AGE protein preparations and immunization protocols were used. For the first preparation, which was used in the studies described in detail in this paper, keyhole limpet hemocyanin (KLH, 2 mg/mL) or rabbit serum albumin (RbSA, 12 mg/mL) was incubated in 0.2 M sodium phosphate buffer, pH 7.5, containing 1 M glucose, for 1 month at 45 °C. The AGE proteins were dialyzed against phosphate-buffered saline (PBS) and stored frozen at −20 °C. Male New Zealand white rabbits were immunized by injection of AGE−KLH (500 μg) at two sites on the back using Freund's complete adjuvant. Booster injections with Freund's incomplete adjuvant (125 μg each side) were given after 1 month, the titer was tested at the end of 6 weeks, and antiserum was prepared at the end of 8 weeks. In the second preparation, AGE−KLH was prepared by incubating KLH (20 mg/mL) in 0.5 M phosphate buffer, pH 7.5, containing 3.3 M glucose, for 1 month at 45 °C. Anti-AGE−KLH was prepared by an alternate immunization protocol: primary injection of 250 μg at one site, followed by secondary injections of 250 μg each week for 10 weeks and then collection of antiserum at 12 weeks. Both antisera yielded a titer of 1:2000 dilution, and for all experiments described in this paper, equivalent results were obtained with both antisera.

Competitive ELISA assays were conducted in Costar (Cambridge, MA) multiwell polystyrene plates, coated with AGE−RbSA solution (200 μL; 20 ng of protein) in 50 mM sodium carbonate buffer, pH 9.6, for 2 h at 37 °C. The wells were washed 7 or 8 times with PBS containing 0.05% Tween 20 using an automatic plate washer, blocked by incubation with 300 μL of PBS-Tween containing 0.5% ovalbumin for 1 h at room temperature, and washed again with PBS-Tween. Competing antigens (AGE−RbSA, AGE−BSA, AGE−NAL, AcCML, CML, CML−BSA) and antiserum (1:2000 dilution) were added sequentially to wells in PBS (final volume = 200 μL), and the plates were incubated at 37 °C for 2 h. After washing, the wells were incubated with a 1:2000 dilution of anti-rabbit IgG (whole molecule) horseradish peroxidase conjugate in 0.05% ovalbumin in PBS-Tween for 1 h at 37 °C. The wells were washed, 200 μL of substrate solution [25 mg of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) and 30 μL of 30% hydrogen peroxide

in 100 mL of 0.1 M sodium citrate, pH 4.5] were added per well, and the absorbance was measured at 420 nm after incubation for 30 min at room temperature. For quantitation of AGEs (AGE−RbSA equivalents) in various samples, a standard curve was prepared using 20−200 ng of AGE−RbSA as competing antigen. Direct and competitive ELISA assays of AGEs in lens proteins were performed by similar procedures except that lens proteins (200 μL; 5 μg of protein) were coated on the plates and competed with various AGE−NAL or AGE protein preparations.

RESULTS

The starting point for our studies was the recent work of Nakayama *et al.* (1991) and Araki *et al.* (1992) using model systems for formation of immunologically detectable AGEs by reaction of carbohydrates with proteins and lysine derivatives in phosphate buffer. In agreement with these reports, using a competitive ELISA assay with an antibody to KLH browned by incubation with glucose, we were able to detect the formation of AGEs in reactions of glucose or fructose with NAL (Figure 1A) or BSA (Figure 1C,D). However, when the browning reactions were performed under antioxidative conditions, i.e., nitrogen atmosphere with chelators, AGEs were not detected (<1 ng of RbSA equivalents) (Figure 1A, C,D). Fructose formed AGEs at 1−10 times the rate of glucose, depending on the sugar concentration, with the rates for fructose and glucose becoming equal at higher (500 mM) sugar concentration (data not shown). A lag phase was consistently observed in the reactions of fructose and glucose with BSA under oxidative conditions, consistent with involvement of an oxidized intermediate in the formation of AGEs from these sugars. From these experiments, we concluded that oxygen and autoxidative reactions were involved in the formation of AGEs from glucose and fructose.

Since glyoxal has been identified as the dicarbonyl product formed on autoxidation of glucose and a precursor of the AGE/glycoxidation product, CML, in proteins under either oxidative or antioxidative conditions (Wells-Knecht *et al.*, 1995), we also studied the reaction of glyoxal with NAL and BSA. As shown in Figure 1A,B, glyoxal yielded AGEs under both oxidative and antioxidative conditions. In replicate experiments, the yield of AGEs from glyoxal, reacted with either NAL or BSA, was always 2−4-fold greater under antioxidative conditions. The yield of AGEs in BSA reactions was also typically 100−1000-greater than that in NAL reactions (Figure 1B−D; compare to Figure 1A). Reaction mixtures of NAL with glucose or fructose remained colorless during the course of 2-week incubations under antioxidative conditions, but turned straw yellow under oxidative conditions, while reaction mixtures with glyoxal turned dark brown within a few hours, more rapidly under antioxidative than oxidative conditions. Overall, our observations indicated that formation of AGEs from hexoses required oxidation and that glyoxal, formed in the autoxidation reaction, could be an intermediate in the formation of AGEs.

Since glyoxal is a precursor of CML in protein (Wells-Knecht *et al.*, 1995), our results suggested that CML might be one of the antigens detected by the anti-AGE antibody. Gel permeation chromatographic analysis of these above reaction mixtures on Bio-Gel P-2 yielded similar absorbance

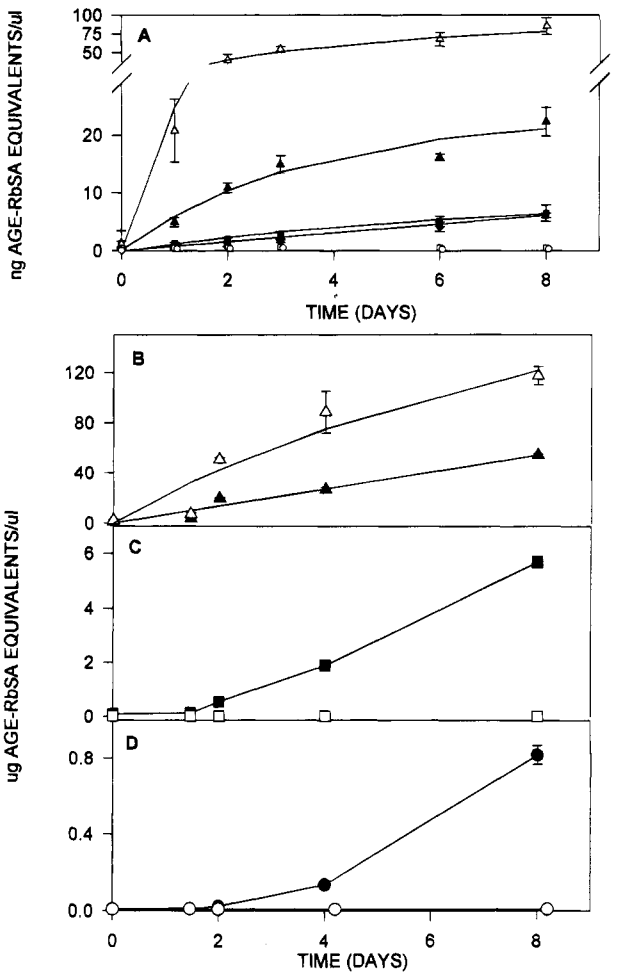

FIGURE 1: Kinetics of formation of AGEs during reaction of sugars and glyoxal with NAL and BSA. (A) NAL (50 mM) was incubated with 100 mM glucose (○, ●), fructose (□, ■), or glyoxal (△, ▲) in 0.2 M sodium phosphate buffer, pH 7.5, at 45 °C under oxidative (closed symbols) or antioxidative (open symbols) conditions. Aliquots (100 μL) were removed at various times and frozen at −70 °C. AGEs were measured by competitive ELISA assay, as described in Experimental Procedures. Similar experiments were conducted by incubation of (B) glyoxal, (C) fructose, and (D) glucose (50 mM each) with BSA (6.7 mg/mL, 1 mM protein, and 57 mM lysine) under oxidative (closed symbols) and antioxidative (open symbols) conditions.

and fluorescence profiles, as illustrated in Figure 2A for glucose under oxidative conditions. The chromatogram is consistent with the results of Nakayama *et al.* (1991), with the major brown and fluorescent products eluting near the exclusion volume of the column. ELISA analysis of column fractions for AGEs indicated that the major AGE formed in the NAL + glucose reaction eluted with a $K_{av} \approx 0.5$ and was separated from the main peaks of brown and fluorescent products (Figure 2B), also consistent with the work of Nakayama *et al.* (1991). A similar-sized AGE was also formed from glucose and fructose under oxidative conditions, eluting slightly before NAL, consistent with formation of a common AGE from both sugars. This product co-eluted with the AGE formed from glyoxal (Figure 2B). We also compared the elution volume of the AGE(s) formed from the various sugars and glyoxal with the elution volume of authentic AcCML, i.e., the product of carboxymethylation of NAL. The AcCML co-eluted with the major AGE fraction detected in the ELISA assays (Figure 2), suggesting that CML was a major AGE antigen formed in these model

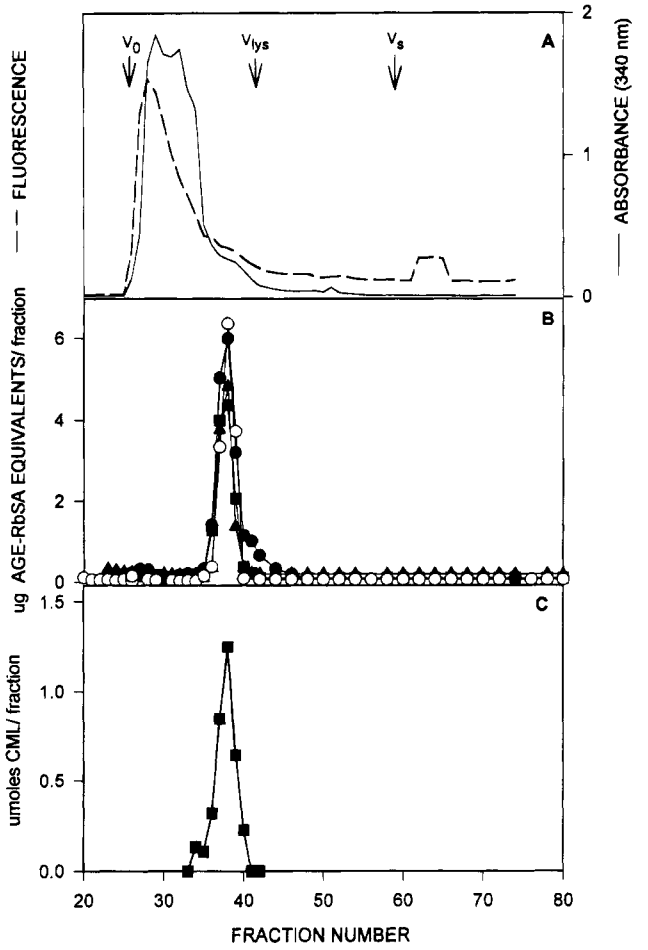

FIGURE 2: Analysis of AGE−NAL preparations by chromatography on Bio-Gel P-2. Comparable amounts of AGEs derived from glucose, fructose, or glyoxal were applied to a Bio-Gel P-2 column (100 × 1 cm) at 4 °C, and fractions (900 μL) were collected. (A) Profiles of absorbance (340 nm) and fluorescence at wavelength maxima (Ex = 398 nm; Em = 450 nm) for the NAL + glucose reaction. Similar profiles were obtained for the fructose and glyoxal reactions. Arrows indicate exclusion volume (V_0), and elution volume of lysine (V_{Lys}) and NaCl (V_s). (B) Equal amounts of AGEs from NAL + glucose (●), NAL + fructose (■), or NAL + glyoxal (▲) reactions, or synthetic AcCML (9.25 μmol) (○), were analyzed consecutively. The AcCML was added to a zero time NAL + glucose reaction to adjust for effects of sample buffer concentration and viscosity. (C) AGE fractions from the NAL + fructose reaction were hydrolyzed (6 N HCl, 2 h at 110 °C) and analyzed by amino acid analysis. Only lysine (a contaminant from side a fraction of the lysine peak fractions) and a single amino acid product were detected, which cochromatographed with an authentic CML standard. Essentially identical results were obtained on analysis of NAL + glucose and NAL + glyoxal AGE fractions.

system reactions. Indeed, when individual chromatographic fractions were subjected to acid hydrolysis and amino acid analysis, CML was identified as a major component under the peak AGE fraction for each carbohydrate precursor, as illustrated in Figure 2C for the fructose reaction. The only other product detected in these fractions was lysine, resulting from contamination from the lysine peak.

To determine the relationship between AGEs formed in model systems and those formed *in vivo*, we tested the ability of model AGEs to inhibit the recognition of AGEs in human lens proteins. As shown in Figure 3, the anti-AGE antibody recognized the age-dependent increase in AGEs in human lens proteins, as reported by Araki *et al.* (1992). As also shown in Figure 3, AGEs isolated by Bio-Gel P-2 chromatography from reactions of NAL with glucose and glyoxal competed strongly and comparably for the recognition of AGEs in human lens proteins. Since the AGEs co-eluted with AcCML on Bio-Gel P-2 chromatography (Figure 2B) and CML was the only identifiable AGE detected in these preparations by amino acid analysis (Figure 2C), the experiment suggested that CML was the major AGE determinant recognized by the anti-AGE antibody and the major AGE antigen in lens proteins. Amino acid analysis of the AGE−KLH and AGE−RbSA used as immunogen and standard, respectively, in our studies indicated that they contained significant quantities of CML. During the browning of KLH (4.9 mol % lysine), 82 and 68% of lysine residues were irreversibly modified in the first and second preparations of AGE−KLH, respectively. The yield of CML accounted for 34 and 24% of the original lysine content of KLH and for 41 and 35% of the lysine lost in the two preparations of AGE−KLH, respectively. Similarly, compared to RbSA (Figure 4A), the AGE−RbSA standard (Figure 4B, prepared according to the first protocol) contained 12.3 mol of CML/mol of protein, representing conversion of 21% of the lysine residues in the protein to CML. The browning of AGE−RbSA resulted in loss of lysine (49%), histidine (7%), and arginine (43%) residues in the protein, and the yield of CML at 30 days accounted for 43% of the lysine loss. Thus, although pentosidine and other uncharacterized AGE products are formed during the browning of the protein, the preparation of AGE proteins by standard procedures (Horiuchi *et al.*, 1991; Nakayama *et al*, 1991; Makita *et al.*, 1992a,b) yields a product rich in CML, consistent with its role as a major antigenic determinant in the protein.

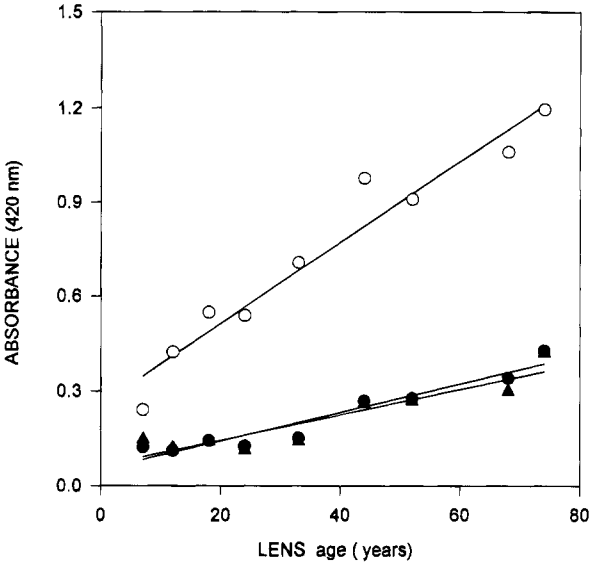

FIGURE 3: Immunological detection of AGEs in human lens proteins and competition by NAL-AGEs. Human lens proteins (5 μg) were coated on multiwell plates for analysis of their AGE content by direct ELISA (○). Competitive ELISA assays were performed by addition of equal amounts of NAL−AGEs [NAL + glucose (●) or NAL + glyoxal (▲), 10 ng of AGE−RbSA equivalents], prepared by Bio-Gel P-2 chromatography.

To assess the quantitative contribution of CML to AGEs in artificial AGE proteins and in lens proteins, we prepared CML−BSA by reaction of BSA with glyoxylic acid and $NaCNBH_3$, obtaining a product with 30 mol of CML/mol of protein, representing 52% conversion of lysine to CML (Figure 4C,D). This protein was white, i.e., not browned

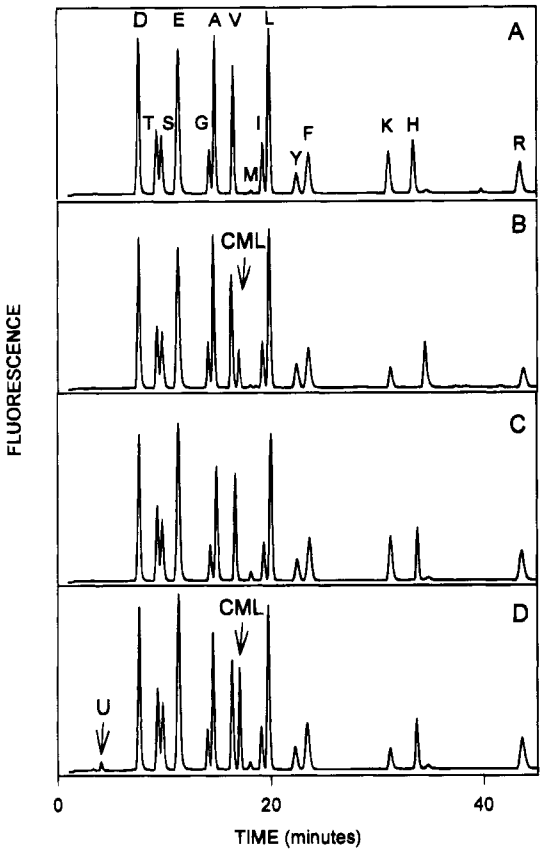

FIGURE 4: Measurement of CML in AGE−RbSA and in CML−BSA preparations. Proteins were hydrolyzed in 6 N HCl for 24 h at 110 °C and then analyzed by cation-exchange amino acid analysis: (A) RbSA, (B) AGE−RbSA, (C) BSA, and (D) CML-BSA. The elution volumes of CML (between valine and methionine) and an unknown acidic product (U) are indicated by arrows.

by Maillard reactions, and there was no loss of histidine or arginine residues during the chemical modification reaction (Figure 4D; compare to 4C). The yield of CML accounted for 86% of the loss of lysine residues during the modification reaction. An additional acidic product, eluting before aspartate on the amino acid analyzer (identified as U in Figure 4D), was also observed. This compound, which yielded approximately 6% of the fluorescence response of CML in the amino acid analysis, could be the bis(carboxymethyl) derivative of lysine, but it was not characterized. When CML−BSA was used in a competitive ELISA assay, it proved to be a potent inhibitor of the recognition of AGE−RbSA by anti-AGE antibodies (Figure 5). Competition by CML−BSA and AGE−RbSA was also comparable when normalized to the CML content of the proteins. AcCML and CML also inhibited recognition of AGE−BSA, but at $\sim 10^3$ higher concentrations than CML−BSA containing equivalent amounts of CML (Figure 5), indicating a significant role for the protein component in the recognition of CML in AGE proteins. CML itself proved to be the weakest inhibitor, about 5−10-fold less potent than AcCML (Figure 5). Significantly, all of the CML-containing competitors, as well as AcCML and CML, yielded essentially complete inhibition of the recognition of AGE−BSA by the anti-AGE antibody, suggesting that CML was the dominant antigenic determinant on AGE proteins and that the polyclonal anti-AGE antibodies were essentially monospecific for CML in protein. The relevance of these observations to the recognition of AGEs in lens proteins was confirmed by the

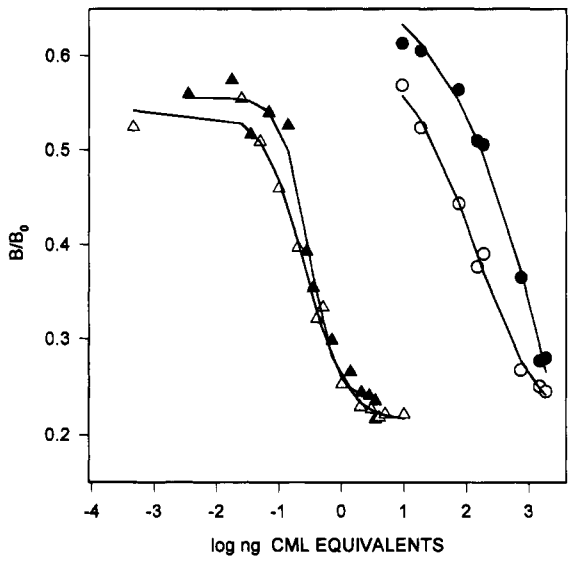

FIGURE 5: Inhibition of anti−AGE antibody recognition of AGE−RbSA by CML (●), AcCML (○), CML-BSA (▲), and AGE-RbSA (△). Sample concentrations are expressed as CML equivalents, measured by amino acid analysis. The data points represent the average of duplicate analyses, which varied by an average of less than 10%.

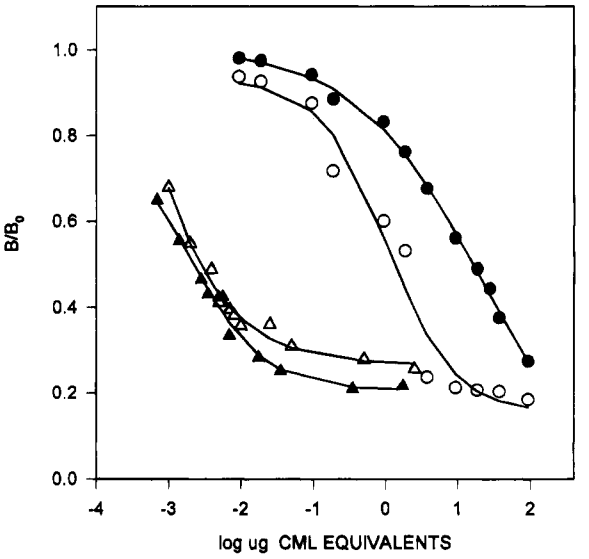

FIGURE 6: Inhibition of recognition of AGEs on human lens proteins by CML (●), AcCML (○), CML-BSA (▲), and AGE-RbSA (△). Competitive ELISA assays were performed by coating plates with normal human lens protein (5 μg, 68 years) and addition of the competing antigens. Similar results were obtained with several lens preparations. The data points represent the average of duplicate analyses, which varied by an average of less than 10%.

experiments shown in Figure 6, indicating that AGE−RbSA and CML−BSA were comparable, when normalized for their CML content, in inhibition of recognition of AGEs in lens proteins. AcCML and CML were much weaker inhibitors, as also observed in Figure 5, but at high concentration, they also completely inhibited recognition of AGEs in human lens proteins.

DISCUSSION

The experiments described above were performed with a single polyclonal antibody preparation, but have been duplicated with a second antibody, prepared by a separate browning and immunization protocol (Experimental Proce-

dures). Both antibodies yielded results similar to those obtained previously in other laboratories, including recognition of an age-dependent increase in AGEs in lens protein (Araki *et al.*, 1992), recognition of artificial AGEs prepared in model systems by reaction of glucose with lysine derivatives (Horiuchi *et al.*, 1991; Nakayama *et al.*, 1991), and identification of a low molecular weight AGE−NAL product eluting from Bio-Gel P-2 at $K_{av} \approx 0.5$ (Nakayama *et al.*, 1991). The major contribution of the present study is the structural characterization of that AGE as CML.

The identification of CML as a principal antigenic determinant in AGE proteins is supported by the observation in Figure 1 that formation of AGEs from glucose and fructose requires oxidative conditions which are also required for the formation of CML. In addition, glyoxal is a product of hexose autoxidation and a precursor of CML under both oxidative and antioxidative conditions (Wells-Knecht *et al.*, 1995), and analyses in Figure 2 establish that a similar AGE product is formed from glucose, fructose, and glyoxal. Further, this AGE elutes in a region of the chromatogram without significant brown color or fluorescence and cochromatographs with authentic AcCML. The identification of CML as a major AGE is also consistent with evidence that AGEs, like CML, are not reducible by $NaBH_4$ (Horiuchi *et al.*, 1991; Makita *et al.*, 1992a). Finally, our results also explain the observation that AGE antigens appear to be destroyed on acid hydrolysis of AGE proteins (Araki *et al.*, 1992; Makita *et al.*, 1992a), since CML, which is released intact during acid hydrolysis, is a much weaker competitor, in comparison to CML protein, in AGE−ELISA assays (Figures 5 and 6). The reason for this difference in competitive activity is not clear, but it may result from stronger interactions between antibody and multivalent AGE proteins or CML−BSA, or from greater charge−charge repulsive interactions between the antibody binding sites and the α-carboxyl or amino groups of CML and AcCML.

Our observations do not exclude the obvious existence of brown and fluorescent AGEs or acid-labile AGEs, other than CML, in tissue proteins, or the possibility that other antibodies may have specificity for AGEs other than CML. However, a number of anti-AGE antibodies have been described for which the AGE proteins used as immunogens were not prepared under rigorously antioxidative conditions. Under these circumstances, CML will be formed during prolonged reaction with sugars or sugar phosphates in phosphate buffers. Our results suggest that the CML content of these proteins should be evaluated and that the resulting antibodies should be screened with AcCML or a carboxymethylated protein, such as CML−BSA. Since CML is already characterized and is measurable by chemical analysis, antibodies which fail to recognize CML should be valuable tools for identification of new AGE products, including those resulting from modification of arginine residues in protein. It appears, however, that other AGEs, in addition to being poorly characterized, may also be poorly antigenic, compared to CML in AGE proteins, perhaps as a result of their heterogeneous structure and the resultant low concentration of individual species in AGE protein immunogens.

In addition to the identification of CML as a major AGE antigen, our results suggest that the recognition of AGE proteins by the scavenger receptor (Takata *et al.*, 1988), which has specificity for polyanionic compounds (Krieger *et al.*, 1993) and alkylated lysine residues (Zhang *et al.*, 1993), may be explained by the presence of CML residues on the surface of AGE proteins. As noted above, significant amounts of CML are formed during the preparation of AGE proteins, providing the requisite anionic character and lysine modification for recognition of AGE proteins by the scavenger receptor. Since it is a major determinant on AGE proteins, CML might also be a candidate for recognition by AGE receptors (Vlassara *et al.*, 1985, 1994; Schmidt *et al.*, 1992, 1994). Bucala *et al.* (1994) noted, however, that AGE−low density lipoprotein (LDL), containing AGEs at a level similar to that observed in diabetic patients with end-stage renal disease, had a longer half-life than the native protein in the circulation. Thus, the concentration of AGEs, CML or otherwise, on LDL *in vivo* was sufficient to inhibit recognition by the Apo-B receptor (Schneider, 1989), but was insufficient to induce clearance of the protein by AGE and/or scavenger receptors. Lyons *et al.* (1994) have shown, in fact, that only very low levels of CML are present on LDL (0.024 mmol of CML/mol of lysine in LDL, or ~8 mmol of CML/mol of LDL), suggesting that extensive, perhaps nonphysiological modification of circulating proteins by CML and other AGE products is required for the recognition and clearance of AGE proteins from the circulation by the AGE or scavenger receptor system(s).

Bucala *et al.* (1993) have also reported the detection of AGEs in both the protein and lipid components of LDL. Using the ELISA assay procedure described here, we have determined that levels of AGEs in plasma proteins are at least 100-fold lower than in lens protein, consistent with the relative concentration of CML in LDL and lens proteins [approximately 0.024 mmol of CML/mol of lysine in LDL (Lyons *et al.*, 1994) vs ~4 mmol/mol of lysine in human lens proteins at age 40 (Dunn *et al.*, 1989)]. Regarding detection of AGEs in the lipid fraction of LDL, Bucala *et al.* (1993) showed that amino lipids, such as phosphatidylethanolamine (PE), also react with glucose to form immunologically detectable AGEs. We have recently detected fructose−ethanolamine, the Amadori adduct of glucose to ethanolamine, in human urine (unpublished data), indicating that PE, and probably phosphatidylserine, residues in lipoproteins and membranes are also glycated *in vivo*. It seems likely, therefore, that the AGE−PE structure in LDL is *N*-(carboxymethyl)-PE, the glycoxidation product derived from glycated PE, which should have sufficient structure homology to CML to cross-react and compete in the AGE−ELISA assay. Bucala *et al.* (1994) also observed that AGE peptides, which are elevated in the serum of patients with diabetes and end-stage renal disease, react with LDL to yield an increase in AGEs in both the protein and lipid fractions of LDL. Although the chemical nature of AGE peptides is uncertain, these preparations certainly contain some glycated peptides, so that, during the extended incubation under oxidative conditions, CML might be formed, either directly by oxidation of Amadori compounds on glycated plasma proteins or lipids or indirectly *via* formation of glyoxal. The *in situ* formation of CML and *N*-(carboxymethyl)-PE provides a reasonable chemical mechanism for the formation of AGEs in the protein and lipid fraction of LDL during its reaction with AGE peptide fractions.

In conclusion, our results demonstrate that CML is a major AGE antigen recognized in tissue proteins by polyclonal anti-AGE antibodies and suggest that measurement of tissue AGEs using polyclonal antibodies may, in fact, be a

measurement of the CML content of the proteins. Because of its charge density and the polarity of its side chain, CML is probably located on the surface of most AGE proteins, making it an exposed and readily recognizable antigenic determinant. In contrast, intermolecular AGE cross-link structures are likely to be buried on the interior of protein dimers and oligomers, while intramolecular AGE cross-links may be present in much lower abundance on proteins because of the requirement for reactive neighboring groups for cross-link formation. Our results re-emphasize the usefulness of CML as a biomarker for Maillard reaction damage to tissue proteins and also suggest that CML may be a major AGE recognized by AGE or scavenger receptors involved in the recognition and catabolism of AGE proteins *in vivo*.

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