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# Conversion of Lysine to $N^{\epsilon}$-(Carboxymethyl)lysine Increases Susceptibility of Proteins to Metal-Catalyzed Oxidation

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**Metal-catalyzed oxidation (MCO) of proteins leads to the conversion of some amino acid residues to carbonyl derivatives, and may result in loss of protein function. It is well documented that reactions with oxidation products of sugars, lipids, and amino acids can lead to the conversion of some lysine residues of proteins to $N^{\epsilon}$-(carboxymethyl)lysine (CML) derivatives, and that this increases their metal binding capacity. Because post-translational modifications that enhance their metal binding capacity should also increase their susceptibility to MCO, we have investigated the effect of lysine carboxymethylation on the oxidation of bovine serum albumin (BSA) by the $Fe^{3+}$/ascorbate system. Introduction of ~10 or more mol CML/mol BSA led to increased formation of carbonyls and of the specific oxidation products glutamic and adipic semialdehydes. These results support the view that the generation of CML derivatives on proteins may contribute to the oxidative damage that is associated with aging and a number of age-related diseases.** 

It is well established that metal-catalyzed oxidation (MCO) of proteins results in the oxidative modification of specific amino acid residues (1, 2). Metal-catalyzed oxidation of proteins has been implicated in a variety of biochemical processes, such as marking of intracellular proteins for proteolytic degradation, regulation of metabolic pathways, and host defense mechanisms (3, 4). The structural disruption caused by the reaction may also result in loss of enzymatic activity (5) and, hence, it has been implicated in the molecular basis of diseases, such as Alzheimer's disease (6), rheumatoid arthritis (7), amyotrophic lateral sclerosis (8), and cataractogenesis (9), among others, and in the functional losses associated with aging (1, 4, 10, 11).

From a chemical point of view, the reaction shows exquisite specificity in that oxidation targets amino acid residues and bonds located in the vicinity of metal binding sites (1, 3, 4). The reaction requires the concurrence of metal ions, typically Fe(III) or Cu(II), oxygen, and an electron donor, which catalyzes the reduction of $O_2$ to $H_2O_2$ and the reduction of metal ions to a lower oxidation state, Fe(II), Cu(I), etc. Binding of the reduced metal to a binding site in the protein is followed by reaction of the complex with $H_2O_2$ with generation of $^{\cdot}OH$, which then attacks neighboring amino acid side chains or peptide bonds (1, 3, 4). Among the most susceptible residues are histidine, degraded to oxo-histidine; aspartate, and other products (4); arginine and proline, oxidized to glutamic semialdehyde (12); and lysine, converted to adipic semialdehyde (12). The latter aldehydes and other carbonyl-containing reaction products can be easily detected spectrophotometrically after their reaction with dinitrophenylhydrazine (DNPH) (13). This technique is widely used to document the occurrence of the reaction in a variety of systems *in vitro* and *in vivo*. Increases in levels of protein carbonyls in animal and human tissues in association with disease and aging, together with the fact that the absolute amounts measured are considerable (in the range of a few moles/mol protein), militate in favor of a role of metal-catalyzed oxidation in the molecular pathology of these processes. Of note, measurement of carbonyls underestimates the extent of damage, since some products of the oxidative reaction are not carbonyl compounds, e.g. oxo-histidine and aspartate derived from histidine, ortho- and metatyrosine derived from phenylalanine, and hydroxy derivatives of valine, leucine and isoleucine.

Amino acid residues that can form complexes with metal ions, such as copper binding histidine or iron binding lysine, are known to facilitate and target oxi-

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dative reactions. It is, therefore, likely that modifications of proteins that enhance their metal ion binding capacity should increase the propensity of proteins to oxidation. Thus, it is noteworthy that $N^{\epsilon}$-(carboxymethyl)lysine (CML) residues of proteins, which are formed by reaction of lysine residues with oxidation products of sugars, lipids, or free amino acids under oxidative conditions (14), were recently shown to bind metal ions (15). Its chemical structure resembles, in fact, that of EDTA and other metal chelating species. In the present communication, we describe studies aimed at determining if CML residues of proteins can enhance their susceptibility to metal-catalyzed oxidation.

## MATERIALS AND METHODS

*Reagents.* Bovine serum albumin (BSA), fraction V, and sodium cyanoborohydride were purchased from Sigma (St. Louis, MO). Glyoxylic acid and DNPH were obtained from Aldrich (St. Louis, MO). Guanidine hydrochloride was purchased from Gibco BRL (Grand Island, NY).

*Synthesis of CML-rich BSA (CML-BSA).* $N^{\epsilon}$-(Carboxymethyl)-lysine-bovine serum albumin was prepared by reductive methylation of BSA by glyoxylic acid and sodium cyanoborohydride in aqueous buffer following the procedure of Reddy *et al.* (16). By increasing the molar ratios of these two reagents with respect to BSA, preparations with different amounts of CML were obtained. $N^{\epsilon}$-(carboxymethyl)lysine-bovine serum albumin samples were dialyzed against de-ionized water at 4°C. The amino acid compositions of CML-BSA and control samples were determined after hydrolysis in 6 N HCl at 155°C for 45 minutes; and hydrolysates were dried *in vacuo* (SpeedVac, Savant, Farmingdale, CA) and dissolved in de-ionized water. Amino acid analysis was performed after OPA derivatization by reverse phase HPLC (17). A standard of pure CML, generously provided by Dr. Suzanne Thorpe, University of South Carolina, was used as reference material.

*Oxidation of BSA and CML-BSA.* Samples of BSA or CML-BSA were diluted in 50 mM Hepes, pH 7.2, to a concentration of 1.25 mg/ml and loaded onto 10000 MW cutoff dialysis cassettes (Slidalyzer, Pierce, IL). Cassettes were introduced in a beaker containing 3 L of pre-warmed oxidizing buffer, prepared by supplementing a solution of 50 mM Hepes, pH 7.2, with 25 mM ascorbic acid/100 $\mu$M $FeCl_3$, added as a freshly prepared 10× mixture. Oxidation proceeded at 37°C with shaking. At appropriate time points, cassettes were withdrawn and their contents were supplemented with EDTA to a final concentration of 1 mM, added as a concentrated, neutral solution. The cassettes were immediately transferred to a beaker containing 1 mM EDTA and dialyzed for 2 hours at 4°C.

*Carbonyl analysis.* Protein was precipitated with trichloroacetic acid (TCA, final concentration, 20%) on ice and spun at 14000 rpm for 10 minutes. The protein pellets were dissolved in 100 $\mu$l of a 6M guanidine/HCl solution in 500 mM sodium phosphate, pH 2.5. Protein was allowed to dissolve overnight, and carbonyls were measured by HPLC after reaction with DNPH as described (13).

*Quantitation of glutamic and adipic semialdehydes.* Glutamic and adipic semialdehydes were measured after their reduction to hydroxyaminovaleric acid (HAVA) and hydroxyaminocaproic acid (HACA), respectively, in protein acid hydrolysates, prepared as described above. Hydroxyaminovaleric acid and HACA were measured by stable isotope dilution SIM-GC/MS as their trifluoroacetyl-methyl ester derivatives. Full details of the analytical method will be described elsewhere (manuscript in preparation).

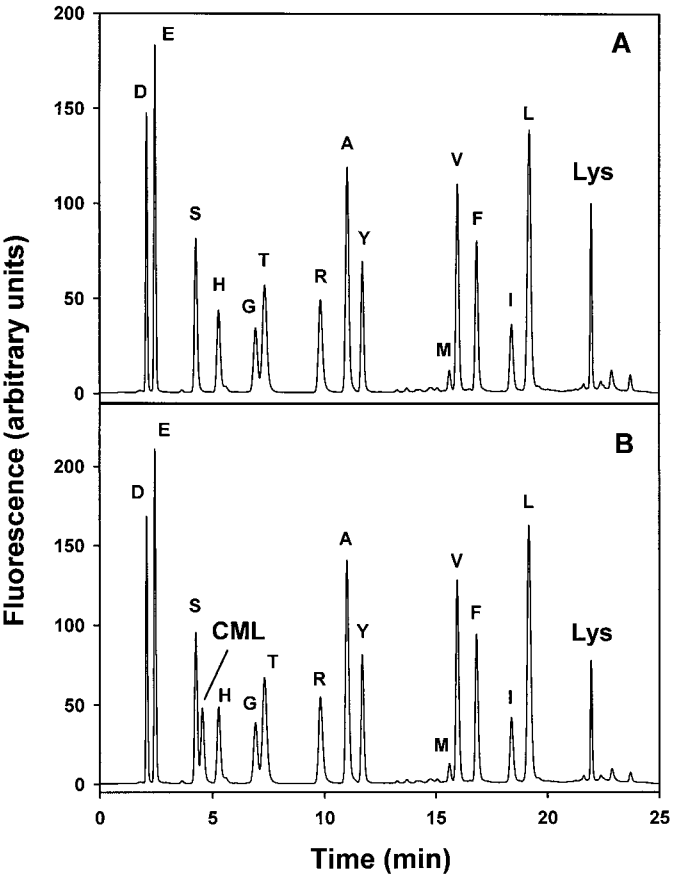


**FIG. 1.** Amino acid analysis of (A) control BSA and (B) 18CML-BSA. Samples were treated as described under Materials and Methods. Labels are the one-letter codes for amino acids, except for lysine and CML.

## RESULTS

Modified BSA samples containing CML ranging from 1 to 29 mol/mol protein were prepared. Amino acid analysis of these samples revealed the appearance of a new amino acid peak with the retention time of CML (which was determined by comparison with a standard of pure CML); a decrease in the content of lysine, closely matching the stoichiometry of CML generation, was also measured. No additional products were detected in the chromatograms (Fig. 1). Preliminary experiments using different metal-catalyzed oxidation systems (copper/ascorbate and iron/ascorbate) at different concentrations indicated that the best conditions to achieve oxidation of BSA was treatment with 100 $\mu$M $FeCl_3$/25 mM ascorbate. These experiments also provided preliminary information on the kinetic course of the oxidative reaction. Using 100 $\mu$M $FeCl_3$/25 mM ascorbate, the time course of oxidation of a CML-BSA sample containing 18 mol CML/mol protein (18CML-BSA) was compared to that of a control BSA sample, whose CML content was below the detec-

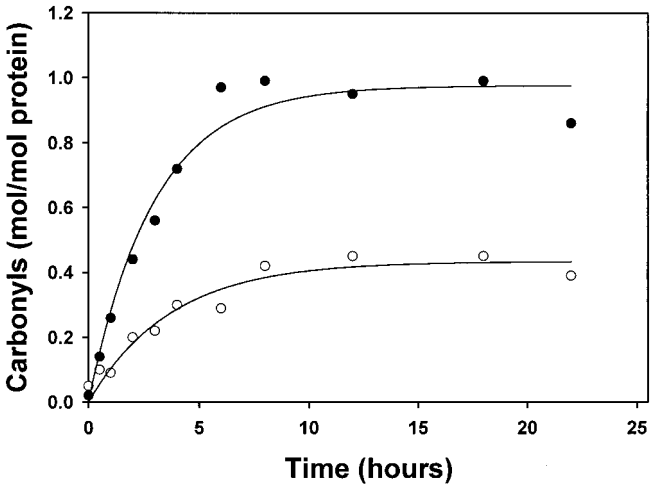


**FIG. 2.** Generation of carbonyls over time upon oxidation of BSA (open circles) and 18CML-BSA (closed circles) by $Fe^{3+}$/ascorbate, 100 $\mu$M/25 mM, as described under Materials and Methods.

tion limit of our analytical method (~0.25 mol/mol). As shown in Fig. 2, carbonyl levels increased during the first ~7 hours, after which their concentration reached a plateau. The shapes of the curves for both control BSA and 18CML-BSA were virtually identical, although the slope of 18CML-BSA during the first ~7 hours was steeper, i.e. the amount of carbonyls was higher for 18CML-BSA at all times. The final amount of carbonyls in 18CML-BSA, when the steady state was reached, was ~1 mol/mol protein, about twice the amount present in the control BSA sample.

In a separate experiment, CML-BSA samples containing different amounts of CML were oxidized under the described conditions for 18 hours, in order to determine the minimum level of CML necessary to effect an increase in the yield of carbonyl content compared to control. In control BSA, the carbonyl content rose from 0.04 ± 0.01 to 0.65 ± 0.05 mol/mol protein. Very similar results were obtained with CML-BSA samples containing 1, 2, or 4 mol CML/mol lys, all of whose carbonyl levels were ~0.6 mol/mol after oxidation. A slightly higher level of carbonyls was seen in samples containing 7 mol CML/mol protein, and increasingly higher levels were measured in samples containing higher amounts of CML (Fig. 3). In the case of CML-BSA samples containing 18 mol CML/mol BSA, the amount of carbonyls after oxidation was 1.31 ± 0.02 mol/mol; an overall slightly higher degree of oxidation was produced in this experiment as compared with the previous one. As illustrated in Fig. 2, CML-BSA samples did not have an increased level of carbonyls before oxidation.

Upon oxidation, the amount of glutamic semialdehyde rose from 8.3 ± 0.6 to 151.7 ± 8.7 mmol/mol protein in BSA and from 10.7 ± 0.6 to 299 ± 16.4 mmol/mol protein in 18CML-BSA. For these samples, adipic semialdehyde increased from 6.3 ± 0.6 to 232.7 ± 56.0 and from 90.7 ± 15.5 to 392.3 ± 31.4 mmol/mol protein, respectively (Fig. 4). The unexpectedly high level of adipic semialdehyde in CML-BSA before oxidation prompted the suspicion that HACA (its reduction product) might be artifactually generated from CML during preparation of samples for analysis. Thus, a sample of pure CML (86 nmol) was treated in the same way as protein samples, and HAVA and HACA measured. While no HAVA was detected, 0.37 nmol HACA was measured. This means that 0.43% of CML converts to HAVA during sample hydrolysis and derivatization. Given 18 moles CML per mol of 18CML-BSA, one should expect a difference of ~77 mmol CML/mol protein between 18CML-BSA and BSA to be artifactually generated during sample work-up, a figure that is in excellent agreement with our experimental results. Comparison of the net increases of HAVA and HACA in oxidized BSA and 18CML-BSA (Fig. 4, insets), leads to the conclusion that HAVA levels experienced a higher net increase in 18CML-BSA; the increase of HACA was also higher in 18CML-BSA, although the difference with respect to BSA was more modest. Of note, HAVA and HACA account for approximately 60% of the total carbonyls in oxidized BSA or CML-BSA.

## DISCUSSION

Metal-catalyzed oxidation of proteins is believed to play a role in disease and aging, as one important effector of oxidative stress. A quite selective reaction, it targets some individual proteins more than others (18) and affects specific amino acid residues rather than

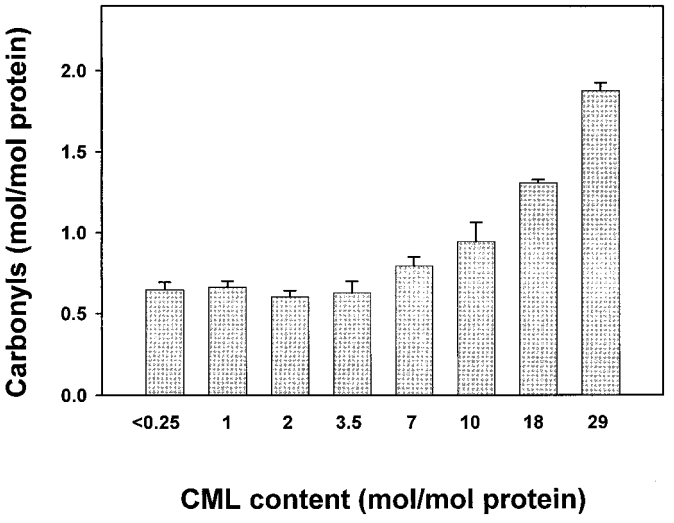


**FIG. 3.** Carbonyls in BSA and CML-BSA samples after oxidation for 18 hours with $Fe^{3+}$/ascorbate, 100 $\mu$M/25 mM, as described under Materials and Methods. Error bars are S.D. of three independent carbonyl measurements of a given oxidized sample.

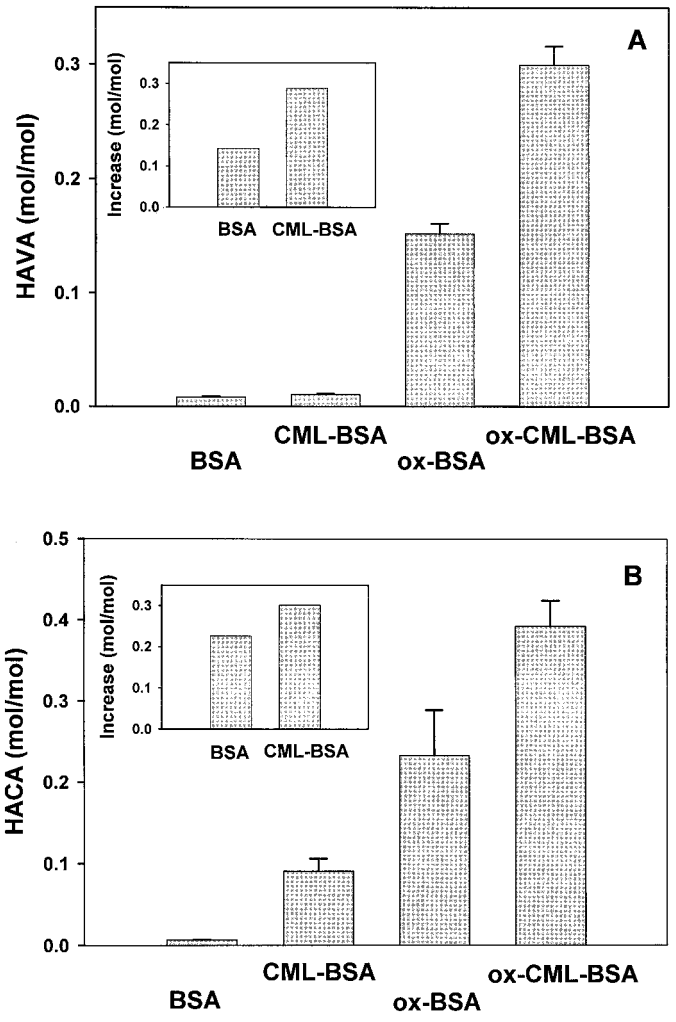


**FIG. 4.** Levels of (A) hydroxyaminovaleric acid (HAVA) and (B) hydroxyaminocaproic acid (HACA) in reduced BSA and 18CML-BSA samples before and after oxidation with $Fe^{3+}$/ascorbate, 100 $\mu$M/25 mM, for 18 hours. Insets: net differences of these levels between samples before and after oxidation. Error bars represent s.d. of three independent measurements.

random ones as numerous studies have characterized (19, 20). However, the possibility that certain post-translational modifications might modulate or enhance the susceptibility of proteins to MCO has received little attention. In the present study, we aimed to test whether this is indeed the case. We show that transformation of lysine residues into CML renders proteins more susceptible to MCO, with the result of an increased yield of protein carbonyls in general and glutamic and adipic semialdehydes in particular. The most likely explanation for our experimental results is increased binding of metal ions via CML. Inspection of CML reveals that its carboxylic and $\epsilon$-amino groups might act in a way similar to the amino and carboxylic groups of glycine and EDTA, which are known metal ion chelators. Copper binding capacity of CML-rich proteins has, in fact, recently been demonstrated by Saxena *et al.* (15). These authors further showed that copper-containing CML-rich proteins are able to oxidize ascorbate. Our results expand these findings showing that the pro-oxidant effect of CML cannot only be exerted towards exogenous substrates, but also results in increased MCO auto-oxidation of the protein bearing it. It has also been recently reported that heavily glycated BSA shows an increased iron and copper binding capacity (21). Under the aerobic conditions used in the study to prepare glycated BSA, CML is known to be the most abundant advanced glycation product formed (16), and therefore invites speculation that CML may be responsible for the increased metal binding observed. An alternative explanation for our observations is that the introduction of a high level of CML in BSA leads to a structural distortion of its structure, which results in increased exposure of metal ion binding sites.

The presence of CML led to an increase in both the rate and total amount of protein carbonyl formation, but the shape of the time-course increase is essentially similar for control BSA and CML-BSA (Fig. 2). This finding suggests the existence of a pool of susceptible sites that are quickly oxidized in CML-BSA during the first hours, while the rest of oxidizable amino acid residues undergo oxidation at an unchanged time rate.

$N^{\epsilon}$-(Carboxymethyl)lysine is a common post-translational modification affecting lysine residues. Originally described as a product of glycoxidation (22,23), it has been subsequently shown that it can be generated as a consequence of the reaction of proteins with products of lipid peroxidation, such as glyoxal (24). Glycolaldehyde, a product of serine oxidation by myeloperoxidase, is another source of CML; this reaction represents an enzymatic pathway for the generation of CML (25). Given such an ample array of sources, CML has been described as a "molecular sink" of oxidative reactions (15). $N^{\epsilon}$-(Carboxymethyl)lysine is elevated in proteins and tissues in association with diabetes (26, 27), atherosclerosis (28), aging (26), and end-stage renal disease (29). Based on the present results it, seems reasonable that CML formation may contribute to the increase in the level of protein oxidation that has been observed in these conditions (although considerable controversy remains on the role of oxidative stress in some of them, 30). In this context, the present findings should be taken cautiously and not be over-interpreted. In our model, a differential effect of CML on protein oxidation was only seen at high levels ($\sim$10 mol/mol protein and higher). Such levels are much higher than the levels that have been documented in tissue proteins even in association with diabetes or end-stage renal disease (26, 29). However, susceptibility of proteins to lysine carboxymethylation and/or its effect on oxidation may vary from one protein to another. Moreover, it is also conceivable that longer pe-

riods of exposure and reaction may partially offset this limitation.

In summary, our results demonstrate that conversion of lysine to CML induces increased susceptibility to MCO in proteins. Under our working conditions, this effect was detected at relatively high levels of CML. Further studies using other MCO systems and model proteins, together with assessments of the iron binding capacity of CML, will help us understand the possible physiological implications of these findings.

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