Amino Acids (2013) 44:361–371
DOI 10.1007/s00726-012-1339-2

ORIGINAL ARTICLE

Formation of *S*-(carboxymethyl)-cysteine in rat liver mitochondrial proteins: effects of caloric and methionine restriction

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Received: 21 November 2011 / Accepted: 4 June 2012 / Published online: 22 June 2012

Abstract Maillard reaction contributes to the chemical modification and cross-linking of proteins. This process plays a significant role in the aging process and determination of animal longevity. Oxidative conditions promote the Maillard reaction. Mitochondria are the primary site of oxidants due to the reactive molecular species production. Mitochondrial proteome cysteine residues are targets of oxidative attack due to their specific chemistry and localization. Their chemical, non-enzymatic modification leads to dysfunctional proteins, which entail cellular senescence and organismal aging. Previous studies have consistently shown that caloric and methionine restrictions, nutritional interventions that increase longevity, decrease the rate of mitochondrial oxidant production and the physiological steady-state levels of markers of oxidative damage to macromolecules. In this scenario, we have detected *S*-(carboxymethyl)-cysteine (CMC) as a new irreversible chemical modification in mitochondrial proteins. CMC content in mitochondrial proteins significantly correlated with that of the lysine-derived analog N^{ε}-(carboxymethyl)-lysine. The concentration of CMC is, however, one order of magnitude lower compared with CML likely due in part to the lower content of cysteine with respect to lysine of the mitochondrial proteome. CMC concentrations decreases in liver mitochondrial proteins of rats subjected to 8.5 and 25 % caloric restriction, as well as in 40 and 80 % methionine restriction. This is associated with a concomitant and significant increase in the protein content of sulfhydryl groups. Data presented here evidence that CMC, a marker of Cys-AGE formation, could be candidate as a biomarker of mitochondrial damage during aging.

Keywords Aging · Carboxymethylated proteins · Dietary restriction · Oxidative stress · Protein damage · Protein cysteine content · Sulfhydryl groups

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Introduction

A low rate of generation of endogenous damage—mainly in mitochondria—and an intrinsically high resistance to modification of tissue macromolecules are key traits of long-lived animal species (Pamplona and Barja 2007, 2011). Caloric restriction (CR) is the most reproducible experimental manipulation that increases maximum longevity of many different animal species and lowers mitochondrial reactive molecular species generation and oxidation-derived molecular damage in rodents (Sanz et al. 2006). Protein and methionine restriction also increase maximum longevity and lower mitochondrial oxidative stress (Pamplona and Barja 2006).

Although all types of biomacromolecules are susceptible to oxidative damage in vivo (Pamplona and Barja 2007), protein damage is thought to have direct physiological consequences due to their role as cellular structural components and bio-catalysts. Oxidative stress-induced modifications on proteins can cause a variety of structural changes including formation of disulfide cross-links, methionine sulfoxide, dy-tirosine cross-links, nitrotyrosine, and carbonyls (Dean et al. 1997). Protein carbonylation can

occur by two different mechanisms: (i) metal-catalyzed oxidation (MCO), which introduces carbonyls into the side chains of certain amino acids (Requena et al. 2001) and (ii) the Maillard reaction (Thorpe and Baynes 2003).

The Maillard reaction between proteins and reducing sugars leads to formation of advanced glycation end-products (AGEs) and contributes to the chemical modification and crosslinking of proteins during aging (Monnier 2003). Exacerbation of this reaction by a hyperglycemic state is mechanistically implicated in the development of chronic complications of diabetes (Negre-Salvayre et al. 2009). Similar chemistry, involving the oxidative chemical modification of proteins by lipids and formation of advanced lipoxidation end-products (ALEs), is also thought to play a role in aging and longevity (Hulbert et al. 2007; Pamplona 2008; Pamplona and Barja 2011), as well as the pathology of diabetes, atherosclerosis, and neurodegenerative diseases (Martínez et al. 2010; Negre-Salvayre et al. 2009).

Up to now, research on the formation of protein AGEs/ALEs in vivo has focused on chemical modification of the N-terminal α-amino group, the ε-amino group of lysine, and the guanidino moiety of arginine residues (Thorpe and Baynes 2003). The described AGEs/ALEs are mostly derivatives of lysine and arginine residues formed by reaction of the amino or guanidino groups on protein with electrophilic intermediates derived from carbohydrate and lipid oxidation or metabolism (Hamada et al. 1996; Nagai et al. 2007). However, the sulfhydryl group on intracellular proteins is a more reactive nucleophile than either amino or guanidino groups so that products of chemical modification of cysteine residues might also be observed as a result of intracellular Maillard reactions. The intracellular space is a reducing environment, rich in cysteine residues that are maintained in the reduced state by an array of reduced intermediates, including thioredoxins, peroxiredoxins, metallothioneins, and glutathione (Kemp et al. 2008). Changes in redox state of thiol/disulfide couples affect protein conformation, enzyme activity, transporter activity, ligand binding to receptors, protein–protein interactions, protein-DNA interactions, protein trafficking, and protein degradation (Kemp et al. 2008).

Presuming that similar modifications of lysine and cysteine residues might occur in parallel during Maillard reactions in vivo, we propose to identify and quantify *S*-(carboxymethyl)-cysteine (CMC), the putative product of reaction of glyoxal or glycolaldehyde with cysteine, in rat liver mitochondrial proteins. CMC is an analog of N^{ε}-(carboxymethyl)-lysine (CML), a major lysine-derived AGE/ALE, formed by reaction of glyoxal or glycolaldehyde with proteins (Wang 2002; Thorpe and Baynes 2003; Zeng and Davies 2005) (Fig. 1), previously described in human plasma proteins (Mostafa et al. 2007) and rat skeletal muscle proteins (Alt et al. 2004). We report here the detection and quantification of CMC in rat liver mitochondrial proteins and the changes in CMC concentrations in mitochondrial proteins as a function of different age-preventing interventions. So, we compare the carboxymethylation state in two different amino acids (lysine and cysteine) and in two different nutritional interventions which are known to decrease mitochondrial free radical generation and specific protein carbonyls (Gómez et al. 2007; Caro et al. 2008). Thus, in this study, male Wistar rats were subjected, for a period of 7 weeks, to two experimental conditions: (i) one about CR, where three groups were defined: control ad libitum, 8.5 % CR, and 25 % CR; and (ii) the second about MetR, with three additional groups: control ad libitum, 40 % MetR, and 80 % MetR. After 7 weeks, the two biomarkers were detected and comparatively measured in liver mitochondrial proteins. The mitochondrial protein sulfhydryl content was also determined.

Materials and methods

Chemicals

Unless otherwise noted, all chemicals were purchased from Sigma–Aldrich Chemical Co. (Madrid, Spain). U–$^{13}C_3$,^{15}N-cysteine, and d_8-lysine were from Cambridge Isotope Laboratories (Woburn, MA, USA). CMC standard was from Sigma-Aldrich (Madrid, Spain). CML, and [$^2H_4$]CML (d4-CML) were prepared as described (Fu et al. 1996). U–$^{13}C_3^{15}N$–CMC was synthesized from U-$^{13}C_3$, ^{15}N-cysteine and iodoacetic acid as described (Wang 2002; Alt et al. 2004).

Animals and diets

Male Wistar rats of 250–300 g of body weight were caged individually and maintained in a 12:12 (light:dark) cycle at 22 ± 2 °C and 50 ± 10 % relative humidity. For caloric restriction experiment ($n = 8$ different animals × group), the control animals were fed ad libitum with a standard rodent diet (Panlab, Barcelona, Spain). The amount of food eaten by the control was measured and their mean food consumption was calculated each week. The two experimental groups received each day an amount of food 8.5 or 25 % lesser that the mean amount eaten by the control animals during the previous week. For methionine restriction experiment ($n = 8$ different animals × group), semipurified diets prepared by MP Biochemicals (Irvine, CA, USA) were used. The composition of the 40 and 80 % MetR diets was similar to that of the control diet except that L-methionine was present at 0.516 and 0.172 %, which

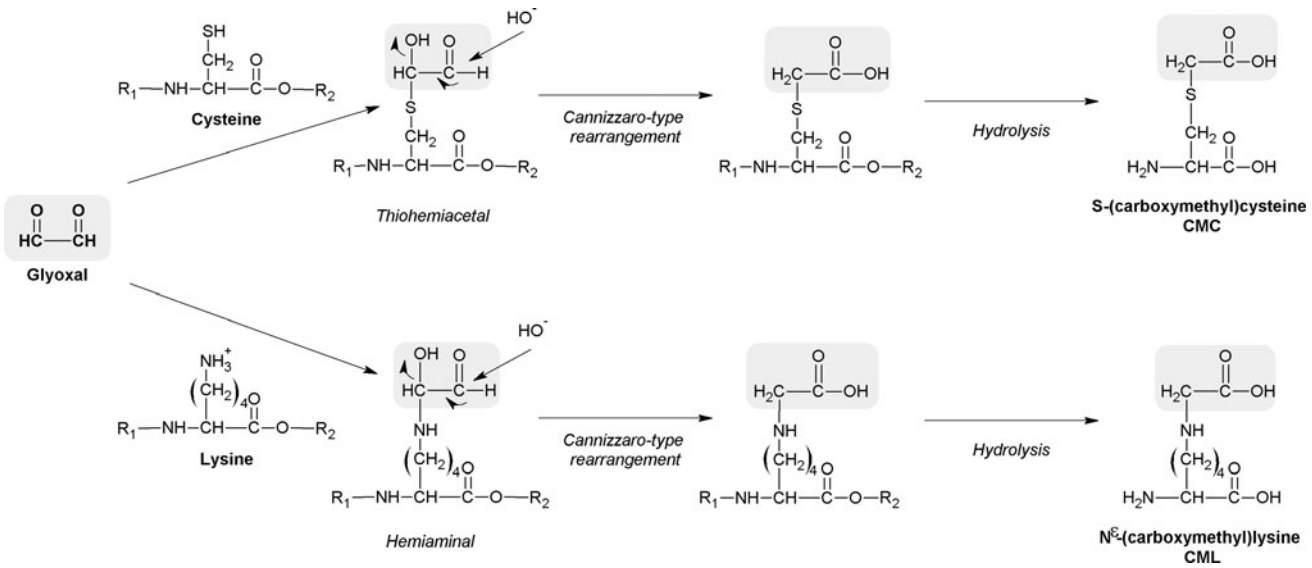

Fig. 1 Structure and proposed mechanisms of formation of *S*-(carboxymethyl)cysteine (CMC) and N^{ε}-(carboxymethyl)lysine (CML). Formation of CMC and CML by reaction of glyoxal with cysteine (*top*) and lysine (*bottom*), respectively, through a Cannizzaro-type rearrangement and then protein hydrolysis to release the protein adduct

corresponds to amounts 40 and 80 % lower than the L-methionine content of the control diet (0.86 %). The % decrease in L-methionine in the 40 and 80 % MetR diets was compensated with increases in all the rest of the dietary components in proportion to their presence in the diet. Since the % absolute decrease in L-methionine was small, with this procedure the % presence of all the rest of the dietary components was almost the same in the three experimental diets. The control and the 40 % MetR groups received each day the same amount of food that the 80 % MetR animals had eaten as a mean the previous week (pair feeding). Daily visual inspection of the rat cages indicated that there were no differences in food spillage between control and treated animals. After 7 weeks of dietary treatment the animals were killed after overnight fasting by decapitation. The liver was immediately processed to isolate mitochondria. All experiments in animal models were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Complutense University of Madrid and University of Lleida.

Liver mitochondria isolation

The liver was rinsed and homogenized in 60 mL of isolation buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, 1 mM EDTA, pH 7.35). The nuclei and cell debris were removed by centrifugation at 1,000×*g* for 10 min. Supernatants were centrifuged at 10,000×*g* for 10 min and the resulting supernatants were eliminated. The pellets were resuspended in 40 mL of isolation buffer without EDTA and centrifuged at 1,000×*g* for 10 min. Mitochondria were obtained after centrifugation of the supernatants at 10,000×*g* for 10 min. After each centrifugation step any overlaying layer of fat was eliminated. The mitochondrial pellets were resuspended in 1 mL of isolation buffer without EDTA. All the above procedures were performed at 5 °C. Mitochondrial protein was measured by the Biuret method. The final mitochondrial suspensions were stored at −80 °C until analyses were performed. The lack of actin was used as indicator of purity in the mitochondrial isolation. No bands were detected by western blot in the mitochondrial fraction when anti-actin antibody (1:5,000, ref. A5441, Sigma) was used (data not shown).

Analytical methods

CMC was detected and characterized by mass spectrometry of the trifluoroacetyl methyl ester (TFAME) derivative using methods described previously (Ayala et al. 2007). Gas chromatography/mass spectrometry (GC/MS) of standards were performed on a Hewlett-Packard (Agilent, Barcelona, Spain) model 6890series gas cromatograph/5973A mass selective detector, equipped with a Rtx-5 column (30 m × 0.25 mm × 0.25 μm) (Restek, Bellefonte, PA, USA). For GC/MS analysis, the injector port was maintained at 275 °C; the temperature program was 5 min at 110 °C, then 2 °C/min to 150 °C, then 5 °C/min to 240 °C, then 25 °C/min to 300 °C, and finally hold at 300 °C for 5 min.

Analysis of mitochondrial protein

CMC and CML concentrations in mitochondrial proteins were determined by selected ion monitoring (SIM)-GC/MS.

Briefly, samples containing 500 μg of protein were (a) delipidated using chloroform:methanol (2:1 v/v) in the presence of 0.01 % butylated hydroxytoluene, (b) precipitated by adding trichloroacetic acid to 10 % (v/v) final concentration, followed by centrifugation, and (c) immediately reduced overnight with 500 mM $NaBH_4$ in 0.2 M borate buffer, pH 9.2, containing 1 drop of hexanol as an anti-foam reagent. Proteins were reprecipitated by adding 1 mL of 20 % trichloroacetic acid (TCA) and subsequent centrifugation. After addition of isotopically labeled internal standards (d_8-lysine, d_4-CML, and U–$^{13}C_3^{15}N$–CMC), the samples were hydrolysed at 155 °C for 30 min in 1 mL of 6 N HCl and were dried in vacuo (Speed-Vac; Savant/GMI Instruments, Barcelona, Spain). The *N*,*O*-trifluoroacetyl methyl ester (TFAME) derivatives of the protein hydrolysates for GC/MS analysis were prepared as previously described (Ayala et al. 2007).

Isotope dilution selected ion monitoring (SIM) GC/MS was used for quantitative analysis of mitochondrial proteins, using standard curves developed from mixtures of varying amounts of natural lysine, CML and CMC with a constant amount of each heavy-labeled compound. The ions used were lysine and [$^2H_8$]lysine, *m/z* 180 and 187, respectively; CML and [$^2H_4$]CML, *m/z* 392 and 396, respectively; CMC and U–$^{13}C_3^{15}N$–CMC, *m/z* 271 and 275, respectively. The amount of products was expressed as the ratio μmol CMC and CML/mol of lysine.

Bioinformatics for amino acid composition of mitochondrial proteome

The NextProt advanced filtering feature in the *nextprot.org* proteome database was used to select liver mitochondrial proteins. A FASTA file was generated and corrected to tab delimited file using the fasta2tab program (*darwin.biochem.ohstate.edu/fasta2tabl*) and then using an amino acid calculator (*proteome.gs.washington.edu/cgi.bin/aa.calc.pl*) the % of individual amino acids in the mitochondrial proteome were estimated.

Measurement of protein sulfhydryl groups content

Thiol concentrations were assayed using spectrofluorimetric method. The thiol fluorescent detection kit (ref. K005-F1; Luminos, MI, USA) determines the extent of free thiol content in samples using a nonfluorescent substrate that covalently binds to free thiol groups to yield a highly fluorescent product. Briefly, the appropriate dilution of the sample or standard (*N*-acetylcysteine) was mixed with the substrate in a 96-well plate and it was incubated at room temperature for 30 min. After incubation, the fluorescent product was read at emission 510 nm with excitation at 390 nm in a microplate fluorimeter (Tecan Infinite M200; Tecan Group Ltd, Switzerland) with the software i-control V1.4 SP1. The sample thiol concentration was calculated using the standard curve of *N*-acetylcysteine, after making a suitable correction for any sample dilution. The thiol content was determined in the whole mitochondrial sample and the supernatants after protein precipitation as previously described (Portero-Otín et al. 1999). The amount of thiol groups in proteins was calculated from the difference of thiol in total sample minus the thiol measured in the supernatants. Results were expressed as nmol SH/mg of protein.

Statistical analysis

Data were summarized throughout as mean ± SEM. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL). The effect of dietary manipulations was analyzed by one way ANOVA. After the ANOVA, the Duncan test was performed for comparisons between pairs of groups. The minimum level of statistical significance was set at $p < 0.05$ in all the analyses. Correlation analysis was performed using the Pearson correlation coefficient.

Results

Detection of *S*-(carboxymethyl)cysteine (CMC) in mitochondrial proteins

The full-scan spectra and structure of the TFAME derivative of the CMC standard and CMC present in mitochondrial proteins, analyzed by gas chromatography/mass spectrometry (GC/MS), are shown in Fig. 2a and b, respectively. The presence of the thiol-aldehyde adduct CMC in mitochondrial proteins was confirmed by detection of several ions derived from CMC at the same retention time and with the same relative ion intensities observed for the unlabeled standard (Fig. 2b). Figure 3 shows the detection of *S*-(carboxymethyl)cysteine (CMC) and N^{ε}-(carboxymethyl)lysine (CML) in rat liver mitochondrial proteins by using selected ion monitoring (m/z 271 and 275 ions correspond to CMC on proteins (Fig. 3a) and labeled internal standard (Fig. 3b), respectively; m/z 392 and 396 ions correspond to CML on proteins (Fig. 3c) and labeled internal standard (Fig. 3d), respectively).

Comparative measurement of carboxymethylation-derived mitochondrial protein markers

The physiological steady-state levels of the carboxymethylation-derived markers in mitochondrial proteins CMC and CML are shown in Fig. 4. CMC was present in mitochondrial proteins in control animals at a level

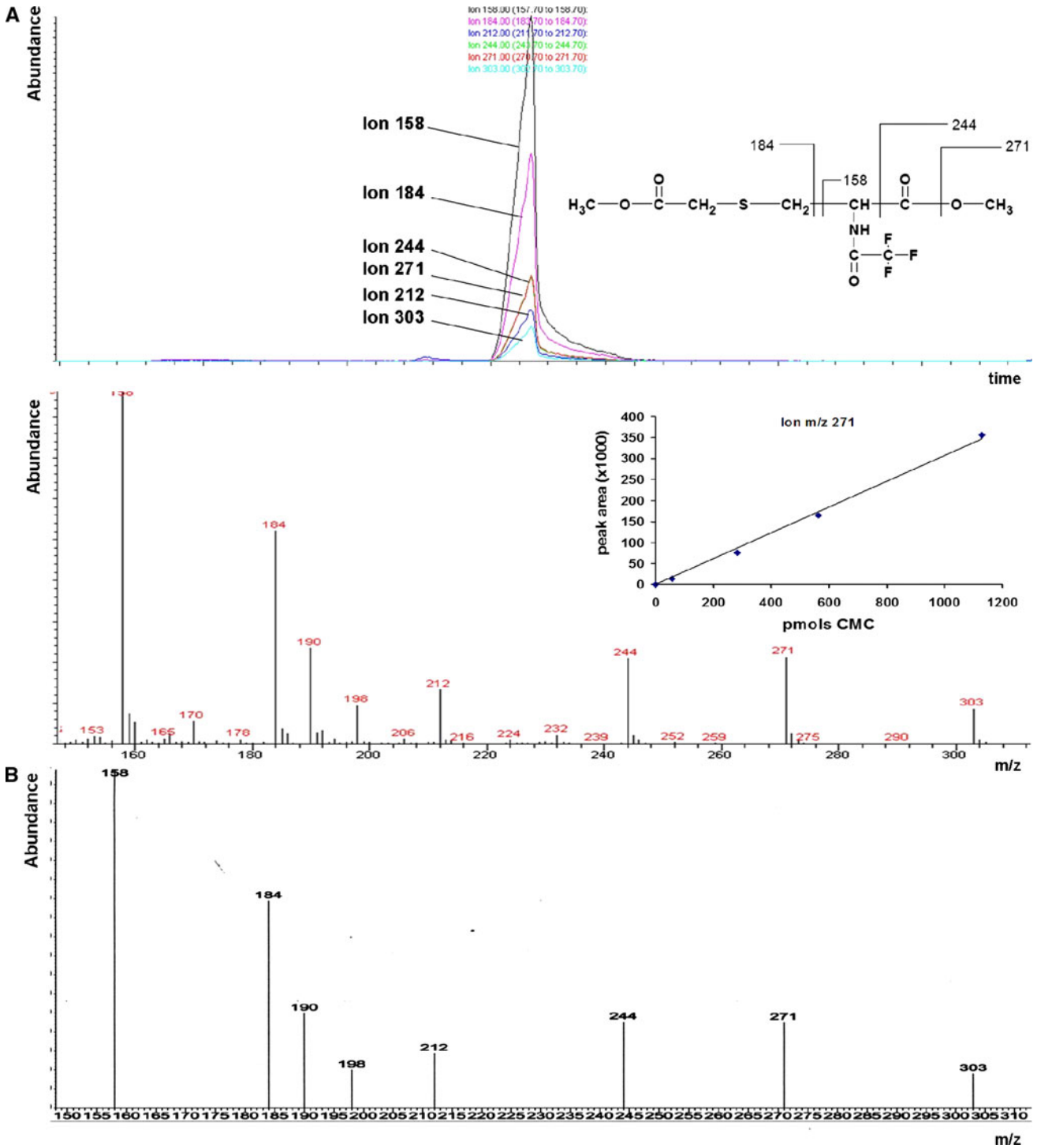

Fig. 2 Structure, mass spectrum, and proposed fragmentation pattern of TFAME derivative of *S*-(carboxymethyl)cysteine (CMC). **a** Specific selected ion monitoring chromatogram (*upper*) and mass spectrum (*lower*) for CMC standard. Insert: Linearity of the CMC signal using the ion m/z 271 for its quantification. **b** Mass spectrum from a peak co-eluting with heavily labeled CMC present in rat liver mitochondrial proteins after protein hydrolysis. The spectrum was obtained on the GC/MSD instrument

representing between 10 and 20 % of CML level (CMC, 185.57 ± 24.91 μmol/mol lys; CML, 1,016.35 ± 50.56 μmol/mol lys; $p < 0.001$). In addition, there was a significant correlation between the CMC and CML content of liver mitochondria proteins (Fig. 5), which was apparent in both caloric- and methionine-restricted (for CR, $r = 0.67$, $p < 0.006$; for MetR, $r = 0.60$, $p < 0.013$) rats suggesting that, irrespective of the experimental intervention,

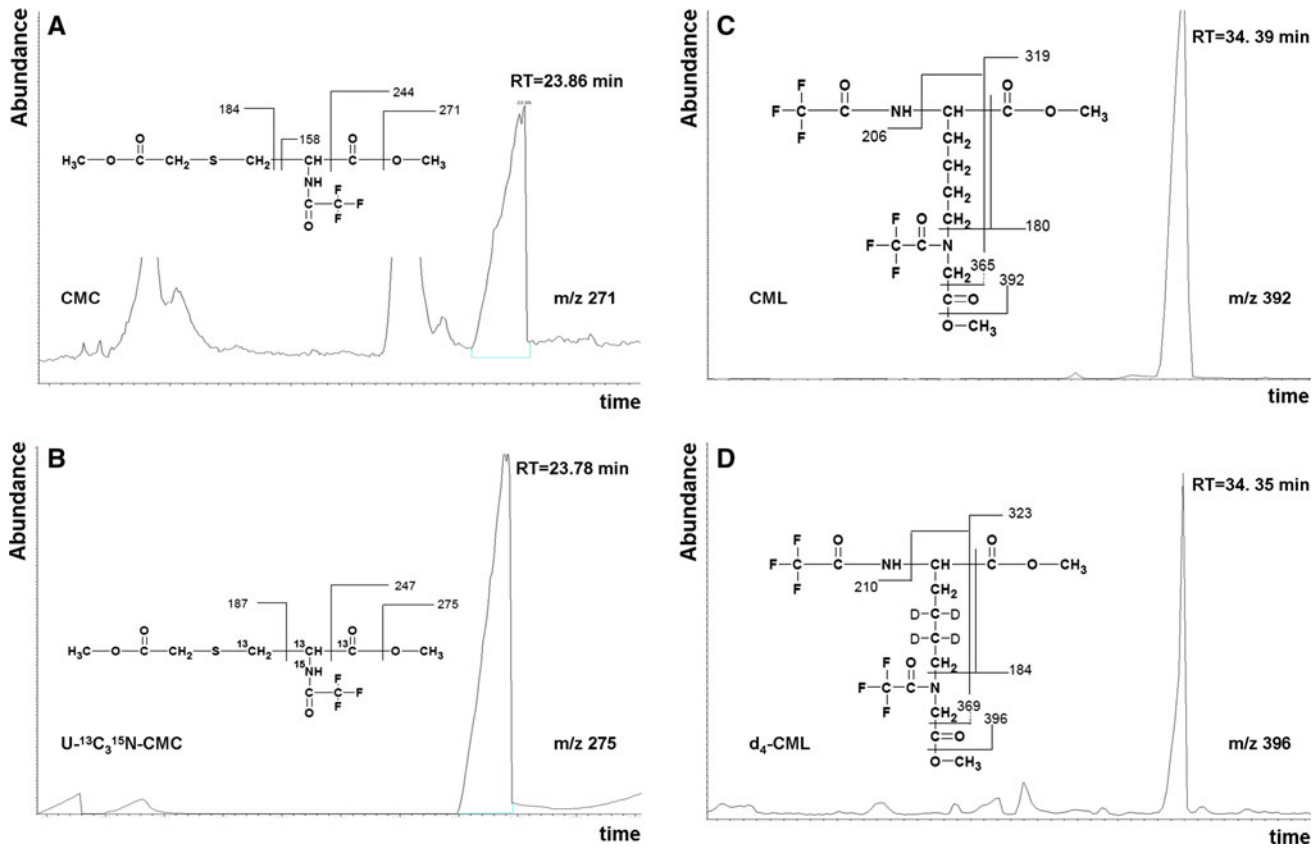

Fig. 3 Quantitative analyses of *S*-(carboxymethyl)cysteine (CMC) and N^{ε}-(carboxymethyl)lysine (CML) in rat liver mitochondrial proteins. Mitochondrial proteins were analyzed by GC/MS as described in "Materials and methods". **a** Selected ion chromatograms for mitochondrial proteins showing the m/z 271 and 275 ions corresponding to CMC protein content and internal standard, respectively. **b** Selected ion chromatograms for mitochondrial proteins showing the m/z 392 and 396 ions corresponding to CML protein content and internal standard, respectively. RT, retention time

these modifications are produced in response to similar stresses, possibly the result of mitochondrial oxidative stress.

Because cysteine and lysine protein contents of the mitochondrial proteome can be a relevant factor in determining the degree of protein nonenzymatic modification and consequently the physiological steady-state level of CMC and CML formation, we proceeded to evaluate the amino acid profile of the whole mitochondrial proteome by means of a bioinformatics approach. Using the Next prot advanced filtering, a total of 678 rat liver mitochondrial proteins were identified. FASTA file was corrected to TAB delimited file and then using the amino acid calculator the % of individual amino acids in the mitochondrial proteome were estimated. The amino acid profile obtained was Ala, 7.84 %; Arg, 5.92 %; Asn, 3.35 %; Asp, 4.64 %; Cys, 1.83 %; Glu, 6.74 %; Gln, 4.47 %; Gly, 6.97 %; His, 2.46 %; Ile, 4.79 %; Leu, 10.46 %; Lys, 5.84 %; Met, 2.50 %; Phe, 3.82 %; Pro, 5.46 %; Ser, 6.96 %; Thr, 5.12 %; Trp, 1.31 %; Tyr, 2.75 %; Val, 6.67 %. As derived from the amino acid profile, the protein lysine content is three times higher that the protein cysteine content.

Effects of caloric and methionine restriction

The effects of caloric and methionine restriction in the physiological steady-state levels of the carboxymethylation-derived markers in mitochondrial proteins (CMC and CML) are shown in Fig. 6. CMC and CML were significantly lower in 8.5 % CR and 25 % CR when compared with the controls. No significant differences for any of the two different protein markers were found when comparing the 8.5 % with the 25 % CR group. In a similar way, CMC and CML were significantly lower in 40 % MetR and 80 % MetR when compared with the controls. No significant differences for any of the two different protein markers were found when comparing the 40 % with the 80 % MetR group. Interestingly, CMC decreases around 15 % (for CR) and 30 % (for MetR) with respect to the control group,

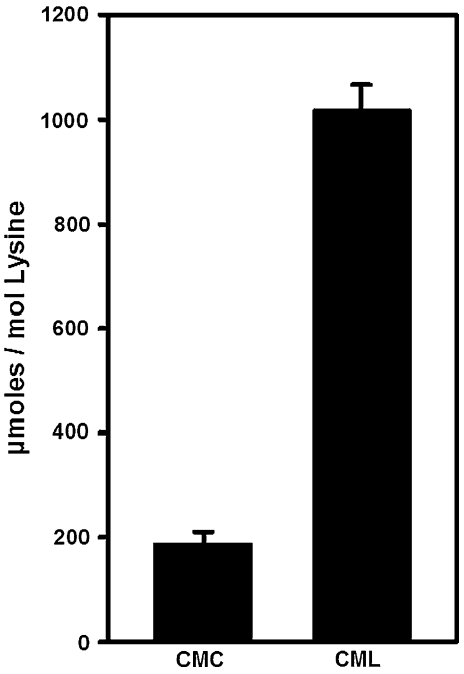

Fig. 4 Physiological steady-state levels of the carboxymethylation-derived markers *S*-(carboxymethyl)cysteine (CMC) and N^{ε}-(carboxymethyl) lysine (CML) in rat liver mitochondrial proteins from control animals. Values are mean ± SEM from $n = 16$ different animals per group. Significant differences were found: $p < 0.001$

while CML decreases around 30 % in both dietary interventions compared with the control group.

Effect of caloric and methionine restriction on mitochondrial protein sulfhydryl content

The formation process of CMC is accompanied by loss of the thiol group and formation of the stable adduct. For this reason we measured the protein sulfhydryl content to evaluate the effect of caloric- and methionine restriction. The values of sulfhydryl content in mitochondrial proteins are shown in Fig. 7. Sulfhydryl content was significantly higher in 25 % CR when compared with the controls ($p < 0.01$), as well as when compared with the 8.5 % CR group ($p < 0.001$). No significant differences were found when comparing the 8.5 % with the control group. In a similar way, sulfhydryl content was significantly higher in 80 % MetR group when compared with the controls ($p < 0.001$) and 40 % MetR group ($p < 0.01$). No significant differences were found when comparing the 40 % MetR group with the control group.

Discussion

In the present study, it has been demonstrated by mass spectrometry methods that cysteine residues, analogously

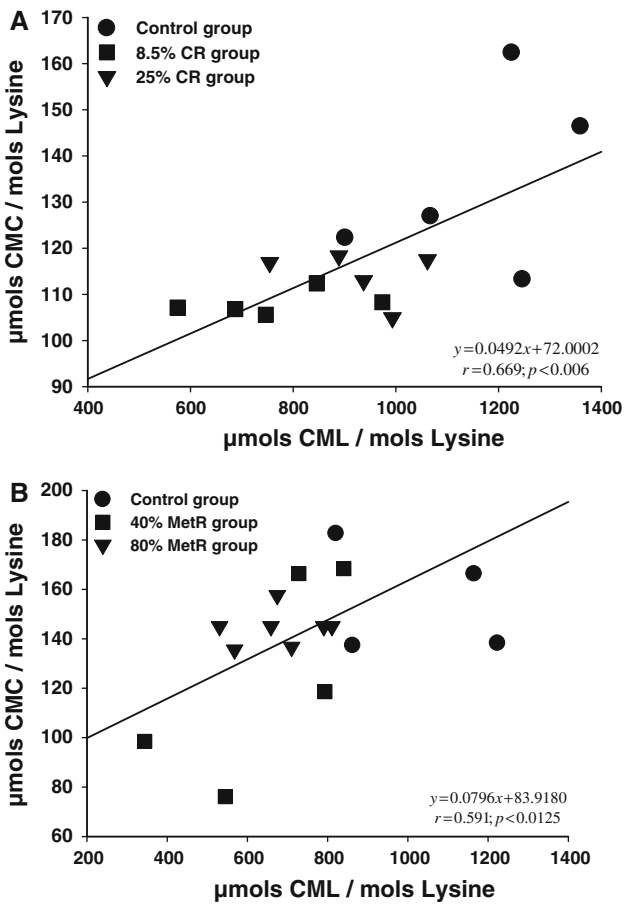

Fig. 5 Correlation between *S*-(carboxymethyl)cysteine (CMC) and N^{ε}-(carboxymethyl) lysine (CML) in rat liver mitochondrial proteins from the (**a**) caloric restriction and (**b**) methionine restriction experiments. Statistical analysis is indicated inside figures

to lysine, are targets for carbonyl compounds in the mitochondrial proteome. Based on its structure and proposed mechanism of formation from in vitro studies (Zeng and Davies 2005), the product CMC is a thioether, not a thioester, adduct to cysteine, and is therefore not reversible by transesterification reactions with glutathione or other cellular thiols. Consequently, on chemical basis, mitochondrial CMC must be considered as an irreversible and stable end product.

The lower level of CMC, ~20 % the level of CML, in mitochondrial proteins is consistent with the low level of cysteine in mitochondrial proteins. Thus, more specifically, in the rat liver mitochondrial proteome Cys accounts for 1.83 % of total amino acids compared with 5.84 % of Lys. So, protein Cys content is around 30 % of the protein Lys content. Accounting that ratio, a more protected Cys environment or the presence of other quantitatively relevant modifications (e.g. from oxidation reactions) could explain this relative lower degree of chemical modifications by carboxymethylation reactions. In any case,

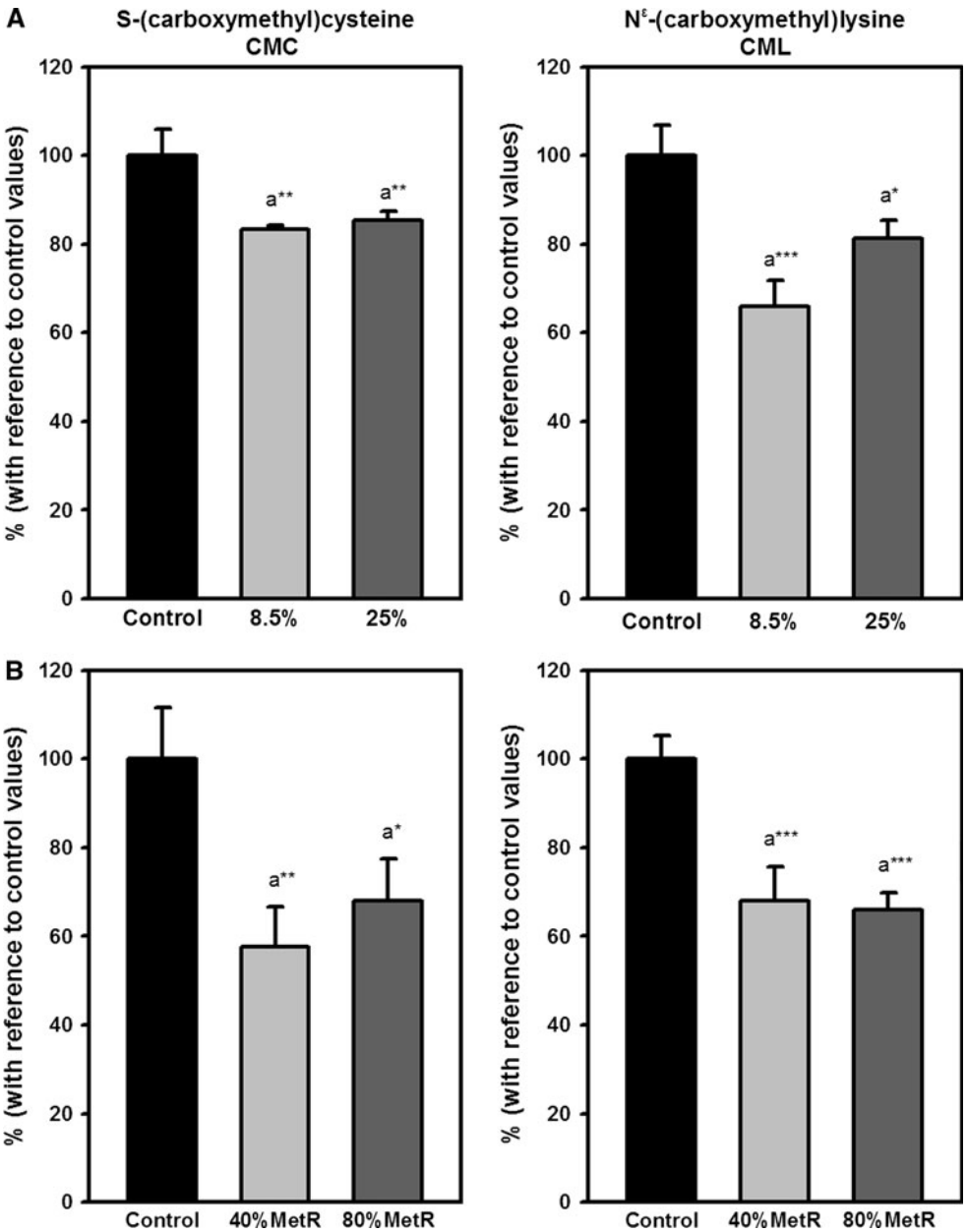

Fig. 6 Steady-state levels of CMC and CML in rat liver mitochondria of (**a**) control, 8.5 and 25 % caloric restricted rats, and (**b**) control, 40 and 80 % methionine-restricted rats. *Asterisks* represent significant differences: *a** compared with control group, *b** between experimental groups. * $p < 0.050$; ** $p < 0.010$; *** $p < 0.001$. Values (μmol/mol lysine; mean ± SEM from 8 different animals × group): **a** CR Experiment: (1) For CMC: control, 131.5 ± 7.75; 8.5 %CR, 109.53 ± 1.31; 25 %CR, 112.25 ± 2.74. (2) For CML: control, 1,160.01 ± 79.73; 8.5 %CR, 765.68 ± 68.04; 25 %CR, 943.84 ± 45.58. **b** MetR experiment: For CMC: control, 200.38 ± 23.26; 40 %MetR, 115.39 ± 18.12; 80 %MetR, 136.01 ± 19.32. For CML: control, 1,026.13 ± 54.3; 40 %MetR, 698.13 ± 78.06; 80 %MetR, 677.63 ± 39.5

cysteine, which is a stronger nucleophile than lysine, can be considered as a primary target of nonenzymatic carboxymethylation of protein and an indicator of mitochondrial (and intracellular) carbonyl formation and oxidative stress.

The significant correlation between CMC and CML in rat liver mitochondrial proteins suggests that these modifications are produced in response to similar stresses, possibly the result of oxidative stress at mitochondrial level. The net flux of mitochondrial oxidative stress results in a physiological steady-state level of damaged mitochondrial proteins that need to be rapidly removed before they accumulate as insoluble aggregates and disrupt mitochondrial function and cellular homeostasis. Quality control of the mitochondrial proteome involves mitochondrial proteases, ubiquitination and proteolysis of outer membrane, mitochondrial fission and fusion, and finally autophagy of damaged mitochondria (mitophagy) (Bota and Davies 2001). AGEs/ALEs formation is a major cause of spontaneous damage to cellular and extracellular proteins in physiological systems, affecting ~0.1–0.2 % of lysine and arginine residues (Thornalley et al. 2003). For proteins with a very slow or even without turnover rate, such as lens proteins, the extent of protein nonenzymatic modification may be up to tenfold higher (Ahmed et al. 2003). Most mitochondrial proteins are short-lived, however, with turnovers from 10 to 30 min to 3–5 days (Bota and Davies 2001). Probably the most important reactive

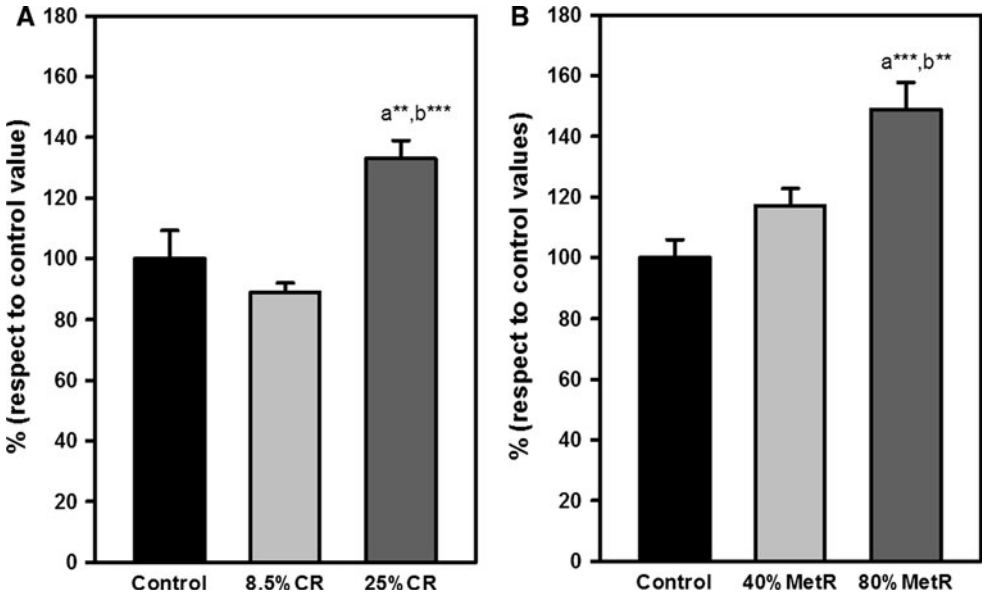

Fig. 7 Effects of (**a**) caloric restriction and (**b**) methionine restriction on amount of protein sulfhydryl content in rat liver mitochondria. *Asterisks* represent significant differences: *a** compared with control group, *b** between experimental groups. * $p < 0.050$; ** $p < 0.010$; *** $p < 0.001$. Values (pmol/μg protein; mean ± SEM from eight different animals × group): **a** CR Experiment: 2.93 ± 0.27; 8.5 %CR, 2.60 ± 0.09; 25 %CR, 3.90 ± 0.17. **b** MetR experiment: control, 3.8 ± 0.22; 40 %MetR, 4.45 ± 0.21; 80 %MetR, 5.65 ± 0.33

compounds to consider for Maillard reaction-derived damage to the mitochondrial proteome are the reactive carbonyls glyoxal and methylglyoxal formed endogenously by lipid peroxidation and the degradation of metabolic intermediates. These carbonyl compounds are potent damaging agents, 200–50,000-fold more reactive than glucose, although physiological concentrations are typically 10,000–50,000-fold lower than glucose (Rabbani and Thornalley 2008).

Protein oxidation is thought to constitute an important trigger of the aging process (Sohal 2002; Stadtman 2004). Animal models of impaired oxidized protein repair have been shown to exhibit premature aging phenotypes (Moskovitz et al. 2001). Cysteine oxidation and the consequent loss of free thiol groups are well known to be one of the first biochemical alterations in proteins after exposure to ROS (Sohal 2002). Thiol loss is an established biomarker of aging (Dröge 2002), notably in membrane proteins (Agarwal and Sohal 1994), and it is known to be induced by hyperoxia and to be attenuated by caloric restriction (Sohal 2002). Long-lived mouse strains maintain higher levels of reduced glutathione, especially in mitochondria, affording the reduction of oxidized protein cysteines (Rebrin and Sohal 2004) and the glutathionylation of free cysteines (Hurd et al. 2005). In addition, supplementation of mice with *N*-acetylcysteine partially prevents the age-associated decline of complex I and IV activity observed in these animals (Miquel et al. 1995). In this line, in the present study mitochondrial CMC content is attenuated by caloric and methionine restriction, with a concomitant increase in protein sulfhydryl content. Considering that CMC is irreversible, this increase in protein sulfhydryl content is probably due to an increased mitochondrial turnover as described recently in caloric restriction (López-Lluch et al. 2006). In addition to the mitochondrial turnover, a decrease in the mitochondrial free radical generation and consequently the oxidative stress-derived damage by both caloric and methionine restriction (Gómez et al. 2007; Caro et al. 2008) probably also plays a relevant role in determining mitochondrial CMC formation.

One important, but to date the almost unexplored element is the potential role of modification of thiol groups in proteins. Most non-enzymatic chemical modifications of protein are products of oxidation and/or Maillard reaction (Thorpe and Baynes 2003). Cysteine and methionine are the only amino acids in proteins which contain elements undergoing reversible oxidation under physiological conditions (Stadtman 2004). These elements are aptly described as "sulfur switches" because the reversible oxidations provide a means to control a broad range of activity and structure of proteins. The sulfur atoms of both cysteine and methionine can undergo multiple oxidations, but the reversible oxidation of thiols to disulfides has been the most extensively studied. Changes in reduction/oxidation (redox) state of thiol/disulfide couples affect protein conformation, enzyme activity, transporter activity, ligand binding to receptors, protein–protein interactions, protein–DNA interactions, protein trafficking, and protein degradation, affecting components in redox signaling, transcriptional regulation, cell proliferation, apoptosis, hormonal signaling, and other fundamental cell functions (Kemp et al. 2008). The ubiquitous and functional nature of these cysteine sulfhydryls also makes them a target for AGE/ALE formation with the potential for widespread impact. In contrast to the reversible character for oxidation, Maillard reaction-derived compounds are stable and irreversible. Considering that mitochondrial membrane is characterised by a uniquely high protein content of

approximately 75 % by mass, it is plausible to suggest that dysfunctional proteins can occur significantly earlier in such a high-protein membrane, and it could have implications for current hypotheses of the cause of mitochondrial dysfunction and oxidative stress in aging. However, we do not yet understand the consequences of increased modification of mitochondrial proteins by CMC.

Interestingly, in a recent research evidence of lifespan extension of the nematode *Caenorhabditis elegans* with overexpression of the gene for glyoxalase 1 (Glo1) (Schlotterer et al. 2009) was found. Silencing of Glo1 decreased lifespan. Glo1 is a glutathione-dependent enzyme that catalyzes the metabolism of reactive carbonyl compounds such as methylglyoxal and glyoxal. It thereby prevents the nonenzymatic modification of proteins by these carbonyl compounds. Proteins of mitochondria were found to be major targets of reactive carbonyl compounds: increased nonenzymatic modification of mitochondrial proteins was associated with increased formation of ROS and increased proteome damage by oxidative and nitrosative processes. There was a decline of Glo1 expression in *C. elegans* and increased formation of mitochondrial ROS in normal aging. Conversely, overexpression of Glo1 in *C. elegans* decreased carbonyl damage of mitochondrial proteins, decreased the formation of ROS and proteome markers of carbonyl modification and also markers of oxidative and nitrosative damage (methionine sulfoxide and 3-nitrotyrosine residues, respectively) with concomitant life extension. This was indicative that chemical modifications derived from carbonyl compounds could critically damage the mitochondrial proteome that triggers increased ROS and oxidative damage in aging.

Mitochondrial respiratory chain complex cysteines are distinguished targets for oxidative attack due to their specific chemistry and localization. Their uncontrolled nonenzymatic modification by reactive species leads to dysfunctional proteins, which entails cellular senescence and organismal aging. For this reason, cysteine depletion does occur on a proteome-wide scale in aerobic unicellular organisms, providing a clear case in point for the evolutionary pertinence of optimizing protein durability by selective amino acid ablation (Schindeldecker et al. 2011). Furthermore, long-lived species avoid employing this amino acid. Indeed, the longer the longevity of a species, the lower is the mitochondrial protein cysteine content (Moosmann and Behl 2008). Consequently, the longer the longevity of a species, the higher is the mitochondrial protein resistance to oxidative damage. The site affected by cysteine depletion seems to be the inner mitochondrial membrane respiratory chain because it renders them more resistant to oxidative attack, being an evidence of the evolutionary pressure towards functionally optimized respiratory chain complexes (Moosmann and Behl 2008). Improved control of the cellular thiol redox state thus constitutes a paradigmatic biochemical adaptation in long-lived versus short-lived animals, which is directed at the prevention or amelioration of the toxic effects of reactive molecular species. Other examples include the use of oxidation-resistant lipids to build mitochondrial membranes (Pamplona 2008) or the minimized radical generation of the mitochondrial electron transport chain in long-lived species (Pamplona and Barja 2007, 2011).

In conclusion, data presented here evidence that CMC, a marker of Cys-AGE formation, could be candidate as a biomarker of mitochondrial defect during aging, a work currently in progress in our laboratory.

Acknowledgments This study was supported in part by I + D grants from the Spanish Ministry of Science and Innovation (BFU2008-00335/BFI and BFU2011-23888), and BSCH-UCM (2009–2010) to G.B; grant from the Spanish Ministry of Health (PI11/01532) to M.P.O; and grants from the Spanish Ministry of Science and Innovation (BFU2009-11879/BFI), and the Generalitat of Catalunya (2009SGR00735) to R.P.P. Caro and J. Gómez received predoctoral fellowships from the Ministry of Education and Science. We thank David Argiles for excellent technical assistance. The authors are grateful to the anonymous reviewers for criticisms and suggestions, which improved the manuscript. No competing financial interests exist.

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