Amino Acids (2003) 25: 221–226
DOI 10.1007/s00726-003-0012-1

Amino Acids
Printed in Austria

Recent advances in the analysis of oxidized proteins

Review Article

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Received February 2, 2003
Accepted May 7, 2003
Published online July 29, 2003;

Summary. Glutamic semialdehyde is a product of oxidation of arginine and proline, and aminoadipic semialdehyde, of oxidation of lysine. These two carbonyl-containing compounds are the main carbonyl products of metal-catalyzed oxidation of proteins, accounting for 55–100% of the total carbonyl value. Accordingly, they are quantitatively very important contributors to the total value of protein carbonyls in tissues as measured by the classic spectophotometric assay. Sensitive gas chromatography-mass spectrometry based analytical methods allow their quantitation in a variety of biological samples, including tissue protein, cell cultures and lipoproteins. These measurements provide specific information on the oxidative status of proteins that is complementary to that afforded by protein carbonyls, and will be useful tools in the ongoing effort to define and assess the role of protein oxidation in pathology and aging.

Keywords: Glutamic semialdehyde – Aminoadipic semialdehyde – Hydroxyaminovaleric acid – Hydroxyaminocaproic acid – Protein carbonyls – Metal catalyzed oxidation

Introduction: protein oxidation and the carbonyl assay

That oxidation of proteins plays an essential role in the pathogenesis of an important number of degenerative diseases and in aging is now widely recognized (Berlett and Stadtman, 1997; Stadtman and Berlett, 1998). Experimental evidence has been gathered in many laboratories over the past two decades showing that proteins are more oxidized, as compared to control samples, in relevant tissues from animals and patients suffering, among other ailments, from Alzheimer's disease (Smith et al., 1991), cataracts (Garland et al., 1994), rheumatoid arthritis (Chapman et al., 1989), atherosclerosis (Uchida et al., 1994; Leeuwenburgh et al., 1997) or amyotrophic lateral sclerosis (Bowling et al., 1993). Also, higher levels of protein oxidation are measured in tissues from older animals and subjects as compared to younger ones (Oliver et al., 1987; Starke-Reed and Oliver, 1989; Sohal et al., 1993), and manipulations that lead to an increased life span, such as calorie restriction, also result in decreased levels of protein oxidation products (Lass et al., 1998). It could be argued that perhaps increased protein oxidation in all these instances is an epiphenomenon that merely shows correlation with the basic pathological or degenerative processes. Such a notion is not consistent, however, with numerous experiments carried on *in vitro* showing that oxidation of specific proteins, particularly that effected by metal catalyzed oxidation (MCO) systems, to levels similar to those found *in vivo*, does result in loss of function, alteration of structure, and altered susceptibility to degradation (Rivett and Levine, 1990; Fucci et al., 1983). Further, and even more significant, structural and functional analysis of individual proteins isolated from old and young animals has shown that increased oxidation and decreased activity in the former go hand in hand (Yan et al., 1997).

Studies on protein oxidation have been greatly facilitated by the availability of a simple, robust, and accurate method to quantify protein oxidation: the carbonyl assay (Levine et al., 1994). The assay detects carbonyl groups spectrophotometrically after their reaction with 2,4-dinitrophenylhydrazine. Carbonyl groups are introduced in proteins by a variety of oxidative pathways, particularly MCO of specific protein amino acid side chains, and also adduction of carbonyl-containing oxidized lipids (4-hydroxynonenal, malondialdehyde) or sugars (Stadtman and Berlett, 1998). Of course, many oxidative modifications do not give rise to carbonyl moieties. Oxidation of

histidyl residues to oxo-histidine and its degradation products (Lewisch and Levine, 1995), of phenylalanine to ortho- and meta-tyrosine (Heinecke et al., 2000), conversion of methionine to methionine sulfoxide (Levine et al., 1999), or oxidative degradation of tryptophan to kynurenines and a variety of other products are examples of protein oxidative modifications which do not result in generation of carbonyl groups. However, the simplicity and convenience of the carbonyl assay has resulted in the widespread measurement of protein carbonyls as a useful and meaningful index of total protein oxidation.

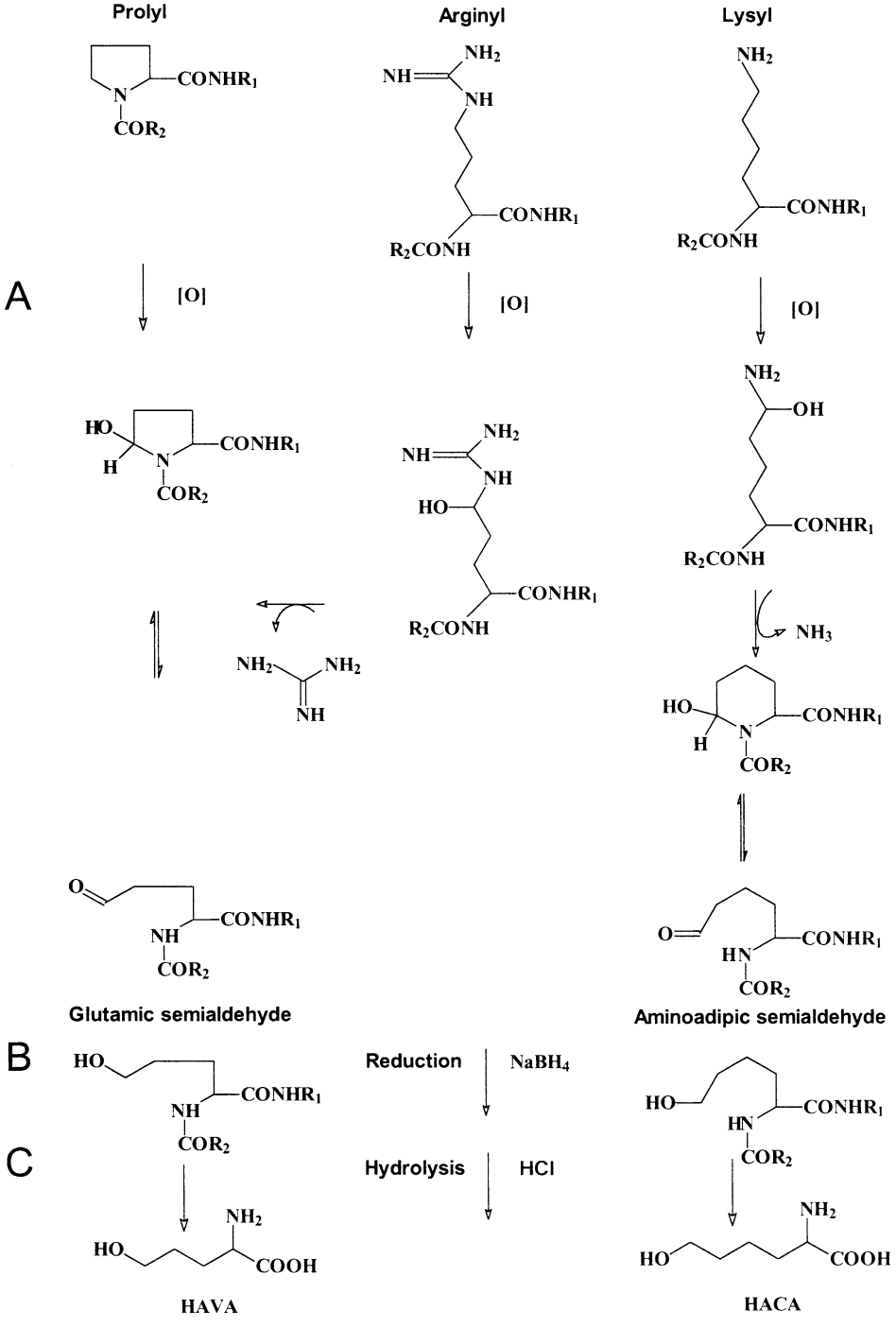

Fig. 1. Scheme of the formation of glutamic and aminoadipic semialdehydes and their reduction products HAVA and HACA. **A** Generation of glutamic and aminoadipic semialdehydes through oxidation of protein prolyl, arginyl and lysyl residues. **B** Reduction with $NaBH_4$. **C** Acid hydrolysis: formation of HAVA and HACA

Specific protein carbonyls: glutamic and aminoadipic semialdehydes

The quantitative and analytical importance of carbonyl-containing protein oxidation aminoacyl residues (protein carbonyls) has prompted several laboratories, including our own, to initiate studies aimed at elucidating the chemical nature of these compounds and at developing specific analytical methods to measure them. Besides the interest in understanding the chemical pathways that lead to formation of protein carbonyls, and acquiring analytical tools complementing the carbonyl assay in the direction of specificity, a third objective guided our efforts: developing a method of quantitating specific carbonyl products in tissue that would provide independent confirmation of analyses based on carbonyl-specific reagents.

Experiments using model proteins and amino acid homopolymers and chromatographic separation of borotritide reduced hydrolysates, had suggested that proline, arginine and lysine were among the most important targets of MCO, and had identified glutamic semialdehyde as an important product of this reaction (Amici et al., 1988). The importance of MCO as the most relevant mechanism of protein oxidation is discussed elsewhere in this monographic issue (Stadtman, this issue). Thus, we anticipated that glutamic semialdehyde, a product of oxidation of arginine and proline, and aminoadipic semialdehyde, a product of oxidation of lysine, would constitute two abundant specific carbonyl products (Fig. 1).

Development of analytical methods to measure glutamic and aminoadipic semialdehydes

The analytical methods that we have developed (Requena et al., 2001) are based on isotope dilution gas chromatography/mass spectrometry (GC-MS). Glutamic and aminoadipic semialdehyde residues are first reduced with $NaBH_4$ to hydroxyaminovaleric (HAVA) and hydroxyaminocaproic (HACA) acids, resistant, unlike their precursors, to acid hydrolysis (Fig. 1). Hydrolysis is then performed in the presence of added deuterated internal standards, d_5-HAVA and d_4-HACA. The hydrolysate is treated to convert HAVA and HACA and their deuterated counterparts to volatile trifluoroacetyl-methyl ester (TFAME) derivatives which are then analyzed by GC-MS in selected ion monitoring (SIM) mode. The use of deuterated internal standards is essential to eliminate the variability introduced during hydrolysis by side reactions that lead to conversion of a fraction of HAVA to proline and HAVA and HACA to the chloroderivatives 5-chloro-2-aminovaleric and 6-chloro-2-aminocaproic acids (Requena et al., 2001; Ayala and Cutler, 1996a; Amici et al., 1989). Another published method to measure glutamic semialdehyde relies on an alternative strategy to overcome this problem: use of enzymatic rather than acid hydrolysis (Pietzsch, 2000), while a third one relies on the assumption that under identical hydrolysis conditions, the extent of conversion of HAVA to side products will be similar in all samples and standards (Ayala and Cutler, 1996a). These methods use, respectively, preparation of trimethylsilyl and N(O)-ethoxy-carbonyl ethyl esters as derivatization strategies. Using electronic ionization as the detection mode, fmol quantities of analytes per injection can be reliably quantified by any of the three methods, which allows analyzing tissue samples containing $\sim$200 μg of protein (obviously sensitivity will vary from instrument to instrument). Negative chemical ionization affords at least a 10 to 20 fold gain in sensitivity, allowing analysis of samples with 10 μg of protein or even less.

Glutamic and aminoadipic semialdehydes are the main products of MCO of proteins

We measured glutamic and aminoadipic semialdehydes in protein samples subjected *in vitro* to MCO and compared their values with those of total carbonyl products obtained for the same samples (Fig. 2). It is evident that although

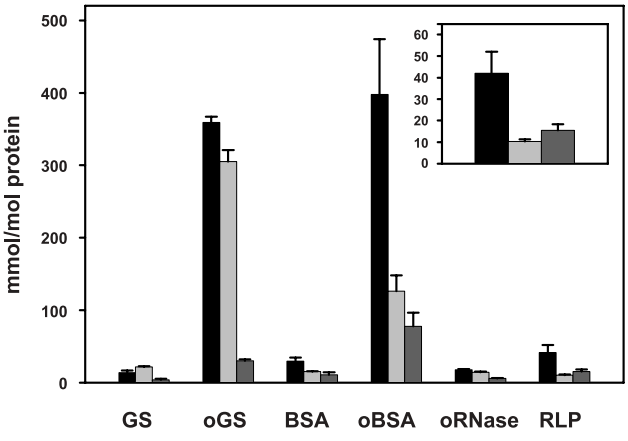

Fig. 2. Contribution of glutamic and aminoadipic semialdehydes to the total protein carbonyl value in different protein samples. Protein carbonyls (black bars); glutamic semialdehyde (light grey bars) and aminoadipic semialdehyde (dark grey bars) in glutamine synthetase (*GS*), oxidized glutamine synthetase (*oGS*), bovine serum albumin (*BSA*), oxidized bovine serum albumin (*oBSA*), oxidized RNase (*oRNase*) and rat liver protein (*RLP*). Oxidized samples were prepared by treatment with 100 μM Fe^{3+}/25 mM ascorbate overnight at 37 °C. Results similar to those seen for oGS and oRNase were obtained for other model proteins (data not shown). Analyses were performed as described (Requena et al., 2001). Results are means of three experiments with S.D. Inset: expanded view of results for rat liver protein

there is some variability from protein to protein, glutamic semialdehyde, and to a lesser extent, aminoadipic semialdehyde, are quantitatively very important carbonyl products in MCO systems, accounting in some cases for the totality of the protein carbonyl value. In fact, the combined value of glutamic and aminoadipic semialdehydes was higher in some cases for native protein samples. This probably reflects an underestimation of the carbonyl value at these very low levels. Bovine serum albumin showed a peculiar behavior, in that only ~55% of its carbonyl value is made up by glutamic and aminoadipic semialdehydes, and that aminoadipic semialdehyde was almost as abundant as glutamic semialdehyde.

Glutamic and aminoadipic semialdehydes in biological samples

Analysis of glutamic and aminoadipic semialdehydes in rat liver extracts shows that they are present at roughly similar levels and account for 60% of the total carbonyl value (Fig. 2), which validates their being quantitatively very important carbonyl products in tissue proteins. This result also establishes that measurements of protein carbonyl by carbonyl-specific reagents such as 2,4-dinitrophenylhydrazine are valid, as emphasized by Sohal and colleagues (Dubey et al., 2002); it is evident that, as a minimum, 60% of the carbonyl value obtained in the measurements described here represents specific chemical structures; since other carbonyl compounds are likely to be generated by a variety of additional chemical pathways, as has already been discussed, it is obvious that the obtained carbonyl value is an accurate measurement of carbonyl containing chemical structures.

We have initiated measurement of glutamic and aminoadipic semialdehydes in a variety of tissues and other biological samples. In mouse liver, kidney, lung and brain protein, glutamic semialdehyde is present at 9–12 mmol/mol protein, with little variation from tissue to tissue. Glutamic semialdehyde was also measured in cultured human fibroblasts and HeLa cells, at 4–5 mmol/mol protein. Application of oxidative stress to HeLa cells, in the form of a H_2O_2 generating system (glucose oxidase/glucose) resulted in an increase in glutamic semialdehyde (Fig. 3). Increased levels of glutamic and aminoadipic semialdehydes have also been found *in vivo* in a situation which is also hypothesized to be associated with increased oxidative stress: proteins from peritoneal fluid obtained from patients during a session of peritoneal dialysis, contains higher levels of these compounds than plasma proteins (Ruiz et al., in press). This finding can be interpreted as the consequence of increased oxidative stress produced by glycoxidation of peritoneal proteins by the high glucose concentration present in dialysis fluid.

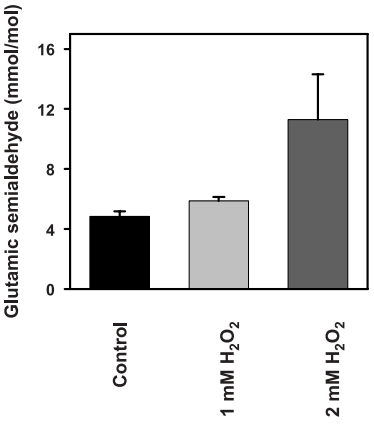

Fig. 3. Increase of glutamic semialdehyde levels in HeLa cells subjected to oxidative stress. Cells were grown to confluency and treated with glucose oxidase/glucose to generate the concentrations of H_2O_2 indicated. Results are expressed considering an average MW of 50 kDa for cell proteins, and represent means of three independent protein samples from a single culture, with S.D. Aminoadipic semialdehyde levels were below the detection limit of the technique for the small sample size available. (From: Requena et al., 2001)

Even though glutamic and aminoadipic semialdehydes are, as has been discussed, quantitatively important carbonyl products in tissue proteins, they do not make up the totality of the carbonyl value in a given sample. This explains cases in which increases in carbonyl values are not accompanied by similar increases in the concentration of these products. As an example, when peptide methionine reductase deficient ($MsrA^{-/-}$) mice, a genetically

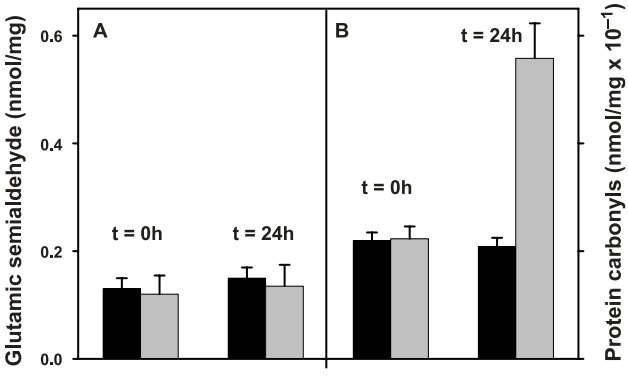

Fig. 4. Glutamic semialdehyde and protein carbonyls in liver protein from $MsrA^{-/-}$ and control mice exposed to oxygen. **A** Glutamic semialdehyde and **B** Protein carbonyls in liver protein extracts from peptide methionine sulfoxide reductase deficient ($MsrA^{-/-}$, grey bars) and control (black bars) mice. Mice were killed after 0 and 24 hours of exposure to 100% oxygen. Results are means of 3 (glutamic semialdehyde) and 5 (protein carbonyls) experiments with S.D.

modified mouse model characterized by increased susceptibility to oxidative stress, were placed in an oxygen atmosphere, there were increases in levels of protein carbonyls, but not of glutamic semialdehyde (Fig. 4), in different tissues (Moskovitz et al., 2001). Unchanged levels of glutamic semialdehyde were seen in kidney, liver and lung protein from control mice subjected to a similar oxygen exposure regimen; these results contrast with those reported by Ayala and Cutler (Ayala and Cutler, 1996b), who detected an increase in levels of glutamic semialdehyde in liver protein from mice exposed to 100% oxygen for 24 and 48 hours.

Glutamic semialdehyde and oxidation of low density lipoproteins (LDL)

Oxidation of LDL is thought to play an essential role in atherogenesis. LDL oxidized *in vitro* by a variety of methods is recognized and taken up by macrophages via scavenger receptors. Lipid laden macrophages derive to foam cells in arterial subendothelial space, one of the key initial events of atherogenesis. While LDL isolated from atheromatous tissue is clearly oxidized, there is not yet agreement on what is the basic oxidative mechanism, and several (myeloperoxidase, lipoxigenases, metal catalyzed oxidation, glycoxidation) have been invoked. The nature of the key oxidation-derived epitopes recognized by scavenger receptors is also controversial. In the meantime, metal catalyzed oxidation has been used as a meaningful model of the process (Esterbauer et al., 1992). In this context, analyses glutamic semialdehyde in LDL are of particular interest. Pietzsch has shown that iron-catalyzed oxidation of LDL *in vitro* results in generation of glutamic semialdehyde, whose levels increased from 0.012 to 16.55 mol/mol protein after 40 hours (Pietzsch, 2000) Such increment represents 65% of the combined losses in proline and arginine measured in the same samples. Accumulation of glutamic semialdehyde and protein carbonyls correlate tightly and show similar kinetics. The final value of protein carbonyls in oxidized LDL was $\sim$6.5 mol/mol protein, also lower than the estimated value of glutamic semialdehyde, but with a discrepancy between these figures higher than that seen in our studies with model proteins. Pietzsch suggests that the loss of positive charge as a consequence of arginine oxidation could contribute to the observed electronegativity of LDL subjected to MCO. Glutamic semialdehyde was measured, in subsequent studies, in circulating LDL from healthy volunteers and patients suffering Familial Defective apoB (FDB), a genetic condition which results in decreased turnover of circulating LDL (Pietzsch et al., 2000). Levels of glutamic semialdehyde were markedly higher in LDL-2, a subfraction of smaller, denser LDL particles, from patients with FDB (374 *vs.* 13 mol/mol protein). With lower content of protective α-tocopherol, LDL-2 would be expected to be more susceptible to oxidation than LDL-1; in fact, no differences in the concentration of glutamic semialdehyde in LDL-1 were detected between groups. A higher yield of glutamic semialdehyde was also obtained by MCO of LDL-1 as compared to LDL-2 *in vitro* (Pietzsch and Julius, 2001). These studies suggest that MCO may be an important mechanism of LDL oxidation *in vivo*. Analysis of glutamic and aminoadipic (LDL apo-B contains 356 lysine residues, more than proline and arginine together) semialdehydes in LDL isolated from atherosclerotic tissue will be of great interest.

Conclusions and future directions

Glutamic and aminoadipic semialdehydes are quantitatively important products of MCO, and make up a sizeable portion of the total protein carbonyl value in biological samples. Obviously, identification of additional individual protein carbonyls will be of great interest. For that purpose, the study of oxidized BSA appears as a very relevant model. Measurement of these compounds affords specific information on the oxidative status of proteins, particularly relevant given the relatively high levels at which these products are found. A number of biological systems appear as attractive candidates for the quantitation of these compounds: tissues from patients suffering from diabetes, Alzheimer's disease and atherosclerosis; cultured fibroblasts from donors of different ages and patients suffering progeria and Werner syndrome, and tissue from animal species of different longevities. In all these instances, the use of negative chemical ionization will allow the analysis of very small samples, while the specific nature of the analytical methods should allow analysis of samples in which interference from absorbing impurities might limit the use of the carbonyl assay. As an example, adaptation of the methods to the analysis of glutamic and aminoadipic semialdehydes in urine, in course, should be cited. Quantitation of glutamic and aminoadipic semialdehydes should become a widespread tool to complement measurement of protein carbonyls, as the two techniques, one more specific, the other more comprehensive, yield information that can be integrated to better characterize protein oxidation.

Acknowledgments

We would like to thank Drs. Lin Tsai and Jakob Moskovitz, from the Laboratory of Biochemistry, NHLBI, NIH, USA.

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