γ-Glutamyl semialdehyde and 2-amino-adipic semialdehyde: biomarkers of oxidative damage to proteins

B. Daneshvar, H. Frandsen, H. Autrup and L. O. Dragsted

Reactive oxygen species are formed in the body by several natural processes and by induced oxidative stress. The reactive oxygen species may react with the various biomolecules of the body, including proteins. In order to assess the impact of oxidative damage to proteins, we have tried to identify oxidized amino acids in blood proteins which might serve as biomarkers of oxidative damage. When oxidative damage is induced into bovine serum albumin by metal-catalysed oxidation systems, the aldehyde groups formed can be derivatized by fluoresceinamine ($FINH_2$). Following acid hydrolysis of $FINH_2$-derivatized protein, two major oxidation products, γ-glutamyl semialdehyde (GGS) and 2-amino-adipic semialdehyde (AAS), were found and identified by HPLC and MS. Isolation and identification of oxidized amino acids from homopolymers (poly-Arg, -Pro, -Lys, -Trp or -Leu) confirmed that GGS can originate from Arg or Pro, while AAS is an oxidation product of Lys. When oxidative stress was induced in rats by treatments with *t*-butyl hydroperoxide or acrolein, rat plasma protein levels of GGS and AAS were found to be significantly higher compared with control rats. The AAS-content in serum albumin or in total plasma proteins collected from eight different mammalian species was found to be inversely proportional to their maximum lifespan potential. The content of AAS in plasma proteins of untreated adult rats showed a positive correlation with the age of the rat. In young rats a negative correlation with age was found for both GGS and AAS. We conclude that GGS or AAS may be useful novel biomarkers of oxidative damage to proteins *in vivo*.

Keywords: *protein oxidation, carbonyl induction, amino acid.*

Abbreviations: *AAS, 2-amino-adipic semialdehyde; BSA, bovine serum albumin; DNPH, 2,4-dinitrophenylhydrazine; EDTA, ethylenediaminetetraacetic acid; $FINH_2$, fluoresceinamine isomer II (6 aminofluorescein); GGS, γ-glutamyl semialdehyde; HPLC, high performance liquid chromatography; HRPO, horseradish peroxidase; MCO, metal-catalysed oxidation; MES, 4-morpholinoethanesulphonic acid; MLSP, maximum lifespan potential; MS, mass spectrometry; SD, standard deviation; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid.*

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Introduction

Introduction of carbonyl groups into amino acid residues of proteins is a hallmark for oxidative modification (Climent *et al.* 1989). Metal-catalysed oxidation (MCO) has been identified as a posttranslational modification of proteins which may be important in several physiological and pathological processes. These include the ageing process, intracellular protein turnover, arthritis, and pulmonary diseases (Levine *et al.* 1990). The mechanism of metal-catalysed oxidation of proteins has recently been reviewed (Stadtman 1993). In brief, protein oxidation is a site-specific process involving the oxidation of Fe^{2+} by oxygen to generate H_2O_2 followed by interaction of H_2O_2 and Fe^{2+} at metal-binding sites on the protein to generate an activated oxygen species which may attack amino acid residues locally. The increase in oxidation of proteins in the presence of EDTA is believed to be due to direct binding of the EDTA–Fe^{2+} complex to the protein (Stadtman 1990).

Oxidative modifications of more than 40 proteins and enzymes have been studied in detail (Amici *et al.* 1989, Levine *et al.* 1990, Miura *et al.* 1992, Stadtman 1993) and the oxidation products of some amino acids have been identified (Stadtman 1993). Most of these studies have assessed the total carbonyl content in purified proteins which were oxidatively modified *in vitro* (Amici *et al.* 1989). Reactive protein carbonyls in crude tissue extracts cannot be reliably measured by these methods, partly because of interference by nucleic acids, and partly because of unspecific binding of the carbonyl-reactive dyes to the protein (Climent *et al.* 1989).

An increase in the amount of oxidatively modified protein with age has been observed. This increase is a result of either a higher rate of protein oxidation, or a lower rate of degradation of oxidized protein, or both (Stadtman *et al.* 1993). We have tried to develop marker(s) for oxidatively damaged proteins by isolation and identification of damaged amino acids in their hydrolysates by HPLC and MS. The identified amino acids were subsequently used as markers to measure *in vivo* induction of oxidative stress by chemicals in rats. We have further investigated whether maximum lifespan potential of mammalian species, or age, were correlated with damaged amino acids in serum albumin.

We have selected albumin since it is easily accessible and abundant, and may be used for assessing relatively recent exposures because of its half-life in the human circulation of 20–25 days (World Health Organization 1993).

MATERIALS AND METHODS

Chemicals

All chemicals were used as supplied, without further purification. Plasma samples and all serum albumins from mammalian species, amino acid homopolymers, horseradish peroxidase, hypoxanthine and xanthine oxidase were obtained from Sigma Chemical Co., Saint Louis, MO, USA; fluoresceinamine (isomer II), sodium cyanoborohydride, 4-morpholinoethane sulphonic acid (MES) and sodium dodecyl sulphate (SDS) were from Aldrich Chemical Co. Steinheim, Germany. Free fluoresceinamine (MW = 347.33), dissolved in 0.1 N NaOH, can be quantified from maximum absorbance at 490 nm, while decarboxylated fluoresceinamine

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($C_{19}H_{13}O_3N$) (MW = 303), dissolved in 6 N HCl, has maximum absorbance at 454 nm. The decarboxylation product of fluoresceinamine was formed quantitatively from free fluoresceinamine by constant boiling of 6 N HCl at 110° C for 24 h. A standard curve for decarboxylated fluoresceinamine from the maximum absorbance at 454 nm was used for quantitation of the derivatized amino acids. Mass spectral analysis confirmed that derivatized amino acids are conjugated with decarboxylated fluoresceinamine. Hydrogen peroxide was from Bei & Berntsen A/S, Rødovre, Denmark; acetonitrile from Rathburn Chemicals Limited, Rathburn, UK; ascorbic acid, formic acid, hydrochloric acid, magnesium chloride, potassium chloride, potassium hydroxide, sodium hydroxide, sodium hypochlorite and ethylenediamine tetraacetic acid (EDTA) were from Merck, Darmstadt, Germany and acrolein, *t*-butyl hydroperoxide, iron(III) chloride and iron(II) sulphate from Riedel-de Haen, Seelze, Germany. Sephadex G-25 (PD-10)^R columns were from Pharmacia LKB Biotechnology, Uppsala, Sweden.

Animals

Male Wistar rats (obtained from Møllegård, Breeding Centre, Køge, Denmark) weighing 150–200 g were used for the *in vivo* study. They were caged individually and had environmentally controlled rooms with an alternating dark (0600–1800h) – light (1800–0600h) cycle and an ambient temperature of 22–25°C. They were given water and food *ad libitum*.

Oxidation of amino acid homopolymers and bovine serum albumin

BSA and the amino acid homopolymer (poly-Arg, -Pro, -Lys, -Trp, or -Leu) were oxidized with different MCO systems (ascorbate/oxygen/iron, AOI systems) as described by Amici *et al.* (1989). Briefly, 20 mg sample dissolved in 2 ml of 50 mM phosphate buffer (pH 7), was mixed with EDTA (2 mM), ascorbic acid (25 mM) and Fe^{3+} (2 mM). Besides the AOI systems, other oxidation systems such as the peroxidase/H_2O_2 system (Nomura *et al.* 1990), aqueous hypochlorite and H_2O_2 (Khan and Kasha 1994) or hypoxanthine and xanthine oxidase (Miura *et al.* 1992) were also used.

All the oxidation reactions were stopped by separating the proteins from low molecular weight molecules by gel filtration. The mixture (2.5 ml) was loaded onto a PD-10 column, which had been equilibrated with 0.25 M MES, pH 6.0, containing with 1% SDS (MES–SDS), and then eluted with the same buffer according to the manufacturer's instructions; i.e. the first 2.5 ml of the effluent were discarded and the next 3.5 ml were collected.

Protein carbonyl group derivatization with $FlNH_2$

The collected MES–SDS solution was heated on a waterbath at 100°C for 1 min. One hundred µl of the solution was conveyed to 10-ml glass vials and 12.8 µl of 0.25 M $FlNH_2$, dissolved in 0.52 M NaOH and 10 µl of freshly prepared 0.4 M $NaCNBH_3$ in 0.25 M MES buffer, pH 6.0, were added. The volume was then adjusted to 160 µl by addition of MES buffer. Unless otherwise stated, the reaction mixture thus contained 20 mM $FlNH_2$ and 25 mM $NaCNBH_3$. The mixture was incubated at 37 °C for 1 h (Climent *et al.* 1989). The volume was adjusted to 2.5 ml by addition of MES–SDS buffer and excess $FlNH_2$ was removed by gel filtration, as described above. The protein was precipitated by the addition of 1 ml 70% TCA, and centrifuged for 5 min at 5000 *g*. The precipitate was dissolved in 1 ml 0.1 M NaOH by incubation for 15 min at 37 °C. Any insoluble material was pelleted by centrifugation for 3 min (11000 *g*). The UV-spectrum of each sample was determined on a Shimadzu UV-160 Spectrophotometer. The carbonyl content was calculated from the maximum absorbance (490 nm) using an ε_M for $FlNH_2$ in 0.1 M NaOH of 86800 M^{-1} cm^{-1} (Climent *et al.* 1989).

PD-10 columns were cleaned by treatment with 3 × 4 ml distilled water, followed by 3 × 4 ml of 20% acetonitrile and finally 3 × 4 ml distilled water and reused one or two times.

Protein determination

The Pierce BCA Protein Assay Reagent ($A_{562\,nm}$) was applied for the spectrophotometric determination of protein concentration, according to the instructions of the manufacturer, using BSA as reference protein.

Acid hydrolysis, HPLC, and mass spectrometric analysis of the $FlNH_2$-carbonyl protein derivative

Native and oxidized BSA and oxidized amino acid homopolymers derivatized with $FlNH_2$ were hydrolysed with 6 N HCl at 110 °C for 24 h. Aliquots of 0.5 ml of samples were purged with argon before heating. The protein hydrolysates in 6 N HCl were filtered through 0.45 µm filters (Hewlett Packard) and analysed directly by HPLC, on a model 1090 liquid chromatograph equipped with a Hewlett-Packard Model 1040A diode array detector. The sample (50 µl) was injected onto a Purospher RP-18 (5 µm) column (Hewlett Packard, Waldbromn, Germany) equilibrated with 2 mM formic acid, pH 3.2, at 1 ml min^{-1}. A linear gradient from 0 to 50% acetonitrile in 2 mM formic acid was developed up to 12.5 min.

The eluent was monitored at both 454 nm (bandwidth 5 nm) and 275 nm (bandwidth 5 nm).

Fractions (15 s) were collected for further analysis as required. Mass spectra were recorded on a Kratos profile mass spectrometer (Manchester, UK), equipped with an electrospray interface.

Stability and reproducibility

Three male Wistar rats (150–200 g; 4 months old) were anaesthetized with carbon dioxide + oxygen (95%+5%) and blood was drawn from the dorsal aorta of each rat. At different time-intervals (5 min, 30 min, 1, 4, and 24 h), aliquots of 1 ml heparinized blood were separated into plasma, white, and red cells by centrifugation on Ficoll Paque^R (Research Grade; Pharamacia Biotech, Uppsala, Sweden) and the samples were stored at –20 °C. The stability of oxidized amino acids or further oxidation of protein during the storage of whole blood was tested by HPLC analysis of $FlNH_2$-derivatized plasma and haemoglobin as described above.

MES–SDS solutions of native or oxidized BSA were stored at 5 °C or room temperature for 7 days and aliquots of 0.1 ml (at 1-day intervals) were derivatized by $FlNH_2$. Oxidized amino acids were determined by HPLC analysis, as described above. Half of these samples were purged with argon before the hydrolysis.

In order to assess reproducibility, aliquots of 0.1 ml of frozen (–18 °C) rabbit plasma were taken at 2-day intervals for 2 months, and the content of oxidized amino acids was measured as described above.

Quantitation of GGS and AAS in plasma proteins of rats dosed with t-butyl hydroperoxide and acrolein

Male Wistar rats (150–200 g) were divided into three groups (a, b and c) each with three animals. Control rats (group c) were given the vehicle (normal saline), group b rats were given *t*-butyl hydroperoxide (1.3 mmol kg^{-1}) subcutaneously (Bauman *et al.* 1991), and group a rats were given acrolein (13 mg kg^{-1}) in normal saline by gavage (Thakore *et al.* 1992). After 4 h they were anaesthetized using carbon dioxide + oxygen (95% + 5%) and blood was drawn from the dorsal aorta of each rat. Blood was separated into plasma, white and red cells by centrifugation on Ficoll Paque^R. Oxidized amino acids in plasma hydrolysate were measured by $FlNH_2$ methods as described above.

Results

In vitro *studies*

Ability of different MCO systems to oxidize BSA

Figure 1 shows the carbonyl content of BSA, oxidized with the various MCO systems. An MCO system consisting of Fe(III),

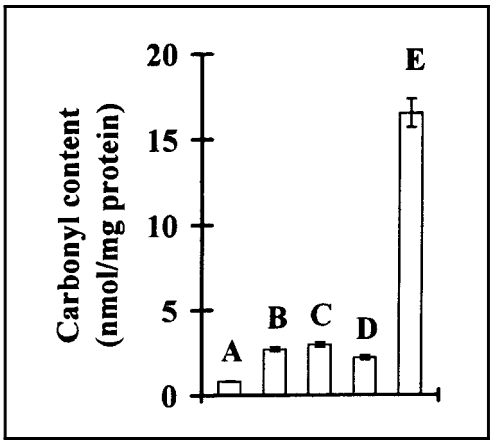

Figure 1. Carbonyl formation in BSA induced by different MCO systems. BSA was exposed to the MCO systems for 60 min at 37 °C and carbonyl formation measured by $FlNH_2$-derivatization. (A) Native BSA; (B) native BSA + Fe(III); (C) native BSA + Fe(III) + EDTA; (D) native BSA + Fe (III) + ascorbic acid; (E) native BSA + Fe(III) + EDTA + ascorbic acid. The values are mean ± SD for $n = 3$.

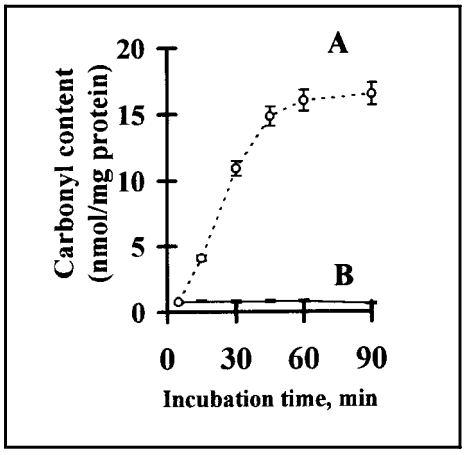

Figure 2. Carbonyl content in BSA as function of oxidation time BSA was exposed to the MCO system containing Fe(III), EDTA, ascorbate and oxygen for up to 90 min at 37 °C. Carbonyl content was determined by the $FlNH_2$-derivatization method. (A) BSA + MCO; (B) control BSA. The values are mean ± SD for $n = 3$.

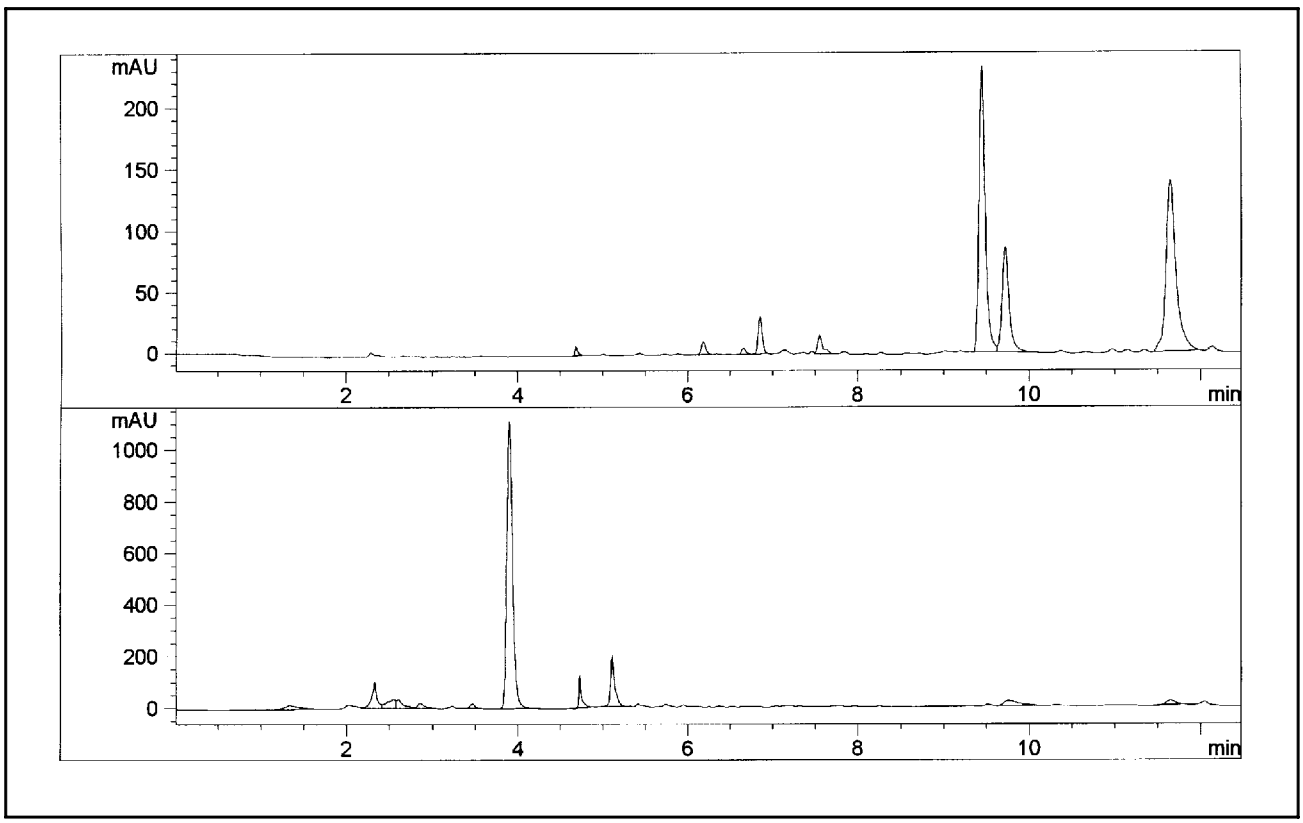

Figure 3. HPLC chromatogram of $FlNH_2$-derivatized BSA after hydrolysis with 6 N HCl at 110 °C for 24 h. The upper tracing is the chromatogram at 454 nm, tracking the fluorescein-containing products. The peaks at 9.4 min and 9.7 min are the decarboxylated fluoresceinamine derivatives of GGS and AAS, respectively. The peak at 11.6 min is decarboxylated fluoresceinamine. This peak was also observed when free $FlNH_2$ was subjected to the HCl treatment used for peptide-bond hydrolysis. The lower tracing is at 275 nm. The peak at 3.8 min is tyrosine.

EDTA, ascorbate and oxygen was more effective than those in which iron, EDTA or ascorbate was omitted. The kinetics of carbonyl group formation showed a linear increase for at least 45 min. The reaction was essentially completed after 1 h (Figure 2).

$FlNH_2$-derivatives of γ-glutamyl semialdehyde and 2-amino-adipic semialdehyde

HPLC analysis of Fe^{3+}/EDTA/ascorbate oxidized BSA, derivatized with $FlNH_2$ and hydrolysed with HCl showed three major peaks at 454 nm in the chromatogram (Figure 3

upper). These peaks were also observed in hydrolysates from BSA treated *in vitro* with other oxidation systems and in derivatized native BSA. The peaks were collected separately. The first and second peaks, at 9.4 min and 9.7 min, respectively, were not seen if cyanoborohydride was omitted from the reaction mixture, indicating these compounds were not artifactual products formed during acid hydrolysis of oxidized BSA and $FlNH_2$. The minor peaks eluting at 6–8 min were only observed in the Fe^{3+}/EDTA/ascorbate oxidized BSA and not *in vivo*.

$FlNH_2$ labelling of several oxidized amino acid homopolymers showed that the first peak can originate from proline and arginine residues, whereas the second peak originated from lysine residues. Mass spectral analysis showed molecular ions m/z of 419 $[M+H]^+$ and 433 $[M+H]^+$, which is 115 and 129 mass units greater than that of decarboxylated $FlNH_2$, indicating that proline and arginine are oxidized to γ-glutamyl semialdehyde (GGS) and lysine is oxidized to 2-amino-adipic semialdehyde (AAS).

The third peak, at 11.6 min, was also observed when free $FlNH_2$ was subjected to the HCl treatment used for peptide-bond hydrolysis. Mass spectral analysis suggested that this compound was decarboxylated $FlNH_2$ ($[M+H]^+ = 304$). The decarboxylation product of $FlNH_2$ was collected, evaporated, weighed and redissolved in 6 N HCl for quantitation by HPLC analysis. A sensitivity of 6 pmol was found. The decarboxylated fluoresceinamine derivative of GGS or AAS content was calculated from the maximum absorbance (454 nm) using: pmol compound = $A_{454}/0.454$, where A_{454} is the area under the peak recorded with the diode array detector.

The peak at 3.8 min at 275 nm (Figure 3, lower) was determined as tyrosine.

Effect of concentration of $FlNH_2$ and protein

The results in Figure 4 show the effect of $FlNH_2$ concentration on conjugation of oxidized amino acids, GGS and AAS. We found that 100 μmol $FlNH_2$ would be sufficient to react with all oxidized amino acids in 16 mg of protein.

The effect of varying the amount of oxidized BSA (1–16 mg) relative to $FlNH_2$ was also tested by measurement of GGS and AAS and a linear relationship was found (not shown). This is in agreement with Climent *et al.* (1989).

Stability and reproducibility

The storage of whole blood from rats at room temperature for up to 24 h has no influence on the GGS or AAS content in plasma and haemoglobin, indicating that these oxidized amino acids are stable and no further oxidation or decomposition happened during the storage.

The levels of GGS and AAS showed no differences between protein samples which had been hydrolysed for 24 h and 48 h. In addition, the GGS or AAS content (100 pmol ml^{-1} HCl, 6 M) in our standard solutions, collected by HPLC analysis of oxidized BSA, was unaffected by the storage at +5 °C for 2 months. There was no difference either in GGS or in AAS contents in oxidized BSA (417 ± 8 pmol mg^{-1} protein) between samples that were not purged with argon and samples which had been purged with argon immediately after oxidation ($n = 18$), indicating that no

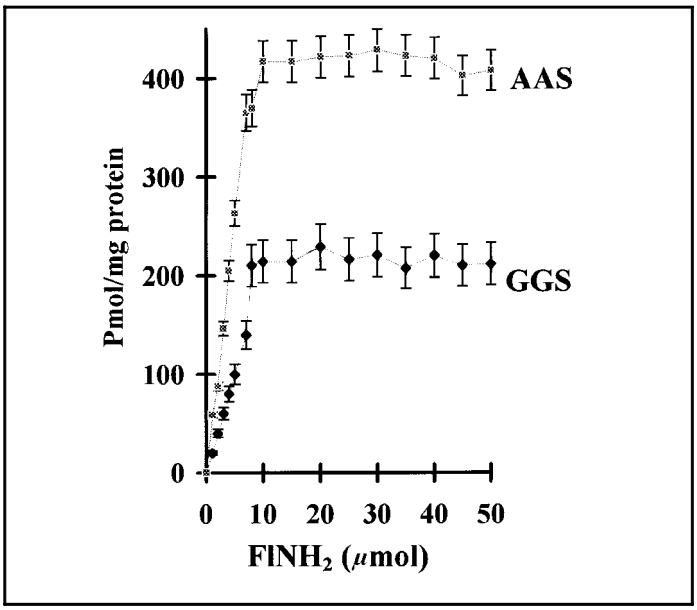

Figure 4. Effect of $FlNH_2$ concentration on labelling of GGS and AAS. Derivatization of 16 mg BSA was carried out in a total volume of 0.8 ml with up to 50 μmol $FlNH_2$. The decarboxylated fluoresceinamine derivative of GGS and AAS are measured in the hydrolysate as described under Materials and Methods. The values are mean ± SD for $n = 3$.

further oxidation took place during storage and acid hydrolysis. The storage temperature had no effect on the GGS or AAS content. The measurements of AAS in rabbit plasma samples which had been stored for 2 months at –18 °C showed no change in AAS content during the 2 months. The mean value was 140 pmol AAS mg^{-1} protein ± 10% ($n = 40$).

Use of tyrosine for determination of protein concentration in protein hydrolysate

Protein concentrations of native and oxidized BSA solutions were determined by the Pierce BCA Protein Assay Reagent. Aliquots of 0.2 ml of the samples were then hydrolysed with 6 N HCl at 110 °C for 24 h, and 50 μl was analysed by HPLC. The L-tyrosine, librated by the hydrolysis, was eluted at 3.8 min (275 nm).

L-Tyrosine was stable during the acid hydrolysis procedure since recovery ($n = 6$) was found to be 98 ± 1%, independently of the tyrosine concentration (1–5 mg ml^{-1}). No other aromatic amino acid had a similar retention time in this system. Figure 5(A) shows a standard curve for absorbance of L-tyrosine at 275 nm obtained by HPLC analysis of L-tyrosine dissolved in 6 M HCl. Figure 5(B) shows that the amount of tyrosine determined by HPLC correlated with the protein concentration, measured by the Pierce assay. A similar correlation curve was obtained with total bovine plasma proteins. We found that the plasma protein concentration can be determined by measurement of the L-tyrosine concentration in their hydrolysates. This serves as a useful control of the protein hydrolysis procedure.

Reactive Oxygen Species generating systems

Table 1 shows that the oxidation products, also present in native BSA, are increased by all oxidation systems. The ratio

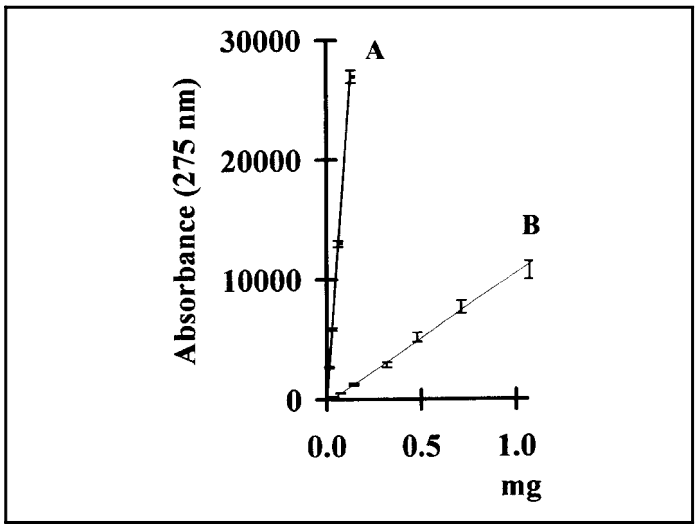

Figure 5. Standard curves for absorbance of L-tyrosine (A) and BSA hydrolysate (B) at 275 nm obtained by HPLC analysis. The equations for the fitted regression lines are $A_{(275)\ tyrosine} = 213867 \times (mg)$ with $r = 0.995$ (A) and $A_{(275)\ BSA\ hydrolysate} = 10792 \times (mg) - 268$ with $r = 0.98$ (B). The values are mean ± SD for $n = 3$.

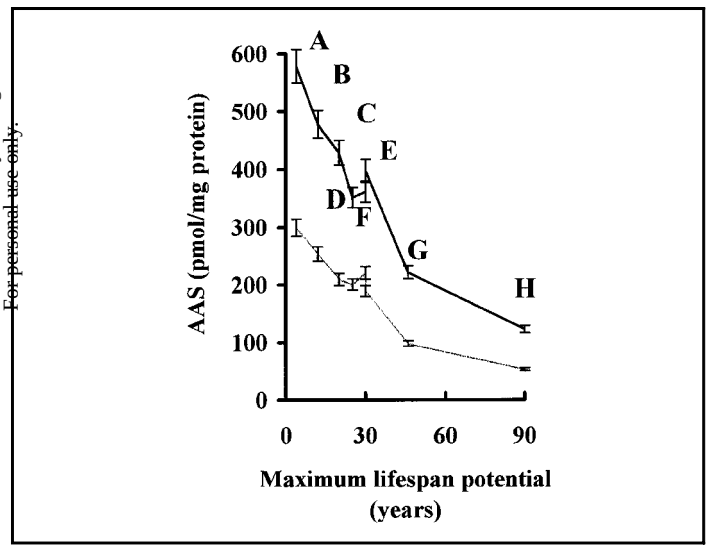

Figure 6. 2-Amino-adipic semialdehyde (AAS) in serum albumins (upper curve) and total plasma proteins (lower curve) of eight mammalian species were derivatized by $FlNH_2$ and measured, as described under Materials and Methods. (A) Rat; (B) rabbit; (C) dog; (D) goat; (E) cow; (F) pig; (G) horse; (H) human. The values are mean ± SD for $n = 3$.

GGS/AAS spans a factor of 9 between these systems. The measurement of carbonyl content of native- and MCO-oxidized BSA showed that the sum of GGS and AAS contents is about 75% of the total aldehyde groups in intact BSA.

In vivo *studies*

Determination of 2-amino-adipic semialdehyde in mammalian species

Serum albumins and total plasma proteins of eight mammalian species were derivatized by $FlNH_2$ and hydrolysed, as described above. The results of measured AAS in serum albumin and total plasma proteins in relation to maximum lifespan potential (MLSP) of each species are shown in Figure 6. The highest and lowest levels of AAS are found for rat and human, respectively. The GGS levels did not show any correlation with MLSP of the mammalian species (data not shown).

Treatment	Total aldehyde (pmol mg^{-1} protein)	GGS (pmol mg^{-1} protein)	AAS (pmol mg^{-1} protein)	GGS/AAS
Native	800 ± 100[a]	214 ± 5	417 ± 8	0.51
Peroxidase + H_2O_2	n.d.	564 ± 11	1665 ± 35	0.34
Fe(III) + EDTA + ascorbate	16500 ± 800	8315 ± 78	2765 ± 43	3.00
NaOCl + H_2O_2	n.d.	588 ± 10	705 ± 13	0.83
Xanthine oxidase + hypoxanthine	n.d.	599 ± 10	918 ± 18	0.65

Table 1. Oxidation of amino acid residues in bovine serum albumin.

[a] The results are expressed as mean ± SD, $n = 3$.

n.d. not detected.

Oxidative stress induced by *t*-butyl hydroperoxide and acrolein in rats

The GGS and AAS content in rat plasma proteins 4 h after *in vivo* treatment with *t*-butyl hydroperoxide, 1.3 mmol kg^{-1}, subcutaneously (group b) or with acrolein, 13 mg kg^{-1}, in normal saline by gavage (group a) is presented in Figure 7. The amounts of GGS or AAS (pmol mg^{-1} protein) increased significantly ($p < 0.05$) by treatment with both chemicals, compared with the control values (group c).

Age-related GGS and AAS in total plasma proteins in rats

Plasma proteins from rats of various ages were analysed for content of GGS and AAS. A positive correlation ($y = 0.31x + 150$, $r = 0.88$) between the amounts of AAS in plasma proteins and age above 3 months was found (Figure 8, upper curves on A and B). Newborns, however, were found to have a much higher level, 460–600 pmol mg^{-1} protein, which declined with an apparent half-life of about 7 days until the adult level is reached after 30 days. The GGS levels (Figure 8, lower curves on A and B) also decrease in the first 30 days, but they increase only marginally during the remaining life ($y = 0.04x + 40$, $r = 0.83$).

Discussion

Carbonyl functions in proteins may be detected by formation of 2,4-dinitrophenylhydrazone (DNPH) derivatives (Levine 1984, Oliver *et al.* 1987, Levine *et al.* 1990), by formation of fluorescent hydrazones or thiosemicarbazone derivatives (Ahn *et al.* 1987), by formation of an alcohol through reduction with tritiated borohydride (Levine *et al.* 1990, Amici *et al.* 1989, Lenz *et al.* 1989), or by derivatization with $FlNH_2$ followed by reduction with cyanoborohydride (Climent *et al.* 1989).

Both DNPH (data not shown) and $FlNH_2$ were used in this study to measure total carbonyl content in native- and oxidized BSA, but DNPH derivatives are not stable during acid

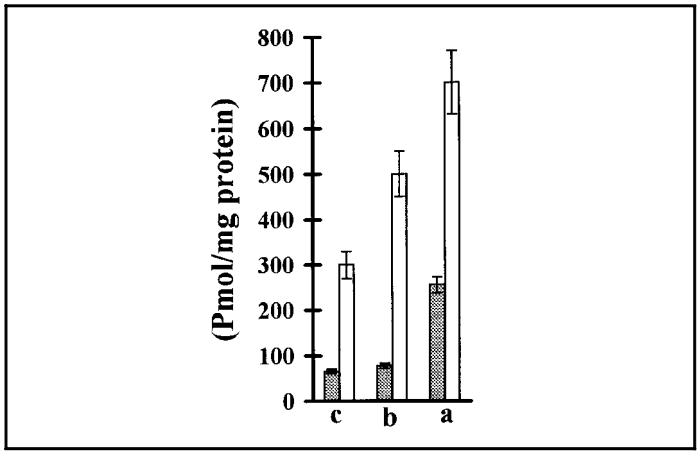

Figure 7. Rat plasma protein GGS (dotted) and AAS (white) measured by $FlNH_2$-derivatization. Control rats (c); rats treated with *t*-butyl hydroperoxide (1.3 mmol kg^{-1}) (b); rats treated with acrolein (13 mg kg^{-1}) (a). The values are mean ± SEM for $n = 3$ rats. The groups a and b are significantly different ($p < 0.05$) from control rats (group c).

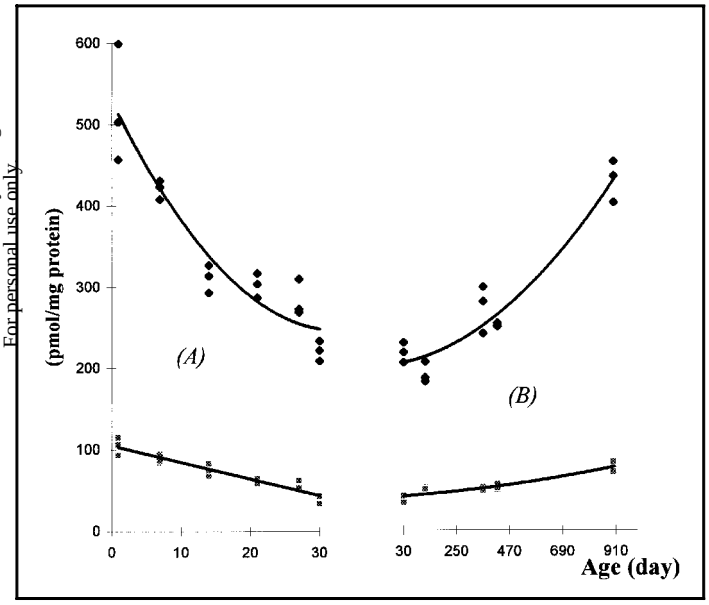

Figure 8. The AAS (upper curves) and GGS (lower curves) in total plasma proteins from rats in different age-groups. The results for rats of age 1 up to 30 days are shown in the curves (A), while curves (B) shows rats of age 30–900 days. The values are observations on the individual rats.

hydrolysis of the protein, while the results presented here demonstrate that $FlNH_2$-derivatization followed by cyanoborohydride reduction generates a stable carbonyl derivative, thereby producing a conjugation product that can be detected with a sensitivity of about 6 pmol carbonyl. A comparison of total aldehyde content in native- and oxidized BSA (measured with $FlNH_2$-derivatization) showed that specific amino acids derivatives account for only about 75% of the measured aldehyde groups in intact BSA, indicating that unspecific or unstable binding of the reagent to protein makes possibly an important part of this difference.

We have identified two major aldehydic oxidation products in serum proteins and compared four different *in vitro* oxidation systems (hydroxyl radical-, superoxide anion-, hypochlorite ion- and Fenton-type systems) with respect to their ability to oxidize amino acid residues in BSA to GGS or AAS. An MCO system based on Fe(III), EDTA, ascorbate and oxygen induces these products more efficiently than the other systems under the conditions employed. Comparison of the relative amounts of GGS and AAS formed indicate that the susceptibility for oxidation of Pro, Arg, and Lys residues varies with the different oxidation systems. Further studies may elucidate their individual sensitivity to oxidation by the different reactive oxygen species.

Both *t*-butyl hydroperoxide and acrolein have been shown to be toxic *in vivo* (Bauman *et al.* 1991, Thakore *et al.* 1992). The former is known as an inducer of oxidative stress that directly produces ROS, whereas the latter is a toxic metabolite of several xenobiotics that probably induces oxidative stress as a consequence of its ability to produce adducts with proteins, glutathione and other cellular constituents. Our results indicate that the GGS and AAS content of plasma proteins can be increased by exposures to chemicals which induce oxidative stress *in vivo*. In addition, the result confirmed also that induction of GGS or AAS varies with the nature of inducer. Urate has been shown to be a strong antioxidant and singlet oxygen scavenger and to protect biological membranes from lipid peroxidation reactions at physiological concentrations *in vitro* (Cutler 1984). In his study on urate and ascorbate, a positive correlation between maximum lifespan potential (MLSP) in mammalian species and the concentration of urate per specific metabolic rate (SMR) was observed (Cutler 1984). Our studies on oxidation products in serum albumins and total plasma proteins of eight mammalian species including rats indicate that the AAS content can be used as a marker of oxidative modification of proteins, with a background level related to the maximum lifespan of the species. It is noteworthy that the levels of the AAS in total plasma proteins are almost half the levels found in serum albumins, indicating that this oxidation product is mainly formed in serum albumin. In contrast, the levels of GGS showed no correlation with MLSP.

There is some influence also by the age on plasma protein GGS or AAS, as seen in Figure 8. The level of both GGS and AAS in plasma proteins decreases with an apparent half-life of about 1 week from the time of birth until it reaches the level of the adult rat after approximately 30 days. This is almost equal to the weight-doubling time in the pups of the stock used, indicating a dilution effect. Since rat serum albumin is replenished with a half-life of 2.5 days, however, the decrease in plasma protein GGS and AAS in the first month of life in the rats cannot be due to a single burst of oxidative damage to serum albumin around birth followed by dilution. It might therefore be due to dilution of another oxidatively damaged protein with a much longer turn-over time or, more likely, to a gradual decrease in oxidative damage to serum albumin due to a decrease in the metabolic rate within the first month. It is well known that a high level of ROS is found in growing pups due partly to a higher formation rate and partly to a less efficient antioxidant defence mechanism (Stadtman *et al.* 1993).

The level of GGS and AAS in plasma proteins reaches steady state after 30 days and increases linearly during the remaining life. Since the basal rate of metabolism does not increase with age, this might indicate that defence towards oxidative damage to proteins is gradually lost in the ageing process (Stadtman *et al.* 1993).

We conclude that conversion of the side chains of the amino acid residues (e.g. Pro, Arg and Lys) in serum albumin to carbonyl derivatives (γ-glutamyl semialdehyde and 2-amino-adipic semialdehyde) might be useful markers to measure *in vivo* and *in vitro* exposure of proteins to reactive oxygen species.

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