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Differential effects of cysteine and methionine residues in the antioxidant activity of human serum albumin

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Abstract

Antioxidant properties of human serum albumin (HSA) may explain part of its beneficial role in various diseases related to free radical attack. In the present study, the antioxidant role of Cys and Met was studied by copper-mediated oxidation of human low density lipoproteins and by free radical-induced blood hemolysis which essentially assessed metal-chelating and free radical scavenging activities, respectively. Mild conditions were set up to specifically modify Cys and Met residues by N-ethylmaleimide (NEM) and chloramine T treatments, respectively. We found that Met and Cys accounted for 40–80% of total antioxidant activity of HSA. Copper binding to HSA was decreased by about 50% with chloramine T treatment of Met whereas no change was observed after NEM treatment of Cys. Although other amino acid residues are likely to be involved in anti-/prooxidant properties of HSA, from our data, we propose that Cys chiefly works as a free radical scavenger whereas Met mainly acts as a metal chelator.

Keywords: *Human serum albumin, oxidation, antioxidant activity, cysteine, methionine, copper binding*

Abbreviations: *ANSA, 1-anilinonaphthalene-8-sulfonique acid; BC, bathocuproinedisulfonic acid; CT, chloramine T; DTNB, 5, 5′-dithiobis, 2-nitrobenzoic acid; HSA, human serum albumin; HT_{50}, 50% of maximal hemolysis time; LDL, low density lipoproteins; NEM, Nethylmaleimide*

Introduction

Albumin represents the most abundant serum protein with normal concentrations between 35 and 50 g/l. Many epidemiological studies have established an inverse relationship between serum albumin level and mortality risk.[1] This association holds also for cardiovascular disease after adjustment for usual risk factors.[2,3] This could be related to the occurrence of a pro-inflammatory status in these diseases. Among the variety of biological mechanisms which have been proposed to explain the beneficial effects of higher albumin concentrations, a direct protective effect of the albumin molecule has been suggested. There is now ample evidence for a significant antioxidant activity of serum albumin. In fact, this molecule may represent the major and predominant circulating antioxidant in plasma which is known to be exposed to continuous oxidative stress. Albumin may thus represent a quantitatively important component of the efficient antioxidant defense that organisms have developed to protect against oxidative attack.[4,5] Antioxidant activity of albumin was principally related to its capacity to bind metal ions and to scavenge free radicals. We have previously reported that glucose and free radicals impair the antioxidant properties of serum albumin.[6] These recent data bring further support to the proposal that, in addition to its plasma concentration, the quality of the albumin molecule may be related to its biological properties.

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Sulfur-containing amino acid residues, namely Cys and Met residues, are particularly sensitive to oxidation. Of the 35 cysteines of the human serum albumin (HSA) molecule, 34 are engaged in 17 S—S-bonded cystines, leaving only Cys-34 available for reactions.[7] It has been suggested that Cys-34 is one of the most reactive thiol groups in serum.[8] Met could also represent an oxidation-sensitive amino acid.[9] In keeping with this finding, it has been recently suggested that oxidation of surface-exposed Met residues to methionine sulfoxide may represent an endogenous antioxidant defense that protects proteins from extensive and irreversible oxidative modification.[10]

In the present study, we investigated the respective involvement of sulfur groups in the antioxidant activity of HSA. Influence and importance of targeted modifications of Cys and Met by N-ethylmaleimide (NEM) and chloramine T (CT) were examined on HSA antioxidant activity assessed by the copper-mediated oxidation of human low density lipoproteins (LDL)[11] and by the free radical-induced blood hemolysis tests.[12]

Materials and methods

Modifications of HSA

The modification of thiol groups of albumin is generated by incubation of 10 mg/ml HSA with 10^{-2} M NEM. After a 30 min incubation at room temperature, NEM in excess was eliminated by repeated dialyses against 0.15 M NaCl. Methionine residues are selectively oxidised by CT reagent according to Shechter et al.[13] Briefly, 5 mg HSA were incubated for 20 min at room temperature in 0.1 M Tris-HCl, pH 8.5, containing 0–200 μM CT. Oxidation was stopped by extensive dialyses against 0.15 M NaCl. Proteins were measured using the bicinchoninic acid technique (Pierce). [14]

Fluorescence studies

As HSA contains one tryptophan residue (Trp-214), we could evaluate the effect of CT to monitor the molecular conformation change by assaying the intrinsic fluorescence of HSA solutions. Fluorescence measurements of CT-modified HSA (0.01 mg/ml) were monitored using a spectrofluorometer (LS50B, Perkin Elmer) at excitation and emission wavelengths of 293 and 340 nm, respectively, as previously described.[6] The CT-induced molecular conformation changes were also investigated by measuring the fluorescence of the 1-anilinonaphthalene-8-sulfonique acid (ANSA) which evaluates variations of probe accessibility to protein hydrophobic sites.[15] After incubation of 0.5 mg/ml native or oxidized HSA with 200 μM ANSA in 0.15 M NaCl, fluorescence was measured at excitation and emission wavelengths of 385 and 463 nm, respectively. Results are expressed as arbitrary units per mg protein.

Thiol group measurement

Thiol groups of native or modified HSA were measured according to the Ellman's assay[16] using 5, 5′-dithiobis, 2-nitrobenzoic acid (DTNB). Briefly, 3.5 mg/ml HSA samples in 0.05 M PBS, pH 7.6 were incubated with 2.5 mM DTNB for 15 min. The free thiol concentration was calculated from absorbance data obtained at 410 nm with the help of a standard curve performed with various native BSA concentrations (0.8–4 mg/ml, corresponding to 19–96 nmol total thiols).

LDL preparation and oxidation

LDL (1.019–1.055 g/ml) were isolated as described[11] by sequential ultracentrifugation of pooled plasma from normolipidemic subjects (Beckman centrifuge). After dialysis against PBS, pH 7.4 to remove KBr, LDL were assayed for protein content by the bicinchoninic acid method (Pierce)[14] and stored at 4°C under argon in the dark no longer than 15 days. LDL oxidation (100 μg/ml) was started by adding 40 μl of $CuSO_4$ (40 μM) in a 500 μl sample volume containing the various HSA preparations (final concentration: 2 mg/ml). Lipid oxidation was measured at 245 nm in a spectrophotometer equipped with a thermostated 6-cuvette holder maintained at 37°C (Beckman DU 640). According to the previous work of Schnitzer et al.,[17] we confirmed that due to interferences at elevated HSA concentrations, the monitoring of the conjugated dienes for lipid oxidation usually measured at 234 nm[11] was improved at 245 nm.

Free radical-induced hemolysis test

The antioxidant properties of the native and modified HSA preparations were examined using a test based on *in vitro* free radical-induced blood hemolysis.[12,18] After an overnight fast, blood samples were taken from rats using 10% (v/v) buffered sodium citrate as anticoagulant. Hemolysis was started by adding 52.4 M AAPH and was assayed by measuring turbidimetry at 450 nm (MRX Dynatech) of diluted rat blood without or in the presence of the various HSA samples (2 mg/ml final concentration). Results were expressed as 50% of maximal hemolysis time (HT_{50} in min). The measurement of HT_{50} was very reproducible: intra- and interassay coefficients of variation: 1.32 and 3.85%, respectively. In human and animal models where oxidative stress has been well documented, HT_{50} was shown to be representative of the total defense against free radicals.[19]

Copper binding

Copper ions were measured spectrophotometrically by use of bathocuproinedisulfonic acid (BC, Sigma). The complex with Cu(I) absorbs strongly at 480 nm. Triplicate samples of native or modified HSA in 0.15 M NaCl were incubated for 2 h with 10 μM $CuSO_4$. After extensive dialysis against 0.15 M NaCl, BC was added to samples to obtain a final concentration of 400 μM, then followed by 1 mM of sodium ascorbate. The mixture was incubated at room temperature for 5 min to allow complete reduction of any HSA-bound Cu(II) to Cu(I) resulting in formation of BC-Cu(I) complex which absorbs strongly at 480 nm. Results were expressed by comparison with a calibration standard curve made with $CuSO_4$ up to 100 μM.

Statistical analysis

Data are expressed as the means ± standard deviation (SD) from at least three experiments performed in triplicate. The main effects of modification of HSA on its antioxidant activity were evaluated using one-way analysis of variance (ANOVA) followed by Bone-ferroni post-test performed with Prism (Graphpad Software Inc., San Diego, CA.).

Results and discussion

First, we established conditions to specifically modify Cys to obtain thiol-blocked HSA. It was realized by incubation of HSA with NEM which is known to bind to reduced —SH groups. By using DTNB, we confirmed that thiol concentration was drastically decreased by 70% from 4.3 ± 1.0 in native HSA to 1.3 ± 0.4 pmol/mg protein for NEM-HSA ($p < 0.01$) with no change in molecular conformation as assessed by ANSA fluorescence (plateau at 416.2 ± 3.7 and 415.5 ± 5.5 AUF/mg prot. for native and NEM-treated HSA, respectively). Second, to selectively modify Met residues, we adapted the previously described experimental conditions of Shechter et al.[13] They reported that only surface-exposed Met residues were oxidised by low concentrations of CT (less than 5 μM) with no major effect on either their conformations or biological functions. Under our conditions, we found no modification of thiol concentration. HSA conformation was monitored using both intrinsic Trp and ANSA fluorescences (Figure 1). Both indicators markedly decreased in a concentration dependent manner with respect to CT. These data indicate that CT treatments induced drastic change in the conformation of HSA molecule because both indicators are sensitive to hydrophilic and hydrophobic environment. These results are in favor of CT-induced protein refolding leading to a reduction in accessibility of hydrophobic sites of HSA.

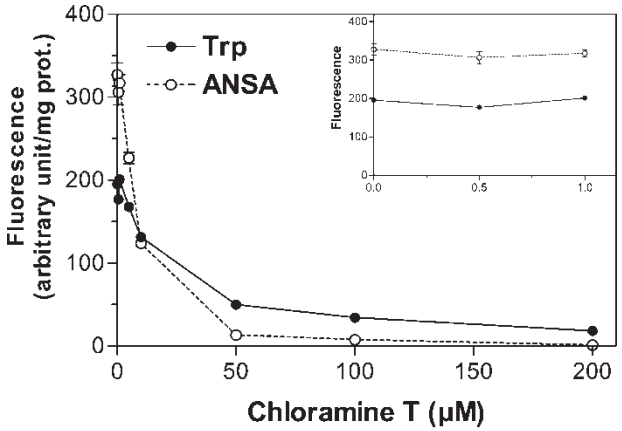

Figure 1. Effect of chloramine T concentration on tryptophan and ANSA fluorescence in HSA. HSA (5 mg/ml) were incubated for 20 min in 0.1 M Tris-HCl, pH 8.5 containing 0 to 200 μM chloramine T. Incubations with ANSA (200 μM) were carried out in 0.15 M NaCl buffer. Fluorescence analyses were carried out using excitation wavelength of 293 nm and emission wavelength 340 nm for Trp and, excitation wavelength of 385 nm and emission wavelength 463 nm for ANSA. Each value corresponds to means and SD of three different HSA preparations. Note that SD bars are sometimes smaller than symbols. The insert illustrates Trp and ANSA fluorescences of HSA modified by low chloramine T concentrations (up to 1 μM).

Moreover, these results are in line with the expected decrease in Trp fluorescence. However, we succeeded in finding gentle conditions by using low concentrations of CT (up to 1 μM) for which Trp and ANSA fluorescences were not impaired (Figure 1, insert). Under our mild conditions, these results indicate that the environment of the Trp residue and the hydrophobic regions probed by ANSA were not altered in the HSA molecule. As evidenced by polyacrylamide gel electrophoresis (data not shown), these conditions did not result in large damage to the protein such as fragmentation or aggregation. These low concentrations of CT are in agreement with those used by Shechter et al. to selectively oxidize Met residues in proteins.[13]

Antioxidant activity of native and modified HSA were compared as their capacity to inhibit copper-induced LDL oxidation. Results of representative oxidation curves in presence of CT-treated HSA are illustrated in Figure 2A. We confirmed that HSA has strong antioxidant activity (+53%, $p < 0.001$ vs. control) and we found that CT-treatment induced a concentration-dependent progressive loss of the property to inhibit LDL oxidation. In addition, higher CT concentration (10 μM) resulted in pro-oxidant effects as shown by the oxidation $T_{1/2}$ which was significantly shorter than the control (Figure 2B).

Mildly modified HSA preparations have also been tested with regard to free radical-induced blood hemolysis (Table I). We found that native HSA has strong free radical scavenging properties as HT_{50}

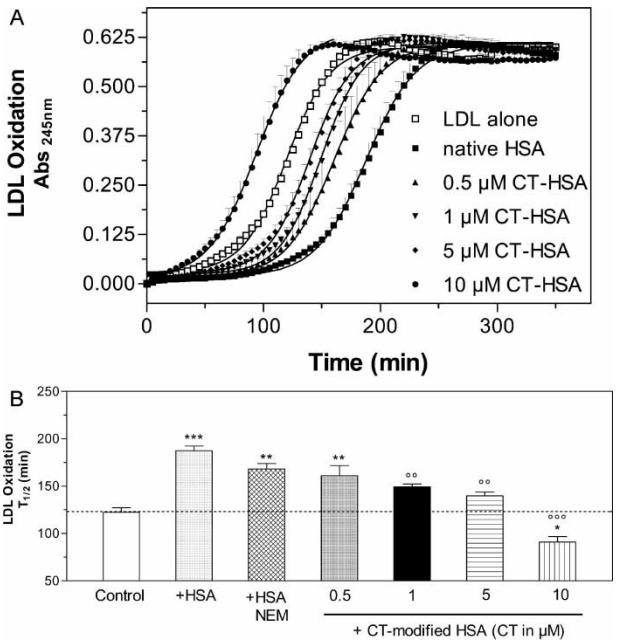

Figure 2. Effect of chloramine T concentrations on HSA antioxidant activity in copper-induced LDL oxidation. (A) Illustration of Cu^{2+}-mediated LDL oxidation curves (100 µg/ml) in presence of (2 mg/ml) native HSA or modified by various concentrations of chloramine T (CT-HSA). Oxidation was started by adding $CuSO_4$ (3 µM) and monitored by absorbance of conjugated dienes (245 nm). (B) Histograms represent half maximum oxidation time ($T_{1/2}$ in min) expressed as means ± SD of three independent experiments. The doted line facilitates comparison with control. Significance of the results using one-way ANOVA followed by Tukey's post-test: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. Control; °°°$p < 0.001$, °°$p < 0.01$ vs. Native HSA.

increased by +28% ($p < 0.001$ vs. control). These data are in keeping with our previous observations dealing with BSA.[6] A 57% decrease in the protective effect of albumin was observed after NEM modification ($p < 0.001$ vs. native HSA). HSA treated with low concentrations of CT also resulted in a marked reduction (−21%, $p < 0.001$) of its antioxidant activity. However, although NEM and CT-modified HSA preparations presented significant reductions in their antioxidant activity, they were not to the same extent. Modification by NEM of HSA induced a 29% reduction ($p < 0.05$ vs. native HSA) in the antioxidant activity of the protein as assessed by the copper-induced LDL oxidation test whereas a 59% decrease ($p < 0.001$ vs. native HSA) was observed in the same test with CT-modified HSA.

HSA is characterised by a unique arrangement of nine loops as the result of 17 disulfide bounds. Although only one cysteine residue is free (Cys-34),[7] albumin is claimed to represent the unique and major source of reactive thiol group in serum, particularly efficient in scavenging reactive oxygen species (ROS).[8] This hypothesis is strongly attested by the 56% decrease in HT_{50} of thiol-blocked HSA. Major part of the reduced antioxidant activity observed in the copper-induced LDL oxidation test could be ascribable to its metal-chelating property. It has been described that a well characterised high-affinity binding site is positioned to domain I of the molecule where Cys-34 is located and is highly specific for Cu(II). Another report from Narazaki et al.[8] also provided evidence that Cys-34 could act as a ligand binding site for various transition metal ions in a reversible manner. Although it is not always the case, it is usually proposed that binding may prevent Cu(II) to be reduced to Cu(I) which is very reactive with peroxides. As suggested by recent work, not only antioxidant activity but also copper handling may depend on the redox status of the albumin molecule.[20] A greater binding and a diminished redox-cycling activity may result in an inhibition of generation of ROS via the Fenton reaction avoiding its subsequent reaction with lipoproteins.[21]

Our attention has also been focused on albumin Met residues (6 in HSA). Met is particularly sensitive to oxidation and a great variety of oxidants such as H_2O_2, hydroxyl radicals, hypochlorite, chloramine and peroxynitrite lead to methionine sulfoxide production.[9,22] Oxidation to sulfone, which is the next step, is only obtained under drastic conditions not usually occurring in biological systems. Methionine sulfoxide is quickly reversed back to Met with mild reductants, whereas sulfone formation is biologically irreversible. There are very few studies reporting on the occurrence of methionine sulfoxide in proteins

Table I. Antioxidant activity of native, NEM- or CT-modified HSA in copper-induced LDL oxidation and in free radical-induced blood hemolysis.

	Control	Native HSA	NEM–HSA	CT–HSA
LDL oxidation $T_{1/2}$ (min)	122.5 ± 8.4^{a,b,c}	187.4 ± 8.7^{a,d,e}	168.7 ± 3.2^{b,d,f}	149.3 ± 5.0^{c,e,f}
Antioxidant activity (%)		100 ± 4.6^{d,e}	71.2 ± 1.3^{d,f}	41.3 ± 3.3^{e,f}
Hemolysis HT_{50} (min)	285.8 ± 1.29^{g}	365.6 ± 1.9^{g}	320.6 ± 7.0^{g}	349.0 ± 1.7^{g}
Antioxidant activity (%)		100 ± 0.5^{g}	43.6 ± 0.9^{g}	79.2 ± 0.4^{g}

Values (mean ± SD of three to six independent experiments) represent data obtained with various HSA preparations after computing kinetic curves. Numbers correspond to the calculated time for 50% LDL oxidation time ($T_{1/2}$) or 50% hemolysis time (HT_{50} in min), respectively. Antioxidant activities were also expressed as per cent of native HSA corrected for control values. Significance of the results are calculated with one way ANOVA followed by Boneferroni post-test. Numbers with identical letters are significantly different: $^{a,b,e,g}P < 0.001$; $^{c}P < 0.01$; $^{d,f}P < 0.05$.

during oxidation-related diseases. This could be due in part to various factors all related to the difficulty and lengthy procedures of specifically analyzing this modified amino acid.[21] Several examples of oxidation of Met residues in proteins have been reported.[23] These data confirm the importance of this process in protein function (see Ref. [22] for review). Levine et al.[24] observed that preferential oxidation with CT of several exposed Met residues in enzymes such as glutamine synthase had little effect on their biological function. They proposed the very attractive hypothesis that oxidation and reduction cycle of Met residues in biological systems could serve as a ROS scavenging system to protect proteins from extensive modifications. As similarly proposed for thiols by virtue of their reversibility, this process may also be involved in the maintenance of redox balance.[25] In the present study carried out with two different tests, we found that mild oxidation of HSA Met residues markedly impaired its potent antioxidant.

Although the precise mechanism by which albumin exerts its antioxidant effect is presently unknown, it may most likely be multifactorial. In LDL oxidation, copper-binding activity of albumin was essentially addressed whereas free radical-induced hemolysis initially considered free radical scavenging property. We found that copper binding to HSA is dependent on the integrity of the surface exposed Met residues. Indeed using bathocuproinedisulfonic acid to assay bound Cu(II), our results indicate that copper binding to CT-treated HSA was reduced by about 50% compared to native HSA whereas no significant change was observed for NEM-treated HSA (Figure 3). In light of our data based on copper binding and on respective residual effects of thiol-blocked and CT-treated HSA compared to native HSA, we propose that Cys residue was chiefly involved in free radical scavenging activity of HSA while Met was more efficient for metal-chelating. However, although strict comparison is difficult essentially because redox cycling activity of HSA should also be considered in relation to copper and other thiols, we may conclude that one of the antioxidant actions of HSA is mainly due to metal-chelating effects. This conclusion is based on the fact that a 53% inhibition prevailed for copper-initiated LDL oxidation whereas only 28% was observed in the hemolysis test (Table I). It is obvious that the\ above percentages of inhibition obtained using *in vitro* tests warrant further studies to evaluate the resulting protection that might occur *in vivo*.

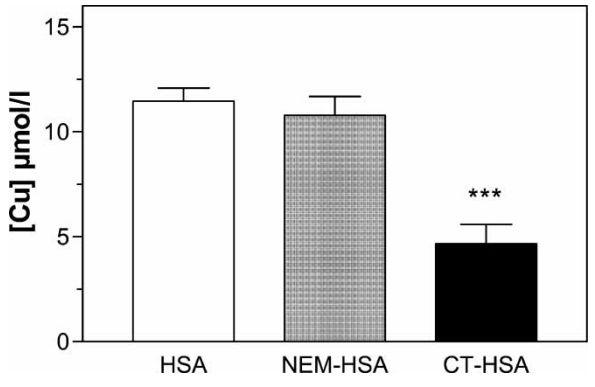

Figure 3. Copper binding of various HSA preparations. Native, NEM- and chloramine *T*-treated HSA were incubated with 10 µM $CuSO_4$ and after dialysis, bound copper ions were assayed using bathocuproinedisulfonic acid as detailed in Materiel and Methods. Data are expressed as means ± SD, ($n = 6$). Significance of the results using one-way ANOVA followed by Tukey's post-test: ***$p < 0.001$ vs. Native HSA.

Our results mainly addressed limited oxidation for which sulfur-containing amino acids afford some protection. For stronger oxidations, beyond sulfur-containing residues, damage can be transferred from thiol group to carbon sites in the protein. Although the mechanism and final products may differ with oxidation conditions, most of the amino acids are susceptible to oxidation by ROS.[26] In this context, Trp appears to be one of the amino acids the most prone to oxidation.[9] It has been shown that the reverse process can also occur: radical damage can be transferred from a carbon-centered site to the thiol group.[27] Spin-trapping studies provided evidence indicating that thiol-blocked BSA was much more susceptible to damage than native protein. Sulfur-containing amino acids may work as a radical sink in protecting protein from denaturation. This idea is in line with our finding that, under more extensive oxidation associated with conformation changes and Trp alteration, radical attack might not be quenched. This might explain why, in these conditions, a prooxidant effect of the protein is evidenced by copper-induced LDL oxidation.

In conclusion, as oxidant stress is thought to play a major role in ageing and the pathogenesis of many disease including cardiovascular disease and cancer,[28] it may be important to consider albumin. Experimental and epidemiological data provided evidences that the circulating albumin concentration was crucial for regulation of important functions.[1,3] In a clinical study, the benefits of administration of albumin to septic patients were attributed to an increase in antioxidant associated to the thiol repletion.[29] We have previously provided ROS results indicating that oxidised LDL and lipid oxidation products (oxysterols) may be responsible for a reduced HSA biosynthesis and secretion in cultured liver cells. We have also reported that glycosylation and oxidation impaired the antioxidant capacity of albumin.[6] The present study provides further evidence that sulfur-containing amino acids may be key players in the metal chelating and redox cycling activities in relation to free radical scavenging properties of albumin.

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