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Research Communication

Quantitative Evaluation of the Role of Cysteine and Methionine Residues in the Antioxidant Activity of Human Serum Albumin Using Recombinant Mutants

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Summary

The importance of cysteine (Cys) and methionine (Met) residues for the antioxidant activity of human serum albumin (HSA) was investigated using recombinant HSA mutants, in which Cys34 and/or the six Met residues had been mutated to Ala. The scavenging activities of the mutants against five reactive oxygen and nitrogen species were evaluated by a chemiluminescence assay, electron paramagnetic resonance spectroscopy, or a HPLC-flow reactor assay. Our results showed that the contributions of Cys34 and the Met residues to the antioxidant activity of HSA were 61% and 29% against O_2·–, 68% and 61% against H_2O_2, 38% and 6% against HO·, 36% and 13% against HOCl, and 51% and 1% against ·NO, respectively. Thus, the findings propose in a direct way that Cys34 plays a more important role than the Met residues in the antioxidant activity of HSA.

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Keywords human serum albumin; antioxidant activity; reactive oxygen species; nitric oxide; cysteine; methionine.

INTRODUCTION

It is well known that reactive oxygen species, such as hydroxyl radical (HO·), superoxide anion radical (O_2·–), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl), and reactive nitrogen species, such as nitric oxide (·NO) and peroxynitrite ($ONOO^-$) contribute to the development of several age-related diseases caused by increasing oxidative stress and oxidative damage. Oxidative stress can result either from low levels of antioxidant and/or from an increased production of reactive species (*1, 2*).

Human serum albumin (HSA) is a single-chain protein synthesized in and secreted from liver cells. The protein is formed by a single polypeptide chain of 585 amino acids and has a molecular mass of ~67 kDa. This highly soluble protein is present in human plasma at normal concentrations between 35 and 50 g/L. It is well known that HSA exhibits numerous biological functions such as maintenance of colloid osmotic pressure and transport of endogenous and exogenous ligands. Recent studies have focused on its antioxidant properties (*3*), and an inverse relationship between HSA level and cardiovascular diseases has been established (*4*). Furthermore, it has been reported that more than 70% of the free radical-trapping activity of serum is due to HSA as assayed by the free radical-induced hemolysis test (*5*). These results indicate that HSA represents the major and predominant antioxidant in plasma.

Sulfur-containing amino acid residues such as cysteine (Cys) and methionine (Met) are particularly sensitive to oxidation. HSA contains one reduced Cys residue (Cys34) which, due to the large amount of albumin in plasma, constitutes the largest pool of thiols in the circulation (*6*). In healthy adults, about 70–80% of the Cys34 in albumin contains a free sulphydryl group, the rest forms a disulfide with several compounds like cysteine,

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homocysteine, or glutathione (*6*). It has been suggested that Cys34 is one of the most reactive thiol groups in serum (*7*). Methionine residues (six in HSA) also represent an oxidation-sensitive amino acid in albumin (*5*). Met is particularly susceptible to oxidation and a wide variety of oxidants lead to production of methionine sulfoxide (*8*), which can be reversed back to Met with mild reductants or by methionine sulfoxide reductases (*9*). It has been 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*).

Recently, Bourdon et al. (*11*) have estimated that the contribution of Cys and Met residues to the antioxidant activity of HSA against HO· is about 40–80%. The estimate was based on a copper-mediated low density lipoprotein (LDL) oxidation assay and an 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH)-induced hemolysis test using Cys- and Met-modified HSA prepared by treatment with *N*-ethylmaleimide and chloramin T, respectively. In addition, they concluded that the antioxidant activity of the Met residues was mainly derived from their copper-chelating properties, not from a free radical scavenging activity. However, it is technically difficult to modify the six Met residues of HSA specifically by chemical modification. Therefore, quantitative evaluation of the role of Met as well as Cys residues in the antioxidant activity of HSA against HO· and other reactive oxygen/nitrogen species are still lacking.

To address these issues, we prepared the following three recombinant mutants of HSA: Cys34 mutated to alanine (C34A), all six Met residues mutated to alanine (M87A/M123A/M298A/M329A/M446A/M548A; 6 point), and Cys34 and the Met residues mutated to alanine (C34A/M87A/M123A/M298A/M329A/M446A/M548A; 7 point). Using these mutants, we assessed the role of Cys and Met residues in the antioxidant activity of HSA against physiologically important reactive oxygen/nitrogen species, namely O_2·–, H_2O_2, HO·, HOCl, and ·NO.

MATERIALS AND METHODS

Electron Paramagnetic Resonance Spin-Trapping Experiments for Hydroxyl Radical Scavenging Activity of Recombinant HSA

Hydroxyl radicals (HO•) were generated by the homolysis of H_2O_2 upon UV-irradiation with a handheld shortwave UV lamp (254 nm, 115 V, 60 Hz, 0.16 amps), and were assayed by electron paramagnetic resonance (EPR) spin trapping with 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO). Briefly, the reaction solution (200 μL) contained 0.5 mM H_2O_2, 100 μM diethylenetnaminepentaacetic acid (DTPA), and 9 mM DMPO, without or with 75 μM recombinant human serum albumin (rHSA), in 0.1 M sodium phosphate buffer (pH 7.4). The mixtures were immediately transferred into quartz EPR flat cells and irradiated with UV (254 nm) for 1 min. Immediately after the UV-irradiation, the ESR spectra were recorded at room temperature on a JES-TE 200 EPR spectrometer (JEOL, Tokyo, Japan). The conditions for the EPR measurement were as follows: scanning field, 335.2 ± 5 mT; sweep time, 2 min; microwave power, 40 mW; time constant, 0.3 sec; gain, 400; modulate width, 0.25 mT; and microwave frequency, 9.43 GHz. After recording the EPR spectra, the signal intensities of the DMPO-OH adducts were normalized against that of a manganese oxide (Mn^{2+}) signal, where Mn^{2+} is an internal control. The scavenging activity was calculated from the relative intensity peak height of the DMPO-OH EPR signal.

The remaining of Materials and Methods can be found in Supporting Information.

RESULTS AND DISCUSSION

O_2·– Scavenging Activity of Recombinant HSAs

The scavenging activity against O_2·– was determined by a luminol-dependent chemiluminescence (CL) assay. As shown in Fig. 1A, significant decreases in chemiluminescence were observed in the presence of wild-type rHSA and allopurinol (a xanthine oxidase inhibitor). Figure 1B shows the relative antioxidant activity of mutant HSAs (% of wild-type rHSA). The reduction of relative antioxidant activity of C34A, 6 point, and 7 point was 61%, 29%, and 93%, respectively, suggesting that the contribution of Cys34 and the Met residues to the O_2·– scavenging activity of HSA is about 60% and 30%, respectively, and that the effects are additive.

H_2O_2 Scavenging Activity of Recombinant HSAs

The scavenging activity against H_2O_2 was evaluated by a lucigenin-dependent CL assay. Significant decreases in chemiluminescence were observed in the presence of wild-type rHSA and catalase (Fig. 1C). Figure 1D shows the relative antioxidant activity of mutant HSAs (% of wild-type rHSA). The reduction of relative antioxidant activity of C34A, 6 point, and 7 point was 68%, 61%, and 86%, respectively, suggesting that the contribution of Cys34 and the Met residues to the H_2O_2 scavenging activity of HSA is about 70% and 60%, respectively.

HO· Scavenging Activity of Recombinant HSAs

The scavenging activity against HO· generated by a H_2O_2/UV system was evaluated by EPR spectroscopy combined with DMPO. The EPR signal was decreased in the presence of wild-type rHSA (Fig. 1E). Figure 1F shows the relative antioxidant activity of mutant HSAs (% of wild-type rHSA). The reduction of relative antioxidant activity of C34A, 6 point, and 7 point was 38%, 6%, and 56%, respectively, suggesting that the contribution of Cys34 to the HO· scavenging activity of HSA is about 40%, whereas that of the Met residues is quite low.

HOCl Scavenging Activity of Recombinant HSAs

The scavenging activity against HOCl was evaluated by the luminol-dependent CL assay. Figure 2A shows the relative antioxidant activity of mutant HSAs (% of wild-type rHSA). The

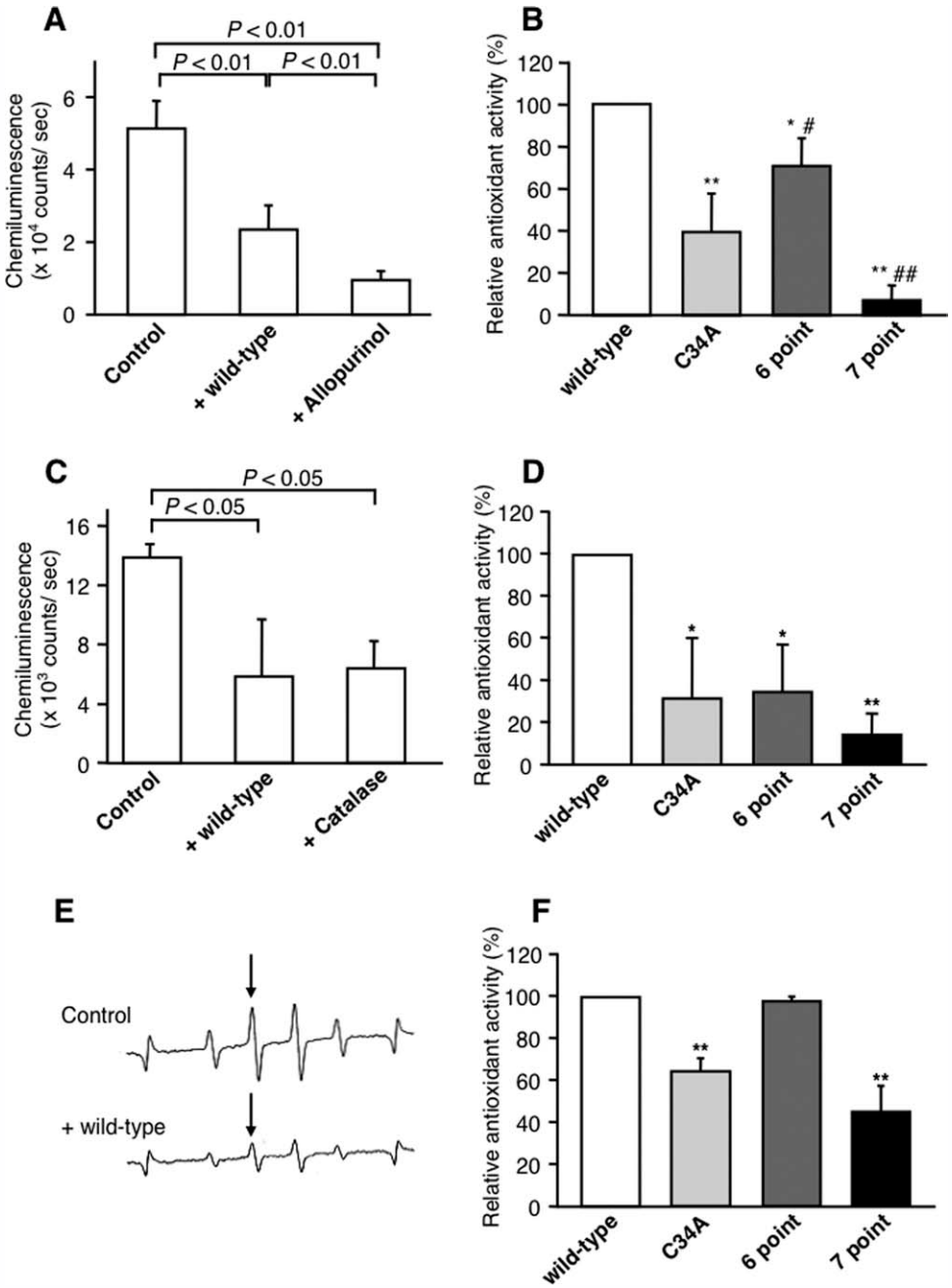

Figure 1. Antioxidant activity of rHSA mutants against O_2·−, H_2O_2, and HO·. (A) Scavenging activity of wild-type rHSA and allopurinol against O_2·− generated by the X/XO system, as assessed by a luminol-CL assay. (B) Relative antioxidant activity of rHSA mutants against O_2·−. (C) Scavenging activity of wild-type rHSA and catalase against H_2O_2 assessed by a lucigenin-CL assay. (D) Relative antioxidant activity of rHSA mutants against H_2O_2. (E) Scavenging activity of wild-type rHSA against HO· generated by a H_2O_2/UV system, as assessed by EPR spectroscopy combined with DMPO. (F) Relative antioxidant activity of rHSA mutants against HO·. Values are expressed as the mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$ as compared with wild-type HSA. #$P < 0.05$, ##$P < 0.01$ as compared with C34A.

reduction of relative antioxidant activity of C34A, 6 point, and 7 point was 36%, 13%, and 49%, respectively, suggesting that the contribution of Cys34 and the Met residues to the HOCl scavenging activity of HSA is about 40% and 10%, respectively, and that the effects are additive.

NO Scavenging Activity of Recombinant HSAs

The scavenging activity against ·NO generated by NOC7 was evaluated by a high performance liquid chromatography (HPLC)-flow reactor assay. Figure 2B shows the relative antioxidant activity of mutant HSAs (% of wild-type rHSA). The

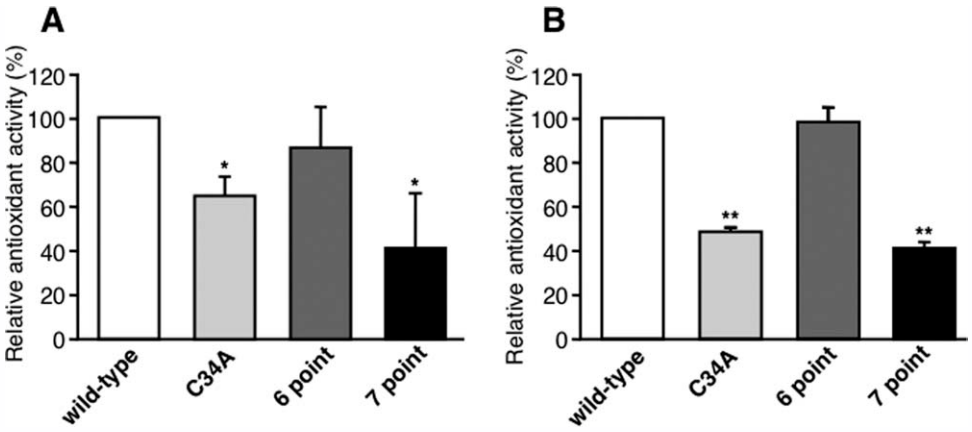

Figure 2. Antioxidant activity of rHSA mutants against HOCl and ·NO. (A) Relative antioxidant activity of rHSA mutants against HOCl, as assessed by luminol-CL assay. (B) Relative antioxidant activity of rHSA mutants against ·NO generated by NOC7, as assessed by a HPLC-flow reactor assay. Values are expressed as the mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$ as compared with wild-type HSA.

reduction of relative antioxidant activity of C34A, 6 point, and 7 point was 51%, 1%, and 59%, respectively, suggesting that the contribution of Cys34 to the ·NO scavenging activity of HSA is about 50%, and that the contribution of the Met residues is negligible.

CONCLUSIONS

The results obtained are summarized in Table 1. Concerning the antioxidant activity against O_2·– and H_2O_2, we found that Cys34 and the six Met residues accounted for more than 90% of the antioxidant activity of total HSA. In accordance with that finding, Finch et al. (*12*), using mass spectrometry, reported that Cys34, Met123, Met298, Met446, and Met548 were selectively modified by H_2O_2. By contrast, HO· is scavenged by Cys34, not by the Met residues, and the contribution of Cys34 to the total antioxidant activity of HSA is ~40% (Table 1). Finally, in the case of HOCl and ·NO, Cys34 and the Met residues contributed with ~50% of the antioxidant activity of HSA. Previously, Salavej et al. (*13*), using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, reported that Met123, Met329, and Met548 are selectively modified by HOCl. However, other types of studies have shown that also tyrosine, tryptophan, and/or arginine residues react with HOCl and ·NO (*14, 15*). Thus, Cys34 seems to possess an universal function as an antioxidant residue in HSA against various reactive oxygen and nitrogen species, whereas the Met residues are more likely to play a supporting role in that respect, because they show specific antioxidant activity against O_2·–, H_2O_2,and HOCl.

Table 1
The percentual contribution of Cys34 and the six Met residues to the antioxidant activity of HSA[a]

	O_2·–	H_2O_2	HO·	HOCl	·NO
Cys34	61 ± 21	68 ± 25	38 ± 5	36 ± 7	51 ± 2
Six Met residues	29 ± 17	61 ± 21	6 ± 4	13 ± 19	1 ± 2

[a]The results are means ± SD ($n = 3$).

HSA has been reported to interact with heme, and this heme-albumin, with heme-protein-like reactivity and spectroscopic properties, showed scavenging activity against reactive nitrogen species such as ·NO (*16, 17*). Therefore, further studies considering the involvement of endogenous compounds which can interact with albumin could shed light on the nature of the antioxidant roles *in vivo* of whole HSA as well as of its Cys and Met residues.

To overcome problems such as a potential risk of contamination with blood-derived pathogens and a limited supply of human plasma from which HSA is fractionated, rHSA that is highly expressed by *Pichia pastoris* has been commercially available in Japan from 2008. Protein engineering will also enable the creation of rHSAs with modified properties such as high antioxidant activity in the circulation. An increased antioxidant activity will be beneficial to several oxidative stress-induced diseases such as arteriosclerosis, multiple sclerosis (*18*), and Alzheimer's disease. This study showed that both Cys and Met residues have significant antioxidant effects against O_2·–, H_2O_2, and HOCl. The O_2·–and H_2O_2 are considered to be initiators of other reactive oxygen species, for example by generating ·OH after reaction of H_2O_2 with metal ions and HOCl by stimulated polymorphonuclear leukocytes and monocytes at inflammatory loci, and they, therefore, play an important role in the progression of diseases. Thus, if additional Cys and Met residues are mutated into HSA, such mutants would be beneficial to suppress the initial and latter parts of oxidative stress. To engineer the rHSA mutants, information regarding the effects of mutations

on antioxidant activity would be desirable. Furthermore, additional studies, especially *in vivo*, are needed to document the modifications in albumin structure and antioxidant properties in pathologic conditions. Albumin has not yet showed all its secrets, and experiments are highly warranted to achieve a better understanding of its important antioxidant properties.

In this study, we constructed the recombinant mutants C34A, M87A/M123A/M298A/M329A/M446A/M548A, and C34A/M87A/M123A/M298A/M329A/M446A/M548A to clarify the effect of the lonely free cysteine residue and the six Met residues on the antioxidant activity of HSA. We found that Cys34 plays the most important role for the antioxidant activity of HSA against $O_2^{\cdot}-$, ·NO, HO·, HOCl, and H_2O_2. On the other hand, the six Met residues seem to play only a supporting role for the antioxidant activity of HSA against $O_2^{\cdot}-$, H_2O_2, and HOCl. These findings imply that in addition to HSA playing a role as a biomarker of various types of oxidative stress, novel rHSAs with superior antioxidant activities can be engineered.

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