Validation of the Chloramine-T Induced Oxidation of Human Serum Albumin as a Model for Oxidative Damage *in Vivo*

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Purpose. The validity of using chloramine-T as a model compound for mimicing oxidative stress was examined using human serum albumin (HSA) as a model. Important sites of oxidation were studied by mild treatment with chloramine-T and by mutating ^{34}Cys for a serine (C34S).

Methods. High-performance liquid chromatography (HPLC) combined with fluorescence detection to confirm the validity of chloramine-T as an oxidizing agent was used. Oxidized amino acid residues were detected by reaction with 5,5′-dithiobis(2-nitro benzoic acid), digestion with cyanogen bromide, followed by capillary electrophoresis. Protein conformation was examined by spectroscopic techniques.

Results. From the HPLC analysis of human serum, the validity of using chloramine-T as an oxidizing agent was confirmed. At low chloramine-T concentrations ($CT_{0.1}$-HSA, CT_1-HSA), ^{34}Cys and Met residues were oxidized, at medium concentrations (CT_{10}-HSA), the tryptophan residue also appeared to be oxidized, and at the highest concentration (CT_{50}-HSA), the net charge of Site II of HSA was found to be more negative. The two highest levels of oxidation of HSA (CT_{10}-HSA, CT_{50}-HSA) resulted in conformational changes with an increased exposure of hydrophobic regions, decreased high-affinity bindings of warfarin and ketoprofen and a reduced esterase-like activity. The latter protein also has a shorter plasma half-life and an increased liver clearance.

Conclusions. We succeeded in imitating oxidative damage to HSA using chloramine-T and the findings show that Site II is more affected than Site I and ^{34}Cys, when HSA is exposed to oxidative stress.

KEY WORDS: human serum albumin; cysteine mutation; chloramine-T; Site II; oxidative damage; pharmacokinetics.

INTRODUCTION

Covalent modification of proteins by oxidative systems has been implicated in various physiological and pathological conditions (1). To protect against this toxicity, organisms have developed a battery of antioxidant defenses. Thus, blood plasma, which is known to be exposed to continuous oxidative stress, contains albumin as a major and predominant circulating antioxidant (2). Human serum albumin (HSA) is a single, nonglycosylated polypeptide of 585 amino acids which is organized in the form of a heart-shaped protein having about 67% α-helix but no β-sheet structure (3). All but one (^{34}Cys) of the thirty-five cysteine (Cys) residues are involved in the formation of stabilizing disulfide bonds. Human serum albumin is also a comprehensive depot and transport protein, and plays an important role with respect to endogenous and exogenous compounds. Therefore, it can be anticipated that, for example, the distribution and half life of ligands might be greatly effected, if the functional properties of HSA were to be modified by disease or aging associated oxidation.

In a previous study, we oxidized HSA using several different procedures and found that several types of amino acid residues are important for its antioxidant activity (4). However, our experimental system was limited to *in vitro* conditions. Thus, it was difficult to judge whether our experimental conditions actually reflect physiological conditions. In a recent study, Era *et al.* (5) reported on the use of a convenient high-performance liquid chromatographic (HPLC) system for the clear separation of reduced and oxidized albumin, using an ES-502N column, and extensively studied the conversion of reduced; HSA to an oxidized form in the elderly and in various pathophysiological states. In general, HSA is a mixture of mercaptalbumin (HMA, reduced form) and nonmercapt albumin (HNA, oxidized form) (6,7). HMA contains one free sulfhydryl group in ^{34}Cys and HNA is comprised of at least three types of molecules, namely a mixed disulfide with cysteine or glutathione (HNA(Cys), HNA(Glut)), and products oxidized HNA (HNA(Oxi)) to a greater extent than mixed disulfide. The chloramine-T method of Shechter *et al.* (8) generates the formation of $HO^{\cdot}$ and chloro radicals and may mimic oxidation reactions that occur under physiological conditions. Preliminary results showed an increase in HNA, when reacting serum was reacted with chloramine-T. Similar results have been found in aging as well as in certain diseased states. Furthermore, by varying the experimental conditions, different types of HSA, in different oxidation states could be prepared easily and reproducibly. Therefore, we chose to further investigate this procedure for oxidizing HSA.

The authors also prepared a recombinant mutant of HSA in which ^{34}Cys was replaced by a serine, because ^{34}Cys represents a potentially important residue of HSA for oxidative stress. The impact of the mutation and four levels of chloramine-T induced oxidations on the structural properties of albumin was investigated using a variety of spectroscopic techniques. The influence of oxidation on various functional properties, namely its ligand-binding and esterase-like ability, were also studied. Finally, the effect of the mutation and oxidations on the plasma half-life and organ clearances of HSA was evaluated in mice.

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ABBREVIATIONS: HSA, human serum albumin; rHSA, recombinant HSA; HMA, mercaptalbumin; HNA, nonmercaptalbumin; $CT_{0.1}$-HSA, CT_1-HSA, CT_{10}-HSA and CT_{50}-HSA is HSA treated with 0.1, 1, 10 and 50 mM of chloramine-T, respectively; DTNB, 5,5′-dithiobis (2-nitro benzoic acid); bis-ANS, 1,1-bis-4-anilinonaphthalene-5,5-sulfonic acid; CNBr, cyanogen bromide; CD, circular dichroism; Cys, cysteine; Met, methionine; Trp, tryptophan; Tyr, tyrosine.

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Based on these data, the validity of chloramine-T treatment as a physiological model and the importance of Site I and Site II in HSA with respect to oxidative damage, are discussed.

MATERIALS AND METHODS

Materials

HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and was defatted by using the charcoal procedure described by Chen (9), deionized, freeze-dried, and then stored at –20°C until used. HSA and the oxidized HSAs prepared in this study gave only one band on SDS-PAGE (data not shown), and the molecular mass of all the albumins were assumed to be 67 kDa. Sera from five healthy young male subjects, who had no renal or hepatic dysfunctions, were obtained from Faculty of Pharmaceutical Sciences, Kumamoto University. Their ages ranged from 21 to 26 years (23.2 ± 1.92. mean ± S.D.), as previously described (10). Blood sera were obtained by centrifugation and stored at –80°C until used for analysis. Chloramine-T, 5,5′-dithiobis(2-nitro benzoic acid) (DTNB) and *p*-nitrophenyl acetate were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). The fluorescence probe 1,1-bis-4-anilino-naphthalene-5,5-sulfonic acid (bis-ANS) and fluoresceinamine (isomer II) were obtained from Sigma (St Louis, MO, USA). Potassium warfarin (Eisai Co., Tokyo, Japan) and ketoprofen (Sanwakagaku Co., Tokyo, Japan) were obtained as pure substances from the manufacturers. $^{111}InCl_3$ (74 Mbq/mL in 0.02 N HCl) was a gift from Nihon Medi-Physics (Takarazuka, Japan). All other chemicals were of analytical grade, and all solutions were prepared in deionized and distilled water. Phosphate buffer, 67 mM and pH 7.4, was used as a standard buffer, and was prepared from sodium phosphate dibasic and sodium phosphate monobasic salts.

Synthesis and Purification of rHSA Forms

The recombinant DNA techniques used to produce wild-type rHSA and the single-residue mutant C34S were essentially those described by Watanabe *et al.* (11). A chimeric plasmid (pJDB-ADH-L10-HSA-A) having a cDNA for the mature form of HSA along with an L10 leader sequence was a gift from Tonen Co. (Tokyo, Japan). The mutagenic primer used (underlined letters indicate mismatches) was 5′-CTTCAGCAG**TCT**CCATTTGAAG-3′. The L10-HSA coding region was amplified by PCR with a forward and a reverse primer carrying a 5′-terminal EcoRI site and cloned into the EcoRI-digested pKF19k vector (Takara Shuzo Co., Kyoto, Japan). Mutagenesis was performed with a site-directed mutagenesis kit (oligonucleotide-directed dual amber method) obtained from Takara Shuzo Co. The mutation was confirmed by DNA sequencing of the entire HSA coding region with the dideoxy chain termination method on a PerkinElmer ABI Prism 310 Genetic Analyzer. For constructing the HSA expression vector pHIL-D2-HSA, an L10-HSA coding region without or with the desired mutation site was incorporated into the methanol-inducible pHIL-D2 vector (Invitrogen Co., San Diego, CA, USA). The resulting vector was introduced into the yeast species *P. pastoris* (strain GS115) to express rHSA. Secreted rHSA was isolated from the growth medium by a combination of precipitation with 60% (w/v) $(NH_4)_2SO_4$, followed by purification on a Blue Sepharose CL-6B column (Amersham Pharmacia Co., Uppsala, Sweden). Isolated protein was defatted using the charcoal procedure described by Chen (9), deionized, freeze-dried and then stored at –20°C until used. The resulting albumins (treated with dithiothreitol) appeared as a single band on SDS/PAGE and all the recombinant proteins migrated at the same position as native HSA. Density analysis of protein bands stained with Coomassie Brilliant Blue showed that the purity of the recombinant albumins was in excess of 97%.

HPLC System

An ion exchange HPLC system was used in this study. The system was used to examine the redox state of albumin in serum and consisted of a Model MIX 100 double-plunger pump and a Model FP-2025 fluorescence detector (excitation wavelength, 280 nm; emission wavelength, 340 nm), in conjunction with a Model PU-2080 system controller, all obtained from Jasco Co., Tokyo, Japan. A Shodex Asahipak ES-502N (Showa Denko Co., Tokyo, Japan) ion-exchange column was used. The column temperature was 37°C. Measurements were carried out by solvent gradient elution with increasing ethanol concentrations from 0 to 5% in 0.05 M sodium acetate-0.40 M sodium sulfate (pH 4.85) and a flow rate of 1.0 mL/min. Samples were injected with fixed volumes of 5 μl of serum. To determine the value for each fraction of albumin, (i.e., f(HMA) = [HMA:(HMA + HNA-1 + HNA-2)], f(HNA-1) = [HNA-1:(HMA + HNA-1 + HNA-2)], and f(HNA-2) = [HNA-2:(HMA + HNA-1 + HNA-2)]) the HPLC profiles obtained were subjected to numerical curve fitting; each peakshape was approximated by a Gaussian function. The Student's *t*-test was used to evaluate the significance of the differences. Values are expressed as mean ± SD (12).

Oxidation of HSA and Serum by Chloramine-T

To prepare CT-HSA, HSA (15 μM) was incubated for 1 h in phosphate buffer (pH 8.0) at 37°C in an oxygen-saturated solution containing 0.1 mM ($CT_{0.1}$-HSA), 1 mM (CT_1-HSA), 10 mM (CT_{10}-HSA) or 50 mM (CT_{50}-HSA) chloramine-T. After incubation, the oxidation reactions were stopped by cooling followed by extensive dialysis of solutions against water. The control involved incubating albumin dissolved in buffer alone, and in all cases the proteins were freeze-dried after dialysis and stored at –20°C until used.

Amino Acid Residues Oxidized

Reactivity of ^{34}Cys Residue with DTNB

Albumin solutions 1.0×10^{-4} M, 2 mL 0.067 M phosphate buffer) were preincubated at 37°C. Absorbance increases at 412 nm were monitored against time after the addition of DTNB (final concentration 2.0×10^{-4} M) (13).

Oxidation of Met Residues

Oxidized HSA, mutated rHSA, and control albumins, all at a concentration of 10 μM, were reduced with dithiothreitol (100 mM) in the presence of EDTA (10 mM) in denaturing

buffer (6 M guanidine hydrochloride in 0.25 M Tris, pH 8.0). The samples were incubated for 16 h at 37°C. After this treatment, the albumins were pyridylethylated and then digested with CNBr (CNBr: Met molar ratio of 200:1) in the dark for 24 h at 37°C. The digestions were stopped by the addition of acetone. After evaporation of the acetone and washing with ethanol, the proteins were dissolved in 0.1% trifluoroacetic acid. Protein concentrations were determined by a Bradford assay (14), and the same amounts of proteins were used in the SDS-PAGE analysis. It is evident that the cleavage of oxidized HSAs was suppressed by the oxidation of Met residues.

Changes in Protein Net Charge

Changes in the net charge of albumin were evaluated by a modification of the capillary electrophoresis method described by Pande *et al.* (15). One mL of an albumin sample (2 μM) was run in 100 mM borate buffer (pH 9.0 and 20°C), and the migration time was determined by means of a CE990/990-10 type capillary electrophoresis from Jasco Co. (Tokyo, Japan).

Carbonyl Group Determination

Protein-bound carbonyl groups were quantitated using the method of Climent *et al.* (1). In summary, the groups were derivatized with fluoresceinamine and their number calculated from the absorbance of the complexes at 490 nm (Jasco Ubest-35 UV/VIS spectrophotometer).

Structural Properties of Native, Oxidized, and Mutant HSAs

Circular Dichroism (CD)

Measurements were done using a Jasco J-720 type spectropolarimeter (Jasco Co., Tokyo, Japan) at 25°C. Far-UV and near-UV spectra were recorded at protein concentrations of 20 μM in phosphate buffer using 1 mm and 1 cm quartz cells, respectively.

Effective Hydrophobicity of Native, Oxidized, and Mutant HSAs

The effective hydrophobicity of the albumins (1 μM), as dissolved in phosphate buffer, was probed with bis-ANS (10 μM) at 25°C. The compound was excited at 394 nm (16), and fluorescence spectra were recorded on a Jasco FP-770 fluorometer (Tokyo, Japan) using 1 cm quartz cells, thermostated devices and 5 nm excitation and emission band widths.

Effect on Aromatic Amino Acid Residues

Steady-state fluorescence measurements were made using a Jasco FP-770 fluorometer with 1 cm quartz cells and thermostated devices. All studies were performed using a protein concentration of 2 μM at 25°C using 5 nm excitation and emission band widths. A fluorescence excitation wavelength of 295 nm (tryptophan (Trp) residue) or 280 nm (tyrosine (Tyr) residues) was employed.

Absorbance spectra (200–400 nm) of the albumins (20 μM) were recorded at 25°C with 1 cm quartz cells by using the Jasco UV/VIS spectrophotometer.

Functional Properties of Native, Oxidized, and Mutant HSAs

Ligand Binding Experiments

To study the binding of ligands to the albumins, warfarin or ketoprofen was added to a solution of albumin (10 μM) in phosphate buffer to give a final drug concentration of 5 μM. The unbound ligand fractions were separated using an Amicon MPS-1 micropartition system with YMT ultrafiltration membranes by centrifugation (2000 g, 25°C, 40 min). Adsorption of warfarin or ketoprofen to the filtration membranes and apparatus was found to be negligible. The concentration of unbound ligand was determined by HPLC. The HPLC system consisted of a Hitachi 655A-11 pump and a Hitachi F1000 variable fluorescence monitor or a Hitachi 655A variable wavelength UV monitor. LiChrosorb RP-18 (Cica Merck, Tokyo, Japan) was used as the stationary phase. The mobile phase consisted of 200 mM sodium acetate buffer (pH 4.5)/acetonitrile (40:60, v/v) for warfarin and of 200 mM sodium acetate buffer (pH 4.5)/acetonitrile (60:40, v/v) for ketoprofen. The flow rates in both cases were 1 mL/min. Warfarin was quantitated fluorometrically by using 320 nm and 400 nm for excitation and emission, respectively, and ketoprofen was detected at 270 nm by means of UV monitoring. The unbound fraction (%) was calculated as follows: Unbound fraction (%) = [ligand concentration in filtered fraction/total ligand concentration (before ultrafiltration)] × 100.

Determination of Esterase-Like Activity

The reaction of *p*-nitrophenyl acetate with the albumins was followed spectrophotometrically at 400 nm (Jasco Ubest-35 UV/VIS spectrophotometer) by monitoring the rate of appearance of *p*-nitrophenol. The reaction mixtures contained 5 μM *p*-nitrophenyl acetate and 20 μM protein in phosphate buffer. Reactions were followed at 25°C. Under these conditions, a pseudo-first-order rate constant analysis could be applied (17), and the apparent hydrolysis rate constants (k_{obs}) were calculated.

***In Vivo* Experiments**

Animals

Male ddY mice (6-weeks old, 25–35 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). The animals were maintained under conventional housing conditions. This study was carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the United States National Institutes of Health.

Biodistribution Experiment

HSAs were radiolabeled with ^{111}In using DTPA anhydride as described previously (18). Each radiolabeled derivative was purified by gel-filtration chromatography using a Sephadex G-25 column eluted with 0.1 M acetate buffer (pH 6.0). The absorbency of the eluants was measured at 280 nm, and the protein-containing fractions were pooled. The resulting solution was concentrated and washed with 0.9% NaCl by ultrafiltration. The specific activity of each derivative was ap-

proximately 37 MBq/mg protein. The mice received a 0.1 mg/kg dose of a ^{111}In-HSA conjugate in saline by tail vein injection and were housed in metabolic cages for urine collection. At given time points, blood was collected from the vena cava with the animal under ether anesthesia, and plasma was obtained by centrifugation. The heart, lung, liver, spleen, and kidney were excised, rinsed with saline, weighed, and examined for radioactivity. The amount of radioactivity in urine was determined by collecting urine both excreted and remaining in the bladder. ^{111}In radioactivity was counted in a well-type NaI scintillation counter (ARC-500; Aloka, Tokyo, Japan).

Pharmacokinetic Analysis

Tissue distribution patterns of the ^{111}In-HSA derivatives were evaluated using organ uptake clearance according to a previous reported method (19). In the early period after injection, the efflux of ^{111}In radioactivity from organs is assumed to be negligible, because the degradation products of ^{111}In-labeled ligands using DTPA anhydride cannot easily pass through biological membranes (19). This assumption was supported by the fact that no ^{111}In was detectable in the urine. Using this assumption, organ uptake clearance was calculated by dividing the amount of radioactivity in an organ at 120 min by the area under the plasma concentration-time curve (AUC) at the same time point. AUC was calculated by fitting an equation to the plasma concentrations of the derivatives using the nonlinear least-squares program MULTI (20). Tissue distribution patterns were evaluated using tissue uptake clearances according to the integration plot analysis. Tissue accumulation at time t was proportional to the $AUC_{0\text{-}t}$. By dividing the tissue accumulation at time t (χt) and the $AUC_{0\text{-}t}$ by the plasma concentration (Ct), CL tissue was obtained from the slope of the plot of $\chi t/Ct$ versus $AUC_{0\text{-}t}/Ct$. A previous report (21) has shown, that ^{111}In is not suitable for evaluating the dynamic phase of a protein for which the *in vivo* half life is long. Therefore, we estimated the plasma half-life and liver clearance within the 120 min, period.

Statistics

Where possible, statistical analyses were performed using the Student's *t* test.

RESULTS

HPLC Studies on Serum and Oxidized Serum From Healthy Young Subjects

Figure 1 shows a representative HPLC profile of serum and oxidized serum from a healthy young subject, obtained by elution from an ES-502N column with an increasing ethanol concentration from 0 to 5% in the acetate-sulfate buffer (pH 4.85). Peaks 1, 2, and 3 in Fig. 1 correspond well to HMA, HNA-1, and HNA-2 with the retention times of 15.8, 25.8, and 31.2 min, respectively (Fig. 1). Increasing the chloramine-T concentration from 0 mM to 0.1 or 1 mM resulted in an increase in, especially, HNA-2 (Table I). When the chloramine-T concentration was increase to 10 or 50 mM (Table I), or the exposure time was increased from 3 min to 1 h (Fig. 1), the three protein peaks disappeared. The reason is that, under these conditions, HSA was modified to such an extent that it remained bound to column and could only be eluted at a high ethanol concentrations (not shown).

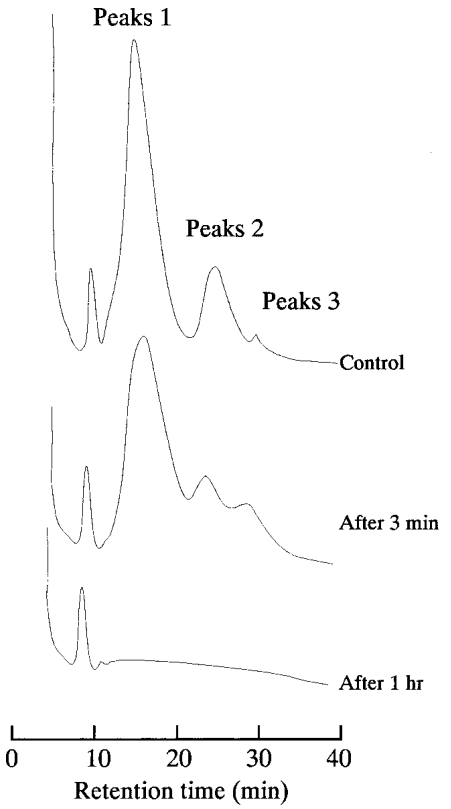

Fig. 1. HPLC profile of control serum and sera exposed to chloramine-T (1 mM) for 3 min or 1 h. The serum was from a healthy young male subject (23 years of age) and was eluted from an ES-502N column with an ethanol gradiant of 0 to 5% in acetate-sulfate buffer (pH 4.85). The ethanol concentration gradient was as follows; 0–5 min, 0%; 5–30 min, linear increase from 0 to 5%; 30–35 min, linear decrease from 5 to 0%; 35–40 min, 0%. Peaks 1, 2, and 3 correspond to HMA, HNA-1 and HNA-2, respectively, and their relative values are included in Table I.

Extent of Oxidation of Amino Acid Residues

The effect of oxidation on the reactivity of ^{34}Cys was examined using DTNB. The reactivity constants for pure HSA and rHSA were found to be the same (i.e., 0.07 min^{-1}). The constant was decreased only slightly for $CT_{0.1}$-HSA (i.e., 0.05 min^{-1} $P < 0.05$), and considerably decreased in the case of CT_1-HSA and CT_{10}-HSA (i.e., 0.02 min^{-1}; $P < 0.001$), and no reactivity at all could be detected for CT_{50}-HSA. As expected, no reactivity was found for the C34S mutant. These results indicate an increased oxidation of the cysteine residue with increasing concentrations of chloramine-T.

To examine the potential effect of the oxidations on the six Met residues of albumin, the proteins were reduced and treated with CNBr and then examined by SDS-PAGE. As seen in Fig. 2, the C34S mutant (lane 2) and $CT_{0.1}$-HSA (lane 3) showed the same fragmentation pattern as normal HSA (lane 1) and wild-type rHSA (not shown). However, in the case of CT_1-HSA (lane 4), a slight decrease in the amount of low-molecular weight fragments and a corresponding increase in high-molecular weight fragments and intact protein were observed. This tendency is more pronounced at higher con-

Table I. f(HMA), f(HNA-1) and f(HNA-2) Values (%) for HSA and Oxidized HSA in Serum from Normal Subjects[a]

	Serum	Serum + CT (0.1 mM)	Serum + CT (1 mM)	Serum + CT (10 mM)	Serum + CT (50 mM)
After 3 min					
f(HMA)	74.9 ± 0.99	72.4 ± 2.50	65.2 ± 3.38*	N.D.[b]	N.D.
f(HNA-1)	22.1 ± 1.51	22.5 ± 1.38	22.4 ± 3.95	N.D.	N.D.
f(HNA-2)	2.97 ± 1.03	2.62 ± 0.78	12.4 ± 1.28*	N.D.	N.D.
After 1 h					
f(HMA)	72.8 ± 2.11	61.2 ± 3.63*	N.D.	N.D.	N.D.
f(HNA-1)	23.9 ± 2.39	23.7 ± 4.23	N.D.	N.D.	N.D.
f(HNA-2)	3.21 ± 1.13	14.6 ± 1.23*	N.D.	N.D.	N.D.

[a] Average values of five experiments (± S.D.).
[b] N.D. denotes for not detectable.
* $P < 0.001$ as compared with Serum.

centrations of chloramine-T (lanes 5 and 6). These results show a concentration-dependent oxidation of methionine residues.

The net charge on the albumins was investigated by determining their migration times by capillary electrophoresis. The migration times for the C34S mutant (18.59 ± 0.11 min), $CT_{0.1}$-HSA (18.66 ± 0.08 min), CT_1-HSA (18.46 ± 0.04 min) and CT_{10}-HSA (18.45 ± 0.04 min) were the same or similar to that of HSA and rHSA (18.59 ± 0.11 min and 18.58 ± 0.19 min). By contrast, the migration time for CT_{50}-HSA was increased significantly (20.03 ± 0.42 min, $P < 0.005$). Thus, the net negative charge on albumin is increased, only at the highest chloramine-T concentrations used.

The oxidation of proteins frequently results in the formation of carbonylated amino acid residues. Therefore, the formation of such groups was measured as a function of chloramine-T concentration. The results (mol carbonyl groups/mol protein) showed concentration-dependent increases in the level of these groups: 0.098 ± 0.045 for $CT_{0.1}$-HSA, 0.158 ± 0.030 for CT_1-HSA, 0.331 ± 0.013 for CT_{10}-HSA and 0.769 ± 0.038 for CT_{50}-HSA. In comparison, the level in control HSA was only 0.045 ± 0.017.

Structural Properties of Native, Mutant, and Oxidized HSAs

The structural properties of the albumins were examined using several different spectroscopic methods. Figures 3A and 3B show the far-UV and near-UV CD spectra, respectively. As can be seen in Fig. 3A, the characteristics of the CD spectra of the C34S mutant, rHSA, $CT_{0.1}$-HSA and CT_1-HSA were similar to that of native HSA. In the case of CT_{10}-HSA and CT_{50}-HSA, the θ_{obs}-values are slightly higher in the range ca. 207–240 nm indicating some loss in α-helix structure. However, in all cases, the spectra were very different from that obtained for albumin, when dissolved in phosphate buffer, pH 7.4, containing 6 M guanidine hydrochloride. Fig. 3B shows that the tertiary structures of CT_{10}-HSA and CT_{50}-HSA, but not that of the other samples, had been modified. However, again the structural changes are not so pronounced as those observed for albumin that had been denatured with guanidine hydrochloride.

The effect of oxidation on the exposure of hydrophobic areas was examined using the fluorescence probe bis-ANS. The results (Fig. 4A) indicate that neither the mutation of ^{34}Cys nor oxidation at the low chloramine-T concentrations had any measurable effect on the accessible albumin hydrophobicity. However, treatment with higher concentrations of chloramine-T (CT_{10}-HSA and CT_{50}-HSA) resulted in an increase in accessible hydrophobic regions.

The effect of mutating ^{34}Cys and of the oxidations on the intrinsic fluorescence of albumin is shown in Fig. 4B. This fluorescence, which mainly is due to the excitation of ^{214}Trp, is only affected to a small extent by the mutation and treatment with 0.1 mM or 1 mM chloramine-T. In contrast, 10 mM

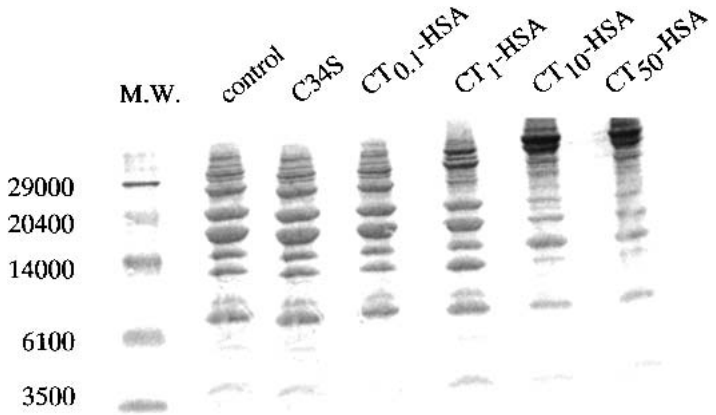

Fig. 2. SDS-PAGE electrophoresis of the reduced forms of control albumin (HSA = rHSA), the C34S mutant and chloramine-T oxidized HSAs treated with CNBr. Left lane, molecular weights of protein markers (M.W.). The gel was stained Coomassie Brillant Blue.

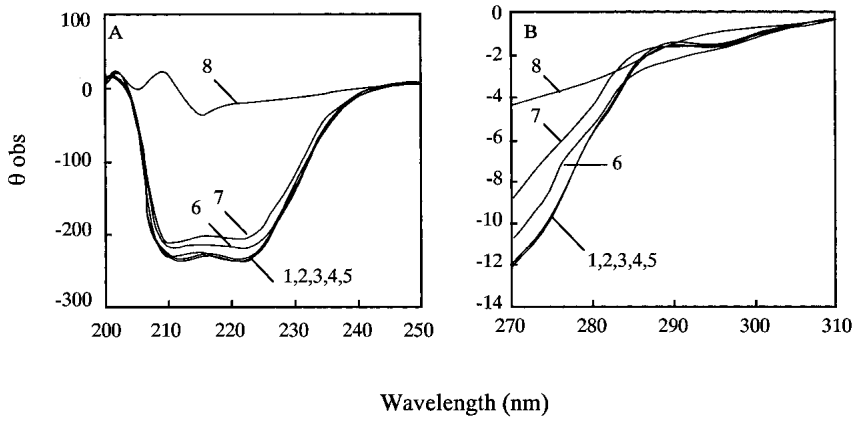

Fig. 3. Far-UV (A) and near-UV CD spectra (B) of native, mutated or oxidized HSAs. (1) native HSA, (2) wild-type rHSA, (3) the C34S mutant, (4) $CT_{0.1}$-HSA, (5) CT_1-HSA, (6) CT_{10}-HSA, (7) CT_{50}-HSA, (8) native HSA dissolved in phosphate buffer, pH 7.4, containing 6 M guanidine hydrochloride. Spectra are the averages of three determinations.

and especially 50 mM chloramine-T had a pronounced effect on the fluorescence spectrum. The fluorescence at λ_{max} was decreased to 76.8% and 23.8% of the normal level, respectively, and the λ_{max} was blue shifted in both cases from 339 nm to 333 nm. These findings suggest that in CT_{10}-HSA and CT_{50}-HSA minor conformational changes occurred in the vicinity of the Trp residue. However, the possibility that the Trp residue itself could have been oxidized cannot be excluded. The type of changes in light absorption spectra (data not shown) suggest that this seems to be the case for CT_{10}-HSA and CT_{50}-HSA (4).

The nature of the Tyr residues in the different albumins was examined by fluorescence measurements using an excitation wavelength of 280 nm and emission wavelengths from 290 nm to 370 nm (data not shown). In this case no effects as the result of the mutation or oxidations seem to have taken place.

Functional Properties of Native, Mutant and Oxidized HSAs

The unique drug binding properties of HSA can, to a great extent, be explained by the presence of two major binding sites; Site I and Site II (22), which are located within specialized cavities in subdomain IIA and IIIA, respectively (3). The potential effects of mutation and oxidation at these sites were examined using warfarin and ketoprofen as representative drugs. As seen in Table II, the high-affinity binding of warfarin which takes place at site I (23), was not affected by the mutation or mild oxidation by chloramine-T. However, binding was slightly decreased by a more severe oxidation of HSA (CT_{10}-HSA and CT_{50}-HSA). Essentially, the same effects, but to a more dramatic extent, were observed for the high-affinity binding of ketoprofen (Table II) which takes place at Site II (24).

Among its many functional properties HSA also possesses an esterase-like activity that is largely due to the close proximity of ^{410}Arg and ^{411}Tyr in Site II (17). Figure 5 shows that this activity is significantly reduced in the case of CT_{10}-HSA and, especially, CT_{50}-HSA, whereas the remaining albumins have comparable activities.

Pharmacokinetic Analysis of Native, Mutant, and Oxidized HSAs

In an attempt to evaluate whether the C34S mutation or the chloramine-T induced oxidations had any effect on the biological fate of HSA, plasma half-lifes and uptake clearance

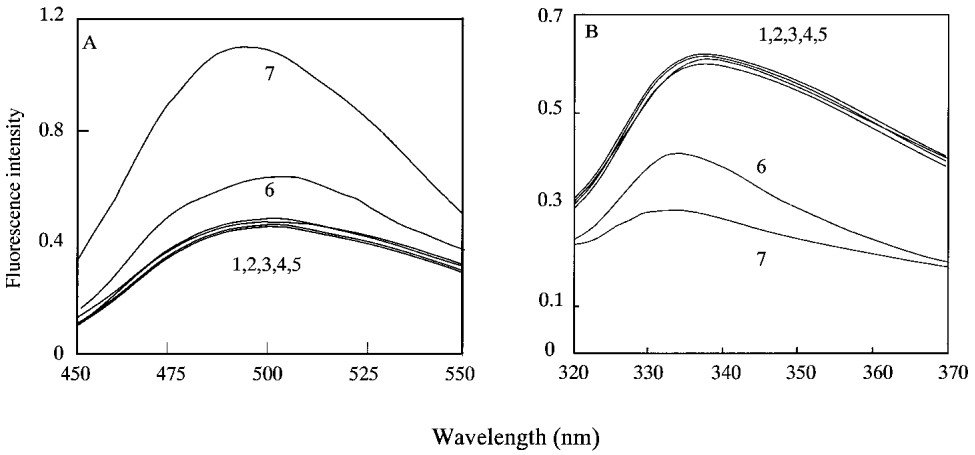

Fig. 4. Effect of different protein modifications on the fluorescence of albumin-bound bis-ANS (A) and on the intrinsic fluorescence of albumin as excited at 295 nm (B). (1) native HSA, (2) wild-type rHSA, (3) the C34S mutant, (4) $CT_{0.1}$-HSA, (5) CT_1-HSA, (6) CT_{10}-HSA, (7) CT_{50}-HSA. Spectra are the averages of three determinations.

Table II. Binding of Warfarin and Ketoprofen to Native, Mutated and Oxidized HSAs at pH 7.4 and 25°C[a]

Protein	Free fraction (%) (warfarin)	Free fraction (%) (ketoprofen)
Native HSA	24.71 ± 2.07	3.78 ± 0.52
Wild-type rHSA	25.48 ± 1.14	3.92 ± 0.67
C34S	25.01 ± 1.33	3.99 ± 1.17
$CT_{0.1}$-HSA	26.54 ± 1.93	3.83 ± 2.11
CT_1-HSA	27.41 ± 0.93	3.89 ± 2.34
CT_{10}-HSA	31.26 ± 1.54***	33.9 ± 2.44**
CT_{50}-HSA	35.60 ± 1.01**	46.5 ± 3.58*

[a] Average values of three experiments (± S.D.). * $P < 0.001$, ** $P < 0.01$ and *** $P < 0.05$ all as compared with native HSA.

Table III. Half-Lifes and Liver Clearances of ^{111}In-HSAs in Mice[a]

Protein	Half-life (min)	Liver uptake clearance (μL/h)
Native HSA	20.7 ± 1.26	13.37 ± 2.57
Wild-type rHSA	20.1 ± 2.85	13.47 ± 2.71
C34S	19.2 ± 1.39	14.00 ± 1.37
$CT_{0.1}$-HSA	20.8 ± 1.40	12.15 ± 1.98
CT_1-HSA	20.5 ± 2.40	13.17 ± 1.86
CT_{10}-HSA	20.3 ± 2.93	12.93 ± 2.12
CT_{50}-HSA	8.69 ± 1.39*	146.0 ± 8.91*

[a] Average values of three experiments (± S.D.). * $P < 0.001$ as compared with native HSA.

of the HSAs were determined in mice (Table III). Except for CT_{50}-HSA, the albumins had the same half-life (i.e., about 20 min). To better understand the reason for the decreased plasma half-life of CT_{50}-HSA (8.69 min) organ uptakes was studied. In Table III, the decreased plasma half-life of CT_{50}-HSA can be explained by a very pronounced increment in liver clearance. By contrast, neither the mutation nor the milder oxidation of albumin had any effect on liver clearance. The uptake by heart, lung, spleen, and kidney of CT_{50}-HSA, and the other modified albumins, were comparable to those of native albumin (results not shown).

DISCUSSION

The Validity of Chloramine-T as an Oxidant

Albumin, which is thought to have antioxidant properties, is the most abundant protein in extracellular fluids. Oxidation of protein sulfhydryl groups to mixed disulfides and their conversion to sulfhydryls by reduction might represent an effective antioxidant system in extracellular fluids. In this sense, albumin, which possess a single sulfhydryl residue (^{34}Cys), is responsible for the largest fraction of reactive sulfhydryl groups in extracellular fluids and the mercapt-nonmercapt conversion (intermolecular sulfhydryl disulfide exchange reaction) of serum albumin might be a component in such a system. In this study, the authors attempted to construction *in vivo* oxidation model of albumin by treatment with CT using on HPLC system. As shown in Fig. 1, in the case of serum treated with CT (1 mM) for 3 minutes, the f(HMA) value of 65.2% was significantly lower than that of 74.9% for healthy young male subjects. A significant decrease in the f(HMA) value as a result of aging and some diseases has previously been reported (10,12). The HPLC profile observed in Fig. 1 was similar to that of serum treated with CT (0.1 mM) for 1 h (Table. I). This finding leads to the suggestion that the oxidation of HSA is dependent on the concentration of oxidant as well as incubation time used for the oxidation and that HSA treated with CT for extended periods is easily oxidized even when low concentration of oxidant are used. Therefore, the authors used this oxidant and carried out a detail examination of the importance of ^{34}Cys and other residues relative to antioxidant properties of HSA.

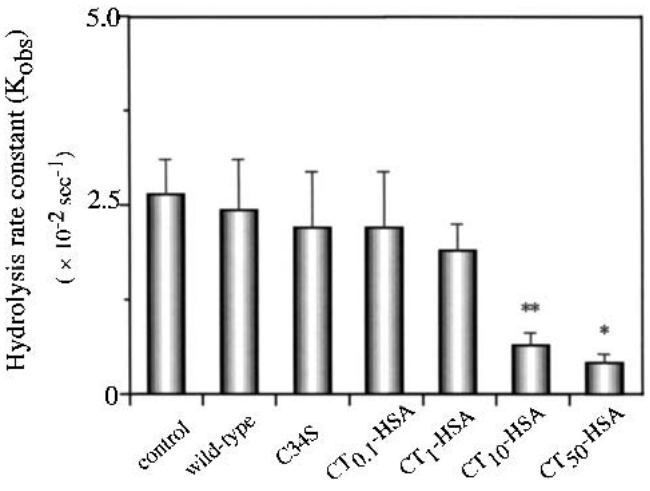

Fig. 5. Effect of mutation and oxidation on the rate constants (K_{obs}) for the hydrolysis of *p*-nitrophenyl acetate by albumin at pH 7.4 and 25°C. The data are average values of three experiments (± S.D.). * $P < 0.001$ and ** $P < 0.01$ both as compared with control.

Amino Acid Residues Oxidized

The type and number of oxidized amino acid residues of HSA increase with the chloramine-T concentration. The active oxidants in this system are $HO^{\cdot}$ and chloro radicals (4). In $CT_{0.1}$-HSA and CT_1-HSA, only ^{34}Cys and Met residues were affected. Increasing the chloramine-T concentration to 10 mM oxidized Met residues were affected to a more pronounced extent and apparently affected ^{214}Trp as well. Finally, in CT_{50}-HSA no reactivity of ^{34}Cys was detected, Met residues and ^{214}Trp (or its surroundings) were intensively modified and the net charge of HSA became more negative. The latter observation is explained by the oxidation of arginine, lysine, or proline residues to λ-glutamyl semialdehyde (1).

Structural Properties

The mild oxidation of HSA ($CT_{0.1}$-HSA and CT_1-HSA) has no measurable effect on its secondary and tertiary structures. However, in CT_{10}-HSA and, especially, CT_{50}-HSA slight decreases in α-helical contents were observed accompanied by tertiary conformational changes. These were detectable by near-UV CD and resulted in an increased exposure of hydrophobic portions of the protein. Large changes in intrinsic fluorescence and light absorption spectra were also observed. Although these changes are consistent with oxidation of ^{214}Trp, they could also, at least partly, reflect conformational changes in the protein in the vicinity of that residue.

In contrast, no conformational changes of rHSA were observed upon mutating Cys in position 34 to a serine.

Functional Properties

The high-affinity binding of warfarin and ketoprofen was studied to examine whether the exceptional drug binding properties of HSA had been affected by the mutation and the oxidations. In both examples, only CT_{10}-HSA and CT_{50}-HSA showed reduced drug binding properties. The decreased binding of warfarin to CT_{10}-HSA can be explained by conformational changes taking place in subdomain IIA and the oxidation of one or more of the Met residues at positions 298, 329 or 446. In CT_{50}-HSA these explanations could be supplemented with oxidation of relevant lysine or arginine residues. However, in both cases, the oxidation of ^{214}Trp could contribute to the diminished binding, because a previous study has shown that mutation of ^{214}Trp to an alanine results in decreased warfarin binding (25). The decreased ketoprofen binding to CT_{10}-HSA and CT_{50}-HSA is most likely the result of conformational changes involving site II in subdomain IIIA (7). The decreased binding to the latter protein can also be caused by oxidation of ^{410}Arg.

The esterase-like activity of HSA is caused by the proximity of ^{410}Arg and ^{411}Tyr in subdomain IIIA (17). This function is only impaired in the case of CT_{10}-HSA and CT_{50}-HSA. The finding is probably the result of conformational changes that affect the orientation of the two residues. In the latter case, however, ^{410}Arg could have been oxidized as well.

Pharmacokinetics

Neither the mutation of ^{34}Cys for a serine nor the relatively extensive oxidation of HSA (CT_{10}-HSA) had any effect on its plasma half-life or liver clearance in mice (Table III). However, CT_{50}-HSA was modified to such a degree that its plasma half-life was decreased to a considerable exist from ca. 20 min to ca. 9 min. This finding can be explained by a ca. 11-fold increase in liver uptake clearance. The increased liver clearance is probably due to an increased endocytosis of the protein by scavenger receptors of endothelial, Kupffer cells (26). In contrast to liver clearance, the uptake by heart, lung, spleen, and kidney were in all cases unaffected by the protein modifications. The authors have not estimated the clearance of other tissues or organs. However, in the future detailed examinations of this will be carried out using cell lines.

CONCLUSION

As illustrated by the column experiments with serum, the authors succeeded in constructing an oxidation model using HSA. Functional studies, performed *in vitro,* with the different albumin preparations indicate that Site II is more effected than Site I and ^{34}Cys in HSA, when it is exposed to oxidative stress.

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