Effect of Oxidative Stress on the Structure and Function of Human Serum Albumin

Makoto Anraku,[1] Keishi Yamasaki,[1] Toru Maruyama,[1] Ulrich Kragh-Hansen,[2] and Masaki Otagiri[1,3]

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Purpose. Human serum albumin (HSA) was mildly oxidized by a metal-catalyzed oxidation system (MCO-HSA), chloramine-T (CT-HSA) or H_2O_2 (H_2O_2-HSA), and the effects of these treatments on the structural, drug-binding and esterase-like properties were studied.
Methods. Protein conformation was examined by calorimetric, chromatographic, electrophoretic and spectroscopic techniques. Drug binding was studied by ultrafiltration method, and esterase-like activity was determined using *p*-nitrophenyl acetate as a substrate.
Results. Far-UV and near-UV CD spectra indicated that significant structural changes had occured as the result of treatment with MCO-HSA and CT-HSA but not with H_2O_2-HSA. However, SDS-PAGE analysis does not provide precise information on gross conformational changes such as fragmentation, cross-linking and SDS-resistant polymerisation. The results of differential scanning calorimetry, the fluorescence of the hydrophobic probe 1,1-bis-4-anilino-naphthalene-5,5-sulfonic acid and the elution time from a hydrophobic HPLC column indicated that MCO-HSA and CT-HSA in particular, have a more open structure and a higher degree of exposure of hydrophobic areas than unoxidized HSA. In all cases, high-affinity binding of warfarin remained unchanged for all the oxidized HSAs. However, high-affinity binding of ketoprofen to CT-HSA and, especially, MCO-HSA was diminished. In addition, the esterase-like activity of these proteins were all decreased to the same low level.
Conclusions. Mild oxidation of HSA has no detectable effect on the binding of drugs to site I in subdomain IIA. In contrast, both the ligand binding property of site II and the esterase-like activity of oxidized HSAs are decreased, most probably due to conformational changes in subdomain IIIA.

KEY WORDS: human serum albumin; oxidative stress; structural changes; warfarin binding; ketoprofen binding; esterase-like activity.

INTRODUCTION

Human serum albumin (HSA) is the most abundant protein in mammalian plasma and is generally considered to be a multifunctional transport protein. Thus, several lines of evidence exist which indicate that albumin also has significant antioxidant activity (1–6) and that the protein, in fact, may represent the major and predominant circulating antioxidant in plasma, which is known to be exposed to continuous oxidative stress (3). A direct protective effect of albumin is indicated from many epidemiological studies (3). The results of *in vitro* experiments also lend support to this hypothesis because they show that albumin protects human low density lipoproteins against copper-mediated oxidation and blood against hemolysis by free radicals (3), protects primary cultures of murine mϕ and renal tubular epithelial cells against reactive oxygen species (4) as well as, in the form of serum, protects porcine thoracic aorta endothelial cells and certain types of porcine lung fibroblasts against affect by free radicals (6). The antioxidant properties of albumin can also help protecting the eyes against contact lens disinfectant solutions which often contain 3% H_2O_2 (7).

HSA is a single, nonglycosylated polypeptide which is organized in the form of a heart-shaped protein having about 67% α-helix but no β-sheet (8,9). The protein has three homologous domains (I–III), each of which is comprised of two subdomains, (A and B), that possess common structural elements. All but one of the thirty-five cysteine (Cys) residues are involved in the formation of stabilizing disulfide bonds. Several studies, on the antioxidant activity of HSA in different clinical situations, have focused on the importance of this single, free sulfhydryl group of ^{34}Cys (10–12). In the present work, we report on an evaluation of the importance of other amino acid residues as well by mild oxidation of HSA by a metal-catalyzed oxidation system (MCO-HSA) (13), chloramine-T (CT-HSA) (14) or H_2O_2 (H_2O_2-HSA) (15). The impact of the oxidative modifications on albumin structure was studied by circular dichroism (CD), differential scanning calorimetry (DSC), the use of a hydrophobic HPLC column and a variety fluorescence methods.

HSA is also able to serve as a depot and transport protein in the circulation, because it can bind reversibly a large number of endogenous and exogenous compounds. Two major drug binding regions, Site I and Site II (16), are located in subdomain IIA and IIIA, respectively (8). In order to test whether the molecular changes associated with the oxidations have an effect on the drug binding properties of albumin, the high-affinity binding of warfarin (Site I-ligand) and ketoprofen (Site II-ligand) was examined for different protein preparations by ultrafiltration. Since ketoprofen binding, in contrast to warfarin binding, was affected in most cases, we also examined the potential effects of oxidation on the esterase-like activity of albumin, an activity which is associated with Site II (17).

MATERIALS AND METHODS

Materials

HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). It was defatted by treatments with an equaous suspension of activated charcoal at 0°C, after which it was acidified with H_2SO_4 to pH 3, deionized, freeze-dried and stored at −20°C until used (18). HSA and oxidized HSAs gave only one band on SDS-PAGE (data

[1] Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan.
[2] Guest professor at Kumamoto University. Permanent address: Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark.
[3] To whom correspondence should be addressed. (e-mail: otagirim@gpo.kumamoto-u.ac.jp)

ABBREVIATIONS: HSA, human serum albumin; MCO-HSA, metal-catalyzed oxidized HSA; H_2O_2-HSA, HSA oxidized by hydrogen peroxide; CT-HSA, HSA oxidized by chloramine-T; DTNB, 5,5′-Dithiobis (2-nitro benzoic acid); Bis-ANS, 1,1-bis-4-anilino-naphthalene-5,5-sulfonic acid; CNBr, cyanogen bromide; CD, circular dichroism; DSC, differential scanning calorimetry; Cys, cysteine; Met, methionine; Trp, tryptophan; Tyr, tyrosine.

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not shown), and the molecular mass of all the albumins were assumed to be 67 kDa. Chloramine-T and 5,5′-Dithiobis (2-nitro benzoic acid) (DTNB) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and 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. 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.

Oxidation of HSA

For preparing MCO-HSA, HSA (300 μM) was incubated in phosphate buffer at 37°C in an oxygen-saturated solution containing sodium ascorbate (100 mM) and $FeCl_2$ (10 μM) (13). Ascorbate was added for reducing the Fe^{3+} formed back to Fe^{2+}. Aliquots were withdrawn after different time intervals, and the oxidative process was terminated by cooling and removing the oxidants by extensive dialysis against water. H_2O_2-HSA (300 μM) and CT-HSA (300 μM) were prepared in phosphate buffer by aerobic incubation at 37°C with hydrogen peroxide (200 mM) (15) and chloramine-T (10 mM) (14), respectively. Aliquots were withdrawn at different time intervals, and the oxidative processes were stopped by cooling and the addition of acetone. After the reactions were stopped the aliquots were dialyzed extensively against water.

Prior to dialysis, all solutions contained 0.05% NaN_3. For all three types of modification, controls were made by 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

Carbonyl Group Determination

Protein-bound carbonyl groups were quantitated using the method of Climent *et al.* (19). In summary, the groups were derivatized with fluoresceinamine and their number calculated from the absorbancy of the complexes at 490 nm (Jasco Ubest-35 UV/VIS spectrophotometer). However, in our hands the reproducibility of the results was improved by increasing the amount of reactants by 40-fold.

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.* (20). One mL of a HSA sample (2 μM) was run in 100 mM borate buffer (pH 8.5 and 20°C), and the migration time was determined by using a CE990/990-10 type capillary electrophoresis from Jasco Co. (Tokyo, Japan).

Oxidation of Methionine Residues

Oxidized HSA (10 μM) and control HSA (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 : Methionine 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 acetone and washing with ethanol, the proteins were dissolved in 0.1% trifluoroacetic acid. The protein concentrations were determined by a Bradford assay (21), and the same amounts of proteins were used in the SDS-PAGE analysis (22). It is evident that the cleavage of oxidized HSAs were suppressed by the oxidation of Met residues.

Reactivity of ^{34}Cys Residue with DTNB

A HSA solution (1.0×10^{-4} M, 2ml 0.067M phosphate buffer) was preincubated at 37°C. The absorbance increase at 412 nm was monitored against time after the addition of DTNB (final concentration 2.0×10^{-4} M) (23).

Structural Properties of Native and Oxidized HSAs

Circular Dichroism (CD)

The 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.

Differential Scanning Calorimetry (DSC)

For a thermodynamic evaluation of the structural properties of native and oxidized HSA, DSC was carried out on a MicroCal MC-2 ultrasensitive DSC (micro-Cal Inc., Northampton, MA, USA) at heating rates of 1 K/min, using sample concentrations of 100 μM. The obtained DSC data were applied to nonlinear fitting algorithms, in order to calculate the thermodynamic parameters, thermal denaturation temperature (Tm), calorimetric enthalpy (ΔH) and the van't Hoff enthalpy (ΔHv), from the temperature dependence of excess molar heat capacity, Cp, by employing Using Origin™ scientific plotting software.

Effective Hydrophobicity of Native and Oxidized HSAs

The effective hydrophobicity of oxidized and control HSAs (1 μM), as dissolved in phosphate buffer, was probed with Bis-ANS (10 μM) at 25°C. The compound was excited at 394 nm (24), 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.

Effective hydrophobicity was also evaluated by HPLC chromatography by using a TSK-Gel Phenyl-5PW column (7.5 mm × 75 mm), obtained from Tosoh (Tokyo, Japan). The HPLC system consisted of a Hitachi 655A-11 pump and a Hitachi 655A variable wavelength UV monitor set at 280 nm. The proteins were eluted using the following ratios of solvent A (1.5 M $(NH_4)_2SO_4$) and solvent B (phosphate buffer): from 0 to 5 min the gradient was changed linearly from 50:50 to 13:87, from 5 to 25 min to 0:100 and then maintained at that value for an additional 15 min.

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 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 were recorded at 25°C with 1 cm quartz cells by using the Jasco UV/VIS spectrophotometer.

Functional Properties of Native and Oxidized HSAs

Ligand Binding Experiments

To study the binding of ligands to HSA and oxidized HSAs, 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 the Amicon MPS-1 micropartition system with YMT ultrafiltration membranes by centrifugation (2000 g, 25°C, 40 min). The 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 HSA and the oxidized HSAs 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, pseudo-first-order rate constant analysis could be applied (17), and the apparent hydrolysis rate constants (k_{obs}) were calculated.

Statistics

Where necessary, statistical analyses were performed by the Student *t* test.

RESULTS

The Oxidation Processes as a Function of Time

Most of the information concerning parameters such as molar ratios between the reactants, the temperature of incubation and how best to stop the reactions is available in the literature (13–15). However, information concerning incubation times, does not appear to be available.

The oxidation of proteins usually results in the formation of carbonylated amino acid residues. Therefore, the formation of such groups was measured as a function of time. As seen in Fig. 1, a clearly detectable increase in the amount of albumin-bound carbonyl groups was found for MCO-HSA and CT-HSA. The formation of these groups reached a plateau after 144 h and 10 h of incubation, respectively, and longer times of incubation resulted in the developement of turbidity, indicating the denaturation of the protein. In contrast, treatment with H_2O_2 resulted in the formation of relatively few carbonyl groups, and a prolonged incubation failed to increase the amount of these groups. Incubation times of 144 h, 10 h, and 12 h for MCO-HSA, CT-HSA, and H_2O_2-HSA, respectively, appear to be a reasonable compromise between the efficiency of oxidation and non-denaturing conditions for the protein.

Extent of Oxidation of Amino Acid Residues

As a first step, we determined the amount of carbonyl groups in the three types of oxidized HSAs after fixed incubation times. The results (mol carbonyl groups/mol protein) showed pronounced increases in the level of these groups in MCO-HSA (0.144 ± 0.007 (S.D.), n = 3) and CT-HSA (0.145 ± 0.004), but only a moderate increase in the case of H_2O_2-HSA (0.056 ± 0.003) as compared with control HSA (0.037 ± 0.002).

The net charge of the albumins was investigated by determining their migration times in capillary electrophoresis (Fig. 2). The migration time for H_2O_2-HSA (7.01 ± 0.09 min (S.D.), n = 3) was only slightly increased as compared with that of normal HSA (6.81 ± 0.21 min). However, the times for MCO-HSA (9.48 ± 0.25 min) and CT-HSA (9.80 ± 0.13 min) were increased considerably more, indicating additional nega-

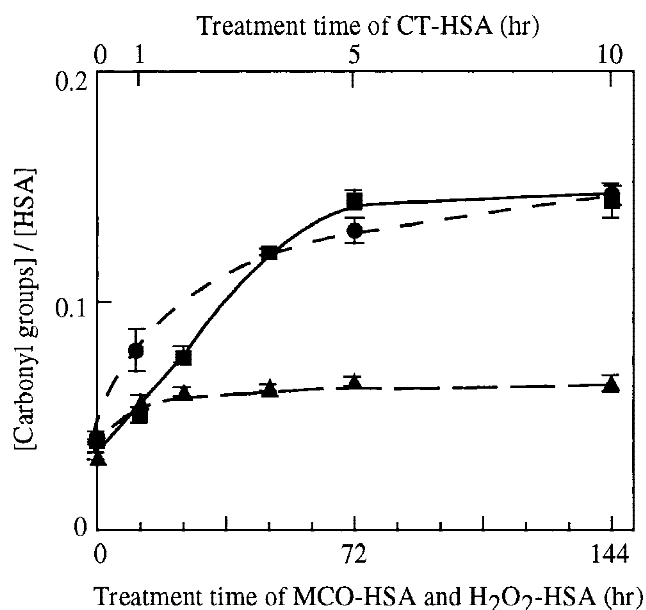

Fig. 1. Carbonyl content of oxidized HSAs as a function of incubation time. ■: MCO-HSA, ●: CT-HSA, ▲: H_2O_2-HSA. The bars represent standard deviations (n = 3).

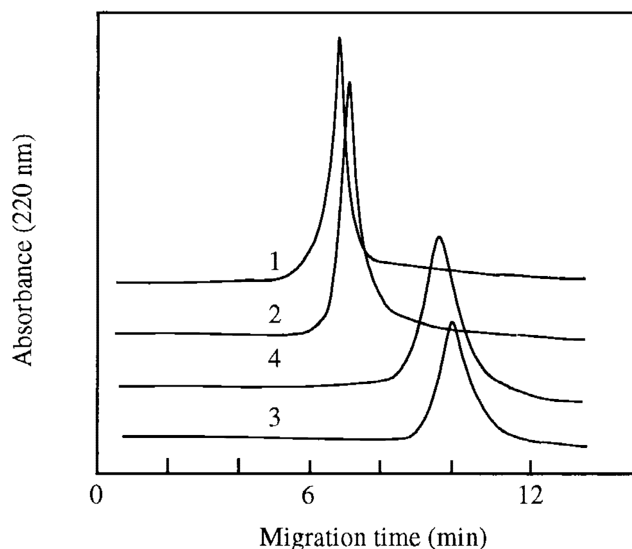

Fig. 2. Electrophoretograms of native and oxidized HSAs. (1) Native HSA, (2) H_2O_2-HSA incubated for 12 h, (3) CT-HSA, (4) MCO-HSA incubated for 144 h, (4) MCO-HSA incubated for 10 h. The absorbance spectra are the averages of three determinations.

tive net charges and, therefore, a greater extent of modification of these albumins.

To examine the potential effect of the oxidations on the six methionine residues of albumin, the proteins were reduced and treated with CNBr and then examined by SDS-PAGE. As seen in Fig. 3, MCO-HSA (lane 2) showed the same fragmentation pattern as normal HSA (lane 1). In the case of H_2O_2-HSA (lane 3), however, a lower amount of low-molecular weight fragments was found. The most pronounced effect was observed for CT-HSA (lane 4). In this case, mainly high-molecular weight fragments and intact protein were detected.

The possible oxidation of only one Cys residue, ^{34}Cys was determined by the reactivity of ^{34}Cys with DTNB on the native and oxidized HSA. CT-HSA and H_2O_2-HSA showed an average reduction in the reactivity of ^{34}Cys of about 3% compared with that of native albumin, whereas only negligeble changes were found in the case of MCO-HSA (data

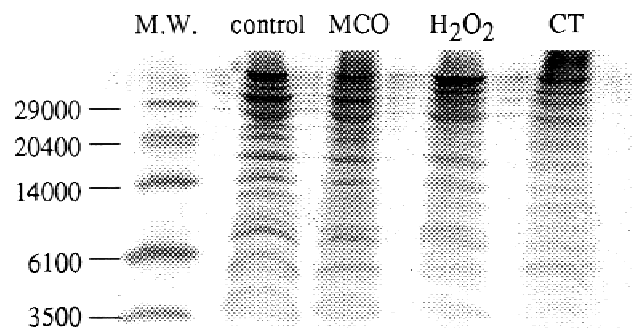

Fig. 3. Pattern of SDS-PAGE electrophoresis of the reduced forms of native and oxidized HSAs treated with CNBr. The lanes represent: control; native HSA, MCO; MCO-HSA, H_2O_2; H_2O_2-HSA, CT; CT-HSA. Left lane, molecular weights of protein markers (M.W.). The gel was stained by using Coomassie Brillant Blue and then computer scanned.

not shown). These results indicate the possibility that ^{34}Cys is oxidized to some extent in the case of CT-HSA and H_2O_2-HSA.

Structural Properties of Native and Oxidized HSAs

The structural properties of the oxidized HSAs were examined by a variety of methods. Thus, Fig. 4A and 4B show the far-UV and near-UV CD spectra, respectively. As can be seen in Fig. 4A, the characteristics of the CD spectra of the three oxidized proteins were similar to that of native HSA. In the case of MCO-HSA and CT-HSA, the θ_{obs}-values are slightly higher in the range ca. 207–240 nm. 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. These findings indicate that the oxidative processes have, at the most, only a slight affect on the secondary structures of albumin. Figure 4B shows that the tertiary structures of MCO-HSA and CT-HSA, but not that of H_2O_2-HSA, have been modified. However, again the structural changes are not so pronounced as those observed for albumin which had been denatured with guanidine hydrochloride.

The effect of the oxidations on the thermal denaturation of albumin was examined by means of DSC measurements. As seen in Table I, the denaturation temperature (Tm) was not affected by oxidation. In contrast, both the denaturation enthalpy (ΔH) and the van't Hoff enthalpy (ΔHv) were diminished as follows: native HSA > H_2O_2-HSA > CT-HSA > MCO-HSA. Therefore in these results, broading of these peaks was made in that order (data not shown). The progressive decrease in the ΔH-values suggests that the oxidized forms are progressively more easily denatured which again implies that the protein structures are more open (i.e., more hydrophobic regions are exposed to the solvent) at 25°C. The parallel diminution in the ΔHv-values indicates that, although the thermal denaturation of the proteins takes place at the same temperature, the denaturation process involves an increasing number of intermediary steps.

The effect of oxidation on the exposure of hydrophobic areas was also examined by using the fluorescence probe bis-ANS and the phenyl-substituted HPLC column. The results obtained with bis-ANS (Fig. 5) indicate that the formation of CT-HSA and of MCO-HSA in particular, invove the formation of increased accessible hydrophobic regions, whereas only small changes take place in the case of H_2O_2-HSA. The results obtained with the hydrophobic column are consistent with these findings, since the elution time of control HSA was 6.35 ± 0.10 min (S.D., n = 3), whereas those of H_2O_2-HSA, CT-HSA and MCO-HSA were increased to 7.72 ± 0.24 min, 9.36 ± 0.19 min and 10.02 ± 0.11 min, respectively.

The effect of the oxidations on the single Trp residue in albumin was monitored by fluorescence measurements (Fig. 6A). Treatment with H_2O_2 resulted in a small increment in the fluorescence maximum (by 3.4%) but it had no effect on λ_{max} which was the same as that observed for native HSA (338 nm). In contrast, MCO-HSA and especially CT-HSA had much lower fluorescence intensities at λ_{max}, 62.1% and 45.8% of the normal, respectively, and λ_{max} itself was, in both cases, blue shifted to 333 nm. These findings suggest that in MCO-HSA and CT-HSA minor conformational changes had taken place in the vicinity of the Trp residue (25). However,

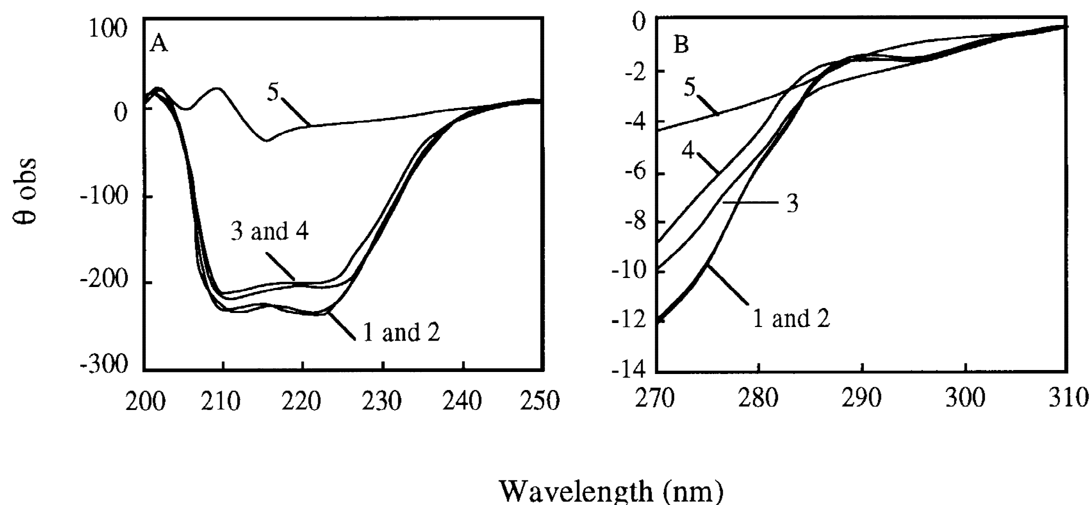

Fig. 4. Far-UV (A) and near-UV CD spectra (B) of native and oxidized HSAs. (1) native HSA, (2) H_2O_2-HSA, (3) CT-HSA, (4) MCO-HSA, (5) native HSA dissolved in phosphate buffer, pH 7.4, containing 6 M guanidine hydrochloride. In all cases, the protein concentration was 20 μM. The spectra are the averages of three determinations.

the possibility also exists that the Trp residue itself could have been oxidized. According to the light absorption spectra shown in Fig. 6B, this seems indeed to be the case for both MCO-HSA and CT-HSA.

The status 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 oxidations appear to have taken place.

Functional Properties of Native and Oxidized HSAs

The unique ligand binding properties of HSA can, to a great extent, be explained by the presence of two major binding sites; Site I and Site II (16), which are located within specialized cavities in subdomain IIA and IIIA, respectively (8). The potential effect of oxidation on these sites was examined by using warfarin and ketoprofen as representative ligands. As seen from Table II, the high-affinity binding of warfarin, which takes place at Site I (2), was not affected by any of the oxidants. In contrast, high-affinity binding of the Site II-ligand ketoprofen (26) was considerably diminished in the case of CT-HSA and even more ($p < 0.02$) in the case of MCO-HSA (Table II). The pronounced difference between the free fractions of warfarin and ketoprofen in the case of native HSA (Table II) is due to differences in the primary binding constants, which are 3.3×10^5 M^{-1} for warfarin (2) and 2.5×10^6 M^{-1} for ketoprofen (27).

HSA also possesses an esterase-like activity which is largely due to the close proximity of ^{410}Arg-and ^{411}Tyr in Site II (17). Since ligand binding to this site, in contrast to Site I, was effected in MCO-HSA and CT-HSA we also examined the enzymic properties of the albumin preparations using *p*-nitrophenyl acetate as a substrate. From Table III, it can be seen that the activity of H_2O_2-HSA is the same as that of native HSA ($p > 0.1$). In contrast, the activities of MCO-HSA and CT-HSA were much reduced and to the same level ($p > 0.1$).

Table I. Thermodynamic Parameters for Thermal Denaturation of Native and Oxidized HSAs[a]

Protein	Tm (°C)	ΔH ($\times 10^5$ J/mol)	ΔHv/ΔH
Native HSA	59.58 ± 0.05	6.91 ± 0.13	0.69 ± 0.01
H_2O_2-HSA	59.45 ± 0.06	5.13 ± 0.19	0.65 ± 0.02
CT-HSA	58.25 ± 0.10	4.04 ± 0.21	0.42 ± 0.03
MCO-HSA	58.10 ± 0.13	3.13 ± 0.11	0.38 ± 0.02

[a] The data are average values of three experiments (± S.D.).

DISCUSSION

Structural Aspects

Covalent modification of proteins and other macrostructures by oxidative systems has been implicated in various

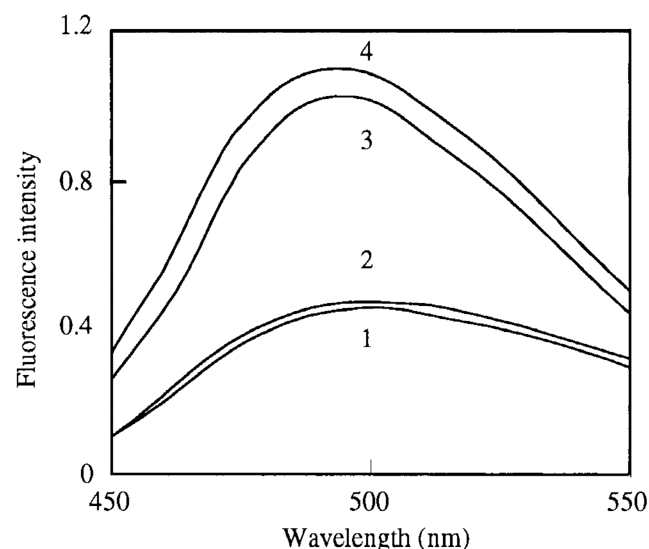

Fig. 5. Effect of native and oxidized HSAs on the fluorescence of bis-ANS. (1) native HSA, (2) H_2O_2-HSA, (3) CT-HSA, (4) MCO-HSA. The concentration of bis-ANS was 10 μM, whereas that of the albumins was 1 μM. The spectra are the averages of three determinations.

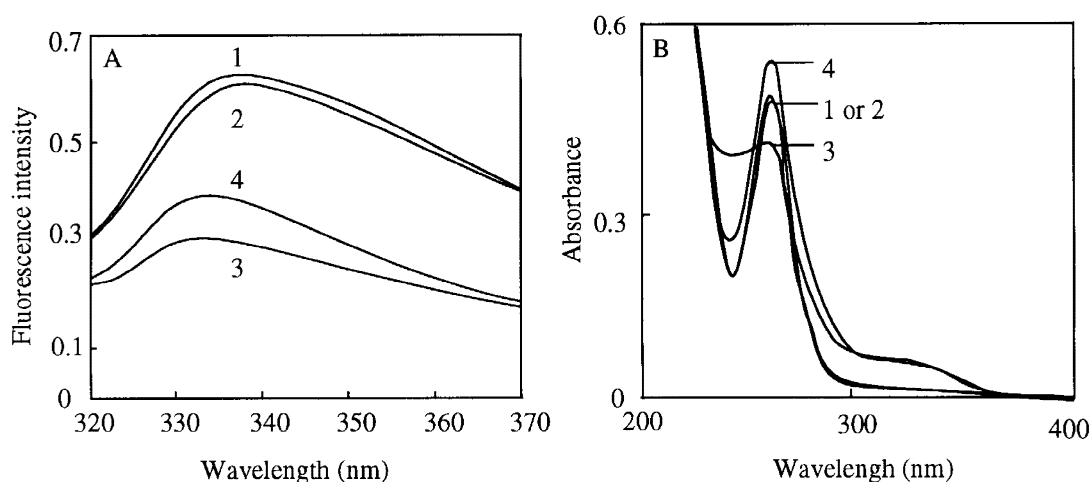

Fig. 6. Intrinsic fluorescence spectra (A) and light absorption spectra (B) of native and oxidized HSAs. The protein concentration was 2 μM (A) or 20 μM (B). (1) native HSA, (2) H_2O_2-HSA, (3) CT-HSA, (4) MCO-HSA. The spectra are the averages of three determinations.

pathological conditions. Albumin seems to be able to provide protection against such systems, and recently the antioxidant activity of HSA has received considerable attention. However, detailed information about how this activity affects HSA itself and its other functions is scarce. Therefore, in an attempt to better understand this situation, we studied the effect of three different oxidative systems on the structure, ligand binding properties and esterase-like activity of HSA.

HSA was mildly oxidized by treatment with a metal-catalyzed oxidation system (MCO-HSA), H_2O_2 (H_2O_2-HSA) or chloramine-T (CT-HSA) because these are widely used as an *in vitro* oxidation model. In general, these oxidants are known to generate different radicals. For example, metal-catalyzed oxidation and chloramine-T generate OH and chloro radicals, and H_2O_2 involves the formation of active oxygen. This was done in order to study the early alterations of albumin structure, and, on an average, less than one carbonyl group was detected per albumin molecule (Fig. 1). In addition, as judged by capillary electrophoresis (Fig. 2), only minor or small changes in the net charge on albumin was introduced. According to the literature, proline, lysine, arginine, and/or histidine residues, but apparently not the Cys residue, have been modified in the case of MCO-HSA (13,28). Our data (Fig. 6) strongly suggest that the Trp residue (^{214}Trp) has been oxidized as well. According to the detailed study of Finch *et al.* (15), the SH-group of ^{34}Cys and Met residues have been oxidized in the case of H_2O_2-HSA. The protocol for preparing CT-HSA was adopted from a study of human high density lipoproteins (Apo AI and Apo AII) (14). According to these authors, chloramine-T also oxidizes Cys and Met residues. Due to its high sensitivity to free radicals (10–12) the Cys residue of HSA is most probably also oxidized in the presence of this compound. Therefore, we examined the effect of chloramine-T on the Met residues of this and the two other albumin preparations by CNBr fragmentation and SDS-PAGE (Fig. 3). This test showed that Met residues were oxidized to methionine sulfoxide in CT-HSA and to a lesser extent in H_2O_2-HSA but not in MCO-HSA. Furthermore, both the intrinsic fluorescence measurements (Fig. 6A) and the light absorption spectra (Fig. 6B) indicated extensive modification of ^{214}Trp in CT-HSA rather than microenvironmental changes around Trp, because the binding of warfarin to oxidized HSA was nearly the same as that for native HSA. Thus, the three oxidized albumin forms have been modified in different manners.

Far-UV CD spectra showed that, in the case of MCO-HSA and CT-HSA, the oxidation of amino acid residues resulted in the same decrease of α-helix content (Fig. 4A). The near-UV CD spectra (Fig. 4B) were also modified, especially that of CT-HSA, and these changes indicate tertiary structural changes in the environment of the disulfide bonds and aromatic amino acid residues (25). However, fluorescence measurements indicated that Tyr residues were not affected in the albumins studied in this work (data not shown). This finding is in accord with other reports (13,25). The conformational changes registered are most probably of a moderate

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

Protein	Free fraction (%) (warfarin)	Free fraction (%) (ketoprofen)
Native HSA	26.31 ± 3.21	3.93 ± 0.89
H_2O_2-HSA	27.56 ± 1.02	4.56 ± 1.23
CT-HSA	30.56 ± 4.02	38.2 ± 2.96
MCO-HSA	27.12 ± 2.45	47.5 ± 2.62

[a] The data are average values of three experiments (± S.D.). The concentration of both ligands was 5 μM, whereas that of the albumins was 10 μM.

Table III. Effect of Oxidation on Hydrolysis Rate Constants (Kobs) of HSA for *p*-Nitrophenyl Acetate at pH 7.4 and 25°C[a]

Protein	Kobs ($\times 10^{-2}$ sec^{-1})
Native HSA	1.981 ± 0.21
H_2O_2-HSA	2.193 ± 0.54
CT-HSA	0.456 ± 0.02
MCO-HSA	0.353 ± 0.08

[a] The data are average values of three experiments (± S.D.). The concentration of both ligands was 5 μM, whereas that of the albumins was 10 μM.

nature, because the changes in CD spectra were smaller than those observed in the presence of 6 M guanidine hydrochloride (Figs. 4A and 4B), and because the results of SDS-PAGE analysis (data not shown) excluded the possibility of gross conformational changes such as fragmentation, cross-linking, and SDS-resistant aggregation. In contrast to MCO-HSA and CT-HSA, no conformational changes in H_2O_2-HSA could be detected by the CD methods used.

The conformational changes of MCO-HSA and CT-HSA, and, to a lesser extent, of H_2O_2-HSA, seem to result in a more open protein molecule with a higher degree of exposure of hydrophobic areas. Such types of molecular changes are indicated by the findings that the fluorescence of the hydrophobic probe bis-ANS is increased considerably in the case of the two former modifications (Fig. 5), that, in addition, these preparations have prolonged elution times from a hydrophobic column, and that the ΔH-values for their thermal denaturation were much decreased (Table I).

Functional Aspects

In addition to its antioxidant activity, HSA possesses a significant esterase-like activity and exceptional ligand binding properties. The latter activity resides to a great extent on the existence of two binding regions, Site I and Site II (16) in subdomain IIA and IIIA (8), respectively. It is widely assumed that ^{214}Trp is important for binding to Site I of ligands such as warfarin (2). Therefore, it was rather surprising to find that the oxidation-induced changes of ^{214}Trp had not effect on warfarin binding (Table II). Fehske *et al.* (29) have selectively and completely modified ^{214}Trp with 2-hydroxy-5-nitrobenzyl bromide or o-nitro-phenylsulfenyl chloride and found, in both cases, that a very pronounced decrease in high-affinity binding of warfarin occurs. The different findings propose that modification of ^{214}Trp as such does not affect drug binding, but rather, it is the introduction of the large chemical groups which results in the effects observed by Fehske and coworkers. Alternatively, or additionally, the conformational changes associated with chemical labelling are more serious for the binding of warfarin than those introduced by the oxidations, employed herein.

Ketoprofen was chosen to represent Site II, because the high-affinity binding of this drug is well-characterized (17,26). Thus, ketoprofen has been shown to interact especially with ^{410}Arg and ^{411}Tyr in helix 2 and with several residues in helix 6 of subdomain IIIA. We found normal drug binding to H_2O_2-HSA but a decreased level of binding to CT-HSA (Table II). This finding cannot be caused by oxidation of ^{34}Cys, ^{214}Trp or Met residues, because the above studies, including a molecular docking model (26), failed to detect such residues as a part of the ketoprofen site. Therefore, the diminished binding must be the result of conformational changes in the binding region. Binding to MCO-HSA was even more decreased than binding to CT-HSA (Table II). Part of this effect must undoubtly be due to conformational changes which render Site II less favorable, as seen from the near UV-CD spectra (Fig. 4B). However, oxidation of ^{410}Arg, and perhaps of ^{485}Arg as well, could also contribute to the observed effect of MCO on ketoprofen binding.

The catalytic activity towards *p*-nitrophenyl acetate was not affected in the case of H_2O_2-HSA, whereas it was depressed to the same low level in MCO-HSA and CT-HSA (Table III). Since this activity of HSA depends on the close proximity of ^{410}Arg and ^{411}Tyr (17), the latter finding most probably is caused by conformational changes which increase the distance between the active groups of these residues. If ^{410}Arg had been modified in MCO-HSA, it would be expected to have a negative effect on esterase-like activity. This observation seems to be in correlated well with the reduced ketoprofen binding. Interestingly, the findings obtained here suggest a decrease in the binding of fatty acids, an endogenous substances to oxidized HSA.

Thus, the oxidation of albumin probably has individual effects on the different parts of the protein, and since these parts possess different functional properties, or have them to different degrees, a variety of effects on the function of albumin can be expected. In the case of the present preparations of H_2O_2-HSA, MCO-HSA, and CT-HSA the functional impairments are especially associated with the ligand binding and the enzymic properties of subdomain IIIA.

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