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Structural and Functional Impacts of Albumin Oxidation by Hypochlorite: Possible Changes in Drug Binding Characteristics upon Myeloperoxidase-Mediated Oxidation *in Vivo*

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Interaction of drugs with serum albumin, the most abundant protein in plasma, has a great significance in pharmaceutical sciences. It can affect the biological activity, toxicity, and pharmacokinetics of drugs and design of dosages. Determination of the impact of chemical modifications of albumin and its structural changes upon interaction with drugs are very important when drugs bind with albumin to a significant degree. Hypochlorite is naturally produced by activated phagocytes *in vivo* at inflammatory conditions. In current study, the effects of hypochlorite-mediated oxidation on the albumin stability, surface hydrophobicity and its interaction with furosemide were investigated using intrinsic and extrinsic fluorescence techniques. Bovine serum albumin (BSA) was chemically modified with sodium hypochlorite under nondenaturing conditions. The Job's plot indicated that the drug binds to the unmodified and modified BSAs with a 1:1 stoichometry. Fluorescence quenching data showed that the albumin affinity for the drug as well as its surface hydrophobicity increases under the effect of protein oxidation. Measurement of conformational stability indicated that oxidized BSA is less stable compared to the unmodified protein. Thermodynamic analyses of the binding process showed that the major forces involved in the interaction of furosemide to the unmodified and oxidized BSA are same (hydrophobic). Increment of protein surface hydrophobicity (PSH) as well as the decrement of protein stability may reminiscent of higher protein structural flexibility upon oxidation which may affect the drug binding site.

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Introduction

Reactive oxygen species (ROS) are present in biological fluids and tissues, both in normal conditions and particularly in oxidative stress scenarios (and also aging), where their rate of production is increased and/or removal rate decreases, leading to an increase in their steady state concentrations. Most biomolecules (lipids, proteins, nucleic acids) are target of reactive species; suffer from oxidative transformations which can modify their structure and capacity of function. ROS can alter structure of proteins, lead to fragmentation or oligomerization, and modify their physiologic function. The last aspect has been extensively studied in several enzymes, but considerably less information is available for plasma soluble transport protein, albumin [1-3].

Serum albumin (SA) is the most abundant protein in plasma. Albumin (HSA and BSA) contains three homologous helical domains (I-III) connected by random coil, each divided into A and B subdomains (Fig. 1), with two main drug-binding sites. During the lifetime (ca. 27 days [4]), it is continuously exposed to different oxidants. This process could be of importance since it has been proposed that albumin could play role as an antioxidant besides its transport function [5]. Several studies have been devoted to describe the changes elicited in proteins by their exposure to biologically relevant oxidants, but only a few have discussed its effect on protein conformation, stability, and binding capacity [6]. In this work, we address this point employing hypochlorite as oxidant.

Hypochlorite is the major strong ROS produced by activated neutrophils and monocytes via the reaction of H_2O_2 with Cl ions catalyzed by the heme enzyme myeloperoxidase (MPO) [7]. Although the production of hypochlorite is a part of host defense mechanism against microorganisms but under certain conditions, it may cause tissue damage [8]. Hypochlorite is considered to be important in progression of some diseases including atherosclerosis, inflammatory bowel diseases, rheumatoid arthritis, coronary artery disease, chronic inflammation and some cancers [7-9]. Methionine, cysteine and tryptophan residues of proteins are the most readily to be oxidized; also, amine groups such as ε-amino of lysine residues can also be oxidized to chloramines by hypochlorite [5]. Indeed, halogenation of amine groups and tyrosyl-residues are the most favored chlorinating reaction of hypochlorite [10]. It has been shown that MPO can react with SA via both sequence specificity and electrostatic interactions. Under pathological conditions, enzyme-mediated release of HOCl in the vicinity of albumin may extensively modify accessible methionine, tryptophan, tyrosines and other susceptible sidchains [5].

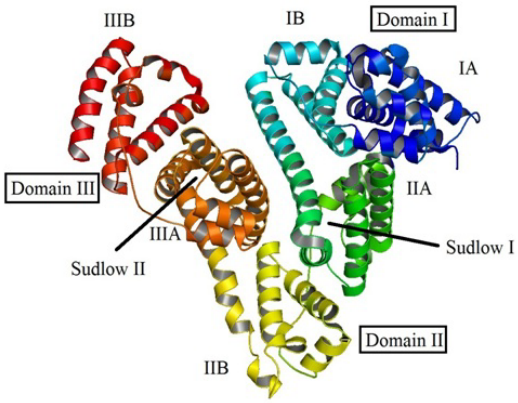
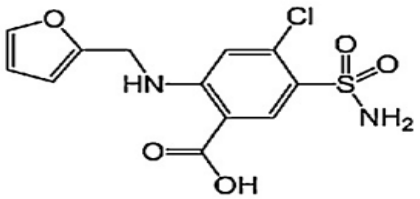


Fig. 1. Main drug binding sites of serum albumin (Sudlow I and Sudlow II) in Ribbon representation.

Furosemide is a commonly used loop diuretic drug with 99% binding at total concentration of 10 µg/ml [11]. It appears that subdomain IIA is the main binding site of furosemide (structure 1) [11, 12].

In current work, after treatment of BSA with sodium hypochlorite, the degree of protein oxidation was estimated. Then, furosemide binding characteristics of unmodified and modified albumins were compared. Changes in both conformation and stability of proteins were also evaluated using extrinsic fluorescence and urea denaturation experiments, respectively.



Structure 1. Chemical structure of furosemide.

Materials and Methods

Materials

ANS (1-Anilinonaphthalene-8-sulfonate) [Sigma, St. Louis, Missouri], bovine serum albumin (BSA) [Applichem, fraction V, fatty acid free, 98% purity], dialysis tube (cut-off 10kDa) [Sigma, St. Louis, Missouri], Dinitropheyl Hydrazine (DNPH) [Merck, Darmstadt, Germany], ethyl acetate [Merck, Darmstadt, Germany], furosemide [Bakhtar Bioshimi, as a gift], sodium borate [Merck, Darmstadt, Germany], sodium hypochlorite solution 6–14% [Fluka, Buchs, Switzerland], sodium phosphate dibasic [Applichem, Darmstadt, Germany], sodium phosphate monobasic [Appliche m, Darmstadt, Germany], Trichloroacetate [Merck, Darmstadt, Germany], urea [Merck] were obtained from companies mentioned in brackets. All reagents were of analytical grade and were used without further purification. The sodium hypochlorite concentration in solution was determined spectrophotometrically at pH 12 (ε_{292}: 350 $M^{-1}cm^{-1}$). Also, concentrations of all protein solutions were assessed according to lowery method. All measurements were carried out at room temperature in 50 mM sodium phosphate buffer, pH 7.6. Each experimental point presented in the figures is average of at least three independent measurements with standard errors less than ±5%.

Methods

Oxidation of albumin with sodium hypochlorite and carbonyl group assay

BSA 2 $mg.mL^{-1}$ (3×10^{-5} M in phosphate buffer) was oxidized by 18×10^{-5} M NaOCl ([NaOCl]/[BSA] 6:1 mol ratio) for 15 min at room temperature. Almost NaOCl is completely consumed in few minutes in the presence of amino acids or proteins. However, measurements were performed after 60 min incubation in the dark at room temperature in order to standardize secondary damage due to the decomposition of the initially formed chloramines [10]. Then, DNPH soulution (15 mM in 2 M HCl) was added up to a final concentration of 4.3 μM/$(mg\ BSA)^{-1}$, and the samples were incubated for 30 min in the dark, mixing them every 10 min. Then, gradually 10% w/v trichloroacetic acid was added the samples and incubated for 10min in the dark and centrifuged at 16000 g for 5 min. The supernatants were discarded; the precipitates were washed with a mixture of ethanol-ethyl acetate (1:1), incubated in the dark for 10min and centrifuged again. Following two additional washes, the precipitates were dissolved by vortexing for 20 min in 100 mM sodium phosphate buffer pH 7, and the absorbance was meas- ured at 360–370 nm by a Cary spectrophotometer (model Bio-100).The carbonyl content was calculated using the molar absorption coefficient (ε) of 22000 [13].

Intrinsic fluorescence measurements

Fluorescence emission spectra of BSA (1 μM) in the absence and the presence of different concentrations of drug were recorded on a Cary Eclipse (Varian) spectrofluorimeter equipped with multicell holder and Peltier temperature control. The protein emission spectra from 300 to 450 nm were collected upon excitation at 290 nm (for avoid the contribution from tyrosine residue) in which the excitation and emission slit widths were 5 nm. Binding constant (K_b) and number of binding sites (n), were determined at 293 K, 298 K, 303 K and 308 K using intrinsic fluorescence.

Intrinsic fluorescence quenching

Quenching of tryptophan fluorescence was used to study furosemide binding as described previously [11]. Briefly, a stock solution of furosemide was prepared in 50 mM sodium phosphate pH 7.6 buffer at a concentration of 1 mM. Different concentrations of furosemide were equilibrated with 1 μM protein in a 10 mm path-length quartz cuvette (3.0 ml) with a polytetrafluorethylene stopper during fluorescence measurements. Fluorescence measurements were performed with a Spectrofluorimeter Cary Eclipse (Varian, Australia). All fluorescence measurements were carried out at temperatures ranging 20-35 ºC with 5 ºC intervals. Excitation and emission slit widths were 5 and 10 nm, respectively. The background fluorescence was subtracted from each emission spectrum. After excitation at 290 nm, emission spectra were recorded from 300 to 450 nm.

Determination of binding stoichiometry

The stoichiometry of drug binding to BSA can be determined using the method of continuous variations [11]. Application of this method requires fluorescence change measurement ($\Delta F = F_{protein} - F_{protein + drug}$) in a

series of protein–drug mixtures under such a conditions that the final drug–protein concentration is constant (10 μM) with different mole fraction of each component. A plot of ΔF versus mole fraction of drug (Job's plot) is used to obtain the stoichiometry of binding from the maximum in the Job's plot [14].

PSH determination

Changes in protein surface hydrophobicity can be monitored using suitable probes, such as ANS [15]. Changes in the ANS binding properties of serum albumin can be detected using ANS-titration experiments in the absence and the presence of furosemide (50 μM). Apparent dissociation constant (K_d^{app}) and maximum fluorescence intensity (F_{max}) for the ANS–SA complex at saturating probe concentration can be obtained from the slope and the x-interceptof Scatchard plot, respectively [16]. In this case, Scatchard plot is the plot of fluorescence intensity/$[ANS]_{free}$ versus fluorescence intensity where $[ANS]_{free}$ is free ANS concentration. Free ANS concentration was calculated according to Moller *et al.* [17]. From these parameters, a Protein Surface Hydrophobicity (PSH) index in the absence and presence of the drug can be calculated and expressed as [16]:

$$\mathrm{PSH_{Index}} = \frac{F_{\max}}{[\mathrm{Protein}] \times K_{\mathrm{D}}^{\mathrm{app}}}$$

ANS was added from a stock solution (4 mM) to a final concentration ranging between 2-180 μM. The protein concentration was 0.3 mg/ml. The increase in fluorescence emission was recorded at 470 nm (with excitation at 380 nm) until no further increase in fluorescence was observed. The observed fluorescence is corrected for dilution using equation:

$$F_{\mathrm{Corr}} = F_{\mathrm{obs}} \times \frac{V_{\mathrm{fin}}}{V_{\mathrm{ini}}}$$

where F_{corr} is the corrected fluorescence, F_{obs} is the observed fluorescence, V_{fin} is the final volume, and V_{ini} is the initial volume [17].

Urea denaturation studies

Urea-induced protein denaturation was studied in 50 mM sodium phosphate buffer, pH 7.5, by placing the protein solution (0.5 mg/ml) in different urea concentrations (0-9.5 M). The stability of the unmodified and modified BSA was determined by monitoring the fluorescence intensity at 335 nm (slit was set at 5 nm for both excitation and emission) in the presence and absence of urea. The measured F_{335} converted to denatured fraction of protein (F_d) using equation:

$$F_{\mathrm{d}} = \frac{F - F_{\mathrm{N}}}{F_{\mathrm{D}} - F_{\mathrm{N}}}$$

Where F is fluorescence intensity at denaturant concentration, F_N and F_D are fluorescence intensity at unmodified and denatured states of protein. Values of F_N and F_D were obtained by linear extrapolation method as described earlier [18]. F_d was plotted as a function of the concentration of urea. Then, denaturation data was fitted to an equation describing the denaturation of the two-state-monomeric protein as follows:

$$F_{\mathrm{d}} = \frac{e^{\left(m[\mathrm{D}]-\Delta G^{\circ}_{\mathrm{H_2o}}\right)/RT}}{1 + e^{\left(m[\mathrm{D}]-\Delta G^{\circ}_{\mathrm{H_2o}}\right)/RT}}$$

Where D is the denaturant concentration, $\Delta G^{\circ}_{\mathrm{H_2o}}$ is the conformational stability of the protein and m is the slope of the unfolding transition [19].

Statistical analyses

The regression analyses of the modified Stern-Volmer curves of protein fluorescence quenching by furosemide, scatchard curves, and other plots were obtained using GraphPad Prism v5.0 for Windows (GraphPad software Inc., California, CA, USA). The K_b and n of furosemide binding, K_d and F_{max} of ANS binding, and ΔG^o_{H2O} and m of urea denaturation were calculated by the same software using the linear and non-linear regression analyses of related curves. Comparisons were performed by one-way analysis of variance, followed by student's t test using the program SPSS (version 14 for Windows; SPSS Inc. Chicago, IL). Data were considered statistically different at $P < 0.05$.

Results and Discussion

In addition of some aspects of drug pharmacokinetics (absorption, metabolism and excretion), distribution is controlled by serum albumin (SA), since most drugs travel in plasma and reach the target tissues as SA-drug complex . Poor or strong binding of drugs to

SA is believed to affect the drug half-life and hence dosage determination also depends on their half-life in plasma [4, 5]. Thus, the SA-drug binding affinity is one of the major factors that determine the pharmacokinetics, halftime and availability of the drug in various tissues. In the present paper, drug binding characteristics and conformational stability of the unmodified and oxidized bovine serum albumin has been studied using furosemide (as a drug model) and sodium hypochlorite (as oxidant).

Protein oxidation has been observed under oxidative stress associated with many (*in vivo*) pathologic conditions such as hypoxemia and inflammation. Hypochlorite is one of the major oxidants which produced within the blood by different vascular and immune cells [7, 8]. Although, previous works have shown that free radical-trapping activity of albumin is due to anti-oxidant activity and redox properties of the Cys-34 residue, the major target amino acids for hypochlorite-induced oxidation of albumin are cysteine, methionine, lysine, tyrosine and tryptophan side chains [5]. On the other hand, oxidation of albumin may alter either its charge or conformation. Thus, identifying the possible effects of this modification on protein structure, stability, and drug binding characteristics have great importance. As stated earlier, *in vivo* oxidative modification of albumin can be mediated by hypochlorite which produced by leukocytes-derived myeloperoxidase activity during immune response. Direct albumin-MPO interaction may accelerate albumin oxidation due to increased concentration of hypochlorite in the vicinity of susceptible amino acid residues. The following results are related to ligand binding characteristics and protein hydrophobicity/stability measurements.

Interaction of myeloperoxidase with human serum albumin

Myeloperoxidase (MPO) is a strongly basic (cationic) protein at physiological pH [4] and has a chance to strongly interact with negatively charged macromolecules, via electrostatic interactions [20] such as albumin, an acidic protein with pI=4.7. MPO-albumin interaction is essential in inducing both MPO binding to the vascular endothelium and its cellular uptake via binding of albumin to its specific binding proteins [20].

Previous reports have demonstrated that a certain part of MPO sequence (425-454) [RLATELKSL NPRWDGERLYQEARKIVGAMV] possesses high affinity binding to albumin where positively charged amino acids R and K were enriched. In the current study, we first docked unmodified MPO to albumin using HEX 6.12 software [21]. Also, to address the possible role of the positive charges, we changed mentioned sequence by replacing K and R by E [ELATELESLNPEWDGEELYQEAEEIVGAMV] and the MPO-SA binding energ ies were compared since interaction energies has been a major challenge for molecular docking,. The results may suggest that unmodified MPO display higher affinity toward BSA with ca. E=-497. The calculated energy values for unmodified MPO-SA interaction are similar to energies of antibody-antigen, receptor-ligand and enzyme-inhibitor complexes, as reported previously [21, 22]. It is noted that the energy value of the unmodified MPO-SA interaction is comparatively more than that value for the modified MPO-SA system (E=-83.57).

Characterization of oxidized form of BSA

Albumin oxidation extent was assessed by following the changes in the protein UV-vis and intrinsic fluorescence spectra and production of carbonyl groups. Hypochlorite can modify a variety of side chains (such as cysteine, methionine, tryptophan, tyrosine, lysine, and arginine) which makes results interpretation to be challenging. It is well known that the oxidation of proteins by OCl^- and some other reactive oxygen species (ROS) generates carbonyl groups [8]. So that, the measurement of protein carbonyls is considered a good indicator of protein damage by free radical reactions both in *in vitro* and *in vivo*. Moreover, chloramines, formed by oxidation of ε-amino group of lysine side chains, can be decomposed to carbonyl groups [10]. Therefore, assessment of carbonyl content of protein before and after albumin oxidation may be a more accurate indicator for extent of protein oxidation. Oxidation of SA under increasing concentrations of NaOCl showed a growth in the carbonyl content of the protein with a linear manner up to 6.85 µmol NaOCl/mg BSA^{-1}.

Furthermore, we compared UV-vis absorption as well as Trp fluorescence signals of unmodified and

modified forms of albumin, since they are good sensors for delicate structural changes of proteins. UV-vis absorption spectra of unmodified and oxidized albumins are represented in figure 2A. The absorption spectrum of the modified BSA differed from unmodified protein with maximum peak at 278 nm. On the other hand, the intrinsic fluorescence behavior of BSA changed upon protein oxidation (Figure 2B). Unmodified BSA contains two tryptophans and 20 tyrosines both of tryptophans are believed to be partially exposed to the aqueous medium, while all of tyrosines are probably buried [4, 12, 23]. Comparisson of Fig. 2A and 2B, showed a bleaching of chromophores/fluorophores in the modified BSA indicating oxidation of some residues (such as tyrosine side chains) by hypochlorite. Moreover, Trp-214 (as main fluorophore of BSA) is located in a chemical microenvironment near Sudlow's site I (Figure 1) and its oxidation may give detailed information about drug binding to subdomain IIA [12, 23]. As indicated in Fig. 2B, it also appears that Trp-214 side chain has experienced no oxidation. Although BSA has two fluorescent tryptophanyl residues in its structure, BSA intrinsic fluorescence originates mainly from Trp-214, after excitation at 290-295 nm [4, 23].

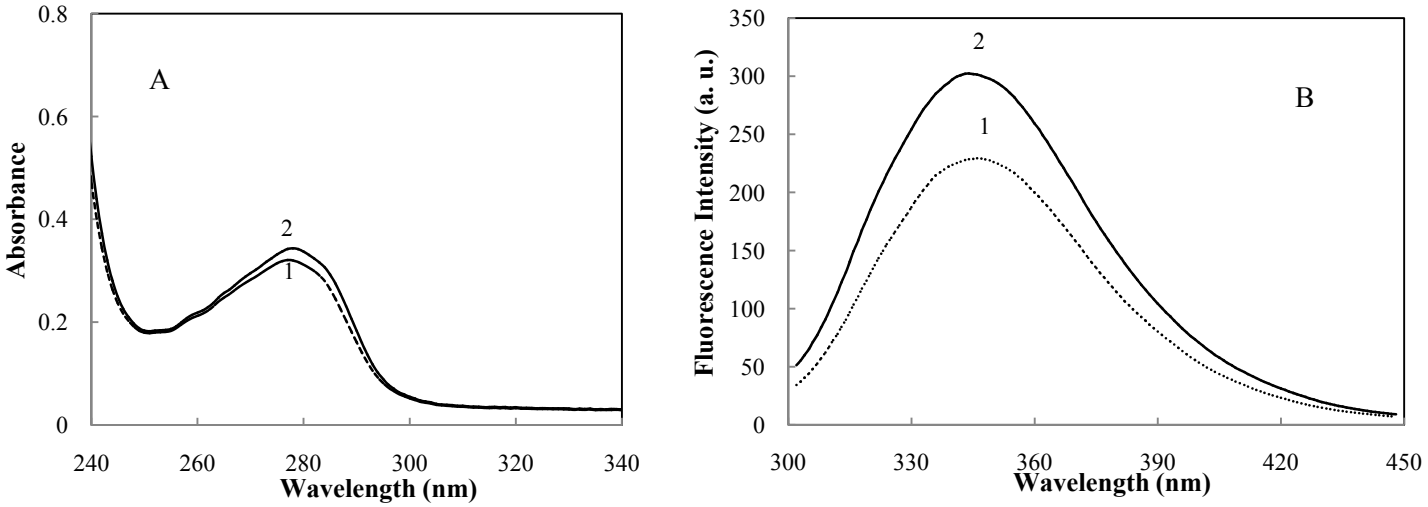


Fig. 2. (A) UV-vis and (B) Fluorescence spectra of oxidized (1) and unmodified BSA molecules (2).

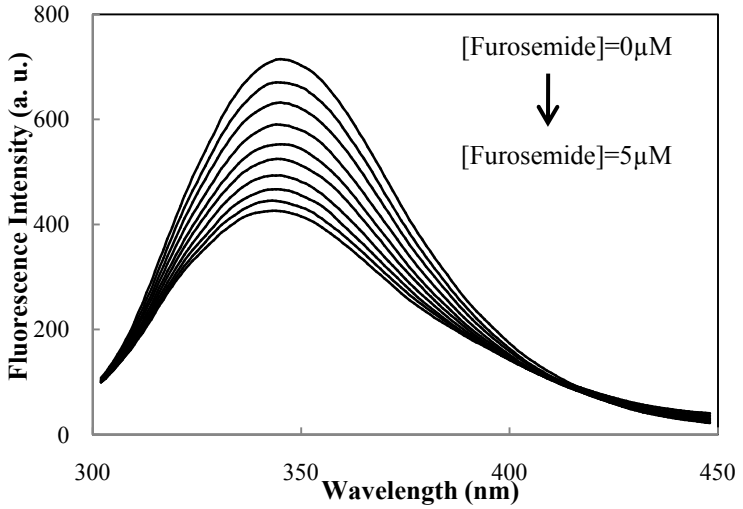


Fig. 3. Fluorescence quenching spectra of the unmodified BSA by different concentrations of furosemide (0-5μM) with the excited wavelength at 290 nm in sodium phosphate buffer solution (pH 7.4) at 25ºC (298 K) and [BSA] was 1 μM.

Furosemide binding studied by fluorescence measurements

The interaction of furosemide with unmodified and modified BSA was studied by fluorescence quenching method. Fig. 3 shows addition of furosemide to the unmodified albumin solutions leads to a progressive decrease in protein intrinsic fluorescence intensity. Additionally, same observations were made for the modified BSA (data not shown).

Since furosemide is capable to accept energy from the excited fluorophores of BSA, there must be some tryptophan residues at or near the furosemide binding site [11]. Trp-214 is located in a chemical microenvironment near Sudlow's site I, so, decrease in protein intrinsic fluorescence intensity, directly related to energy transfer from the protein to bound drug. Quenching of the oxidized protein fluorescence by furosemide would suggest the similar drug binding sites in unmodified and modified BSAs. It is noteworthy that we used a limited concentration range of furosemide (0-1 μM) with constant [BSA]. Based on the earlier reported and our results, we postulated that the drug occupies only sudlow site I [11, 24].

Binding constant (K_b) and average number of binding sites (n)

The value of K_b is essential to our understanding of drug distribution in plasma; since weak binding can lead to a short life-time or poor distribution, while strong binding can decrease the concentration of free drug in plasma.

The values of binding constant (K_b) and the number of binding sites (n), at different temperatures, are obtained by the modified Stern-Volmer plots (data not shown) and listed in table 1. As it is evident, K_b increases with increasing temp- erature, resulting in higher stability of the furosemide-protein complex in both unmodified and modified BSA. The binding constant value (at 308 K) is in a good agreement with previous studies [24, 25]. Upon protein oxidation, the stability of furosemide-BSA complex increased while the n values at different temperatures were kept around unity (see table 1). Also n was estimated by continuous variation to confirm the values obtained by modified Stern-Volmer plots. , In the Job's plot (data not shown), showed maximum change in fluorescence intensity of the complex occurs at 0.5 mol fraction of furosemide, i.e., the stoichiometry of the binding is 1:1 [11]. So, one molecule of drug binds to one molecule of BSA at concentration ranges used in this study. Furthermore, the drug-BSA stoichiometry has not undergone any changes upon protein modification as demonstrated in Table 1.

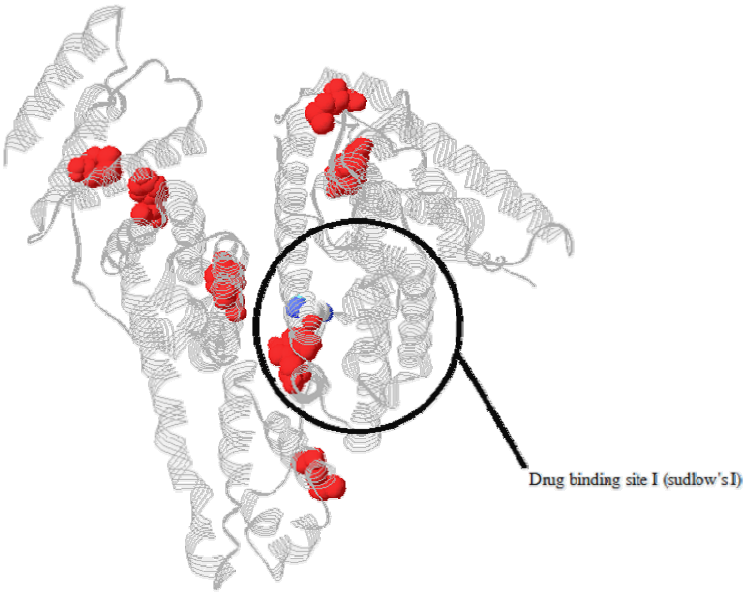


Fig. 4. Position of susceptible amino acid side chains, modified by hypochlorite (in red colour); [28]. Trp-214 and Lys-199 (blue) are located near to Sudlow site I (circle)

Table 1. Binding constant (K_b) and number of binding sites (n) for interaction of furosemide with unmodified/modified BSA, at different temperatures.

BSA	T (K)	$K_b \times 10^{-5}$ (M^{-1})	*n*	R^2
Unmodified	293	2.49632±0.02995	0.9673	0.999
	298	3.13979±0.12559	0.9721	0.999
	303	5.02227±0.10044	0.9961	0.999
	308	6.69730±0.33487	1.0047	0.999
Modified	293	3.15791±0.07895*	0.9177	0.997
	298	3.74025±0.16831*	0.9138	0.998
	303	5.76103±0.08621	0.9299	0.999
	308	7.39265±0.22178	0.9332	0.999

Data are expressed as mean ± SD of three measurements.
R^2 is the correlation coefficient for the K_b values.
*$P < 0.05$ versus unmodified BSA.

Mode of drug binding to the unmodified and modified albumins

Four types of non-covalent interactions could play key role in drug binding to proteins. In order to elucidate the binding mode, the binding constants for furosemide-BSA complexes formation were evaluated at four different temperatures, then the thermodynamic parameters of the binding processes were obtained from van't Hoff plot (data not shown) and then Gibbs energy were calculated using equation: $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ (Table 2).

The negative values of ΔG° support the spontaneous nature of the drug binding process to the unmodified and modified proteins. Ross and Subramanian (1981) reported three states for drug binding as 1) $\Delta H^\circ \leq 0$ $\Delta S^\circ > 0$ 2) $\Delta H^\circ < 0$ and $\Delta S^\circ < 0$, and 3) $\Delta H^\circ > 0$ and $\Delta S^\circ > 0$ where "electrostatic force", "van der Waals interactions/hydrogen bond" and "hydrophobic interactions" dominate for each situation respectively.[26] Since the enthalpy and entropy changes of drug binding to the unmodified and modified BSAs are positive, so the binding is mainly entropy-driven and hydrophobic interactions are the main forces in this process. The similar behaviors in fluorescence quenching and binding mode of furosemide on the unmodified and modified proteins suggest that furosemide binding sites on the unmodified and oxidized BSA molecules are the same (sudlow site I). These observations are in good accordance with pervious published data [27].

Table 2. Thermodynamic parameter for the binding of furosemide to unmodified and modified BSA.

BSA	T (K)	ΔH (kJ.mol^{-1})	ΔS (kJ.mol^{-1}.K^{-1})	ΔG (kJ.mol^{-1})
Unmodified	293	51.4371±2.0575	0.2785±0.0111	-30.1739±1.2070
	298			-31.5666±1.2626
	303			-32.9592±1.3183
	308			-34.3519±1.3741
Modified	293	44.7243±1.1181	0.2575±0.0064	-30.7333±0.7683
	298			-32.0209±0.8005
	303			-33.3086±0.8327
	308			-34.5963±0.8649

Data are expressed as mean ± SD of three measurements.

Although, structural details of the interaction between furosemide and albumin are not known. But, analysis of the crystal structure of the protein has revealed that this binding region (on subdomain IIA) is formed as a pocket, with the inside wall being formed by hydrophobic side chains and the entrance to the pocket being surrounded by positively charged residues [4, 11]. Based on spectroscopic (Fig. 2A) and thermodynamic observations, the location of interacting side chains within binding pocket may have delicate changes due to protein oxidation.

Fig. 4 shows that side chains of some susceptible amino acid to hypochlorite-oxidation (Met, Tyr, and Trp) are far from furosemide binding site in subdomain IIA [28]. According to fluorescence quenching and non-radiation energy transfer between proteins and drug, one may conclude that treatment of protein with hypochlorite had no effects on Trp-214 located within the hydrophobic part of the binding pocket. Since albumin oxidation has not reduced stability of drug-protein complex, the results led the authors to conclude that though Trp-214 could be part of the high-affinity binding site of furosemide.

In an earlier report, the presence of Lysine, especially Lys-199, in subdomain IIA (Sudlow I) has been suggested to be important for maintaining the correct structure of the furosemide binding site [11, 29]. Apparently Lys-199 remains unmodified in oxidized BSA resulting in the maintenance of the *n* value, drug binding capacity. The similarity of furosemide binding site on the unmodified and modified BSA molecules can be future explained by PSH data.

Table 3. Surface hydrophobicity parameters for unmodified and modified BSA in the absence and presence of furosemide.

BSA	K_d (μM)	PSH	ΔPSH (%)
Unmodified	0.5435±0.0163	4776.39±143.3	100
Unmodified+Furosemide	0.9768±0.0195	1669.87±33.39	65
Modified	0.2344±0.0047	6023.43±120.5	26 ↑
Modified+Furosemide	0.5787±0.0289	2265.68±113.3	62

PSH determination

ANS fluorescence is used to monitor possible changes in protein surface hydrophobicity (PSH) induced upon drug binding and chemical modification [11]. ANS, as an extrinsic fluorescent probe, fluoresces very weakly in aqueous solution, but its fluorescence intensity increases significantly in a hydrophobic environment [15].

At a fixed concentration (0.33 mg/ml) of BSA and increasing concentrations of ANS (0–35 μM), fluorescence intensity of ANS was measured in the absence and presence of 50 μM furosemide. A typical hyperbolic response is observed which demonstrates the saturation nature of ANS binding to BSA (data not shown). The surface hydrophobicity and K_d^{app} of modified and native proteins are obtained in the absence or presence of drugs using the Scatchard plot as tabulated in Table 3.

The value of K_d^{app} for ANS binding decreased upon modification, showing the tighter binding of ANS to the modified BSA. Based on PSH Calculation, the surface hydrophobicity of protein increases about 26% upon hypochlorite-mediated modification. Increased PSH upon drug binding may also corroborate the assumption that drug dosages in multi-drug treatments may need to be reconsidered.

As mentioned earlier, UV-vis absorption and fluorescence spectra analyses revealed that the overall structure of the oxidized BSA differs from unmodified BSA. Also, drug binding induced quenching of ANS fluorescence and the PSH decrement in both the unmodified and modified proteins. This suggests similar drug binding modes in modified and native BSA.

Conformational stability

The impact of the oxidation on the structural properties of albumin was also investigated by urea denaturation of the proteins using fluorescence spectroscopy. The urea denaturation profiles were obtained by following difference spectral changes at 335 nm at different urea concentrations. The transition curves (denatured fraction (F_D) versus urea concentration) are shown in Fig. 5. The transition curves for the unmodified and modified albumins appear to be simple cooperative with two-state transition. The shift of the transition curve of oxidized BSA towards lower urea concentrations may reminiscent of its lower stability compared to unmodified BSA.

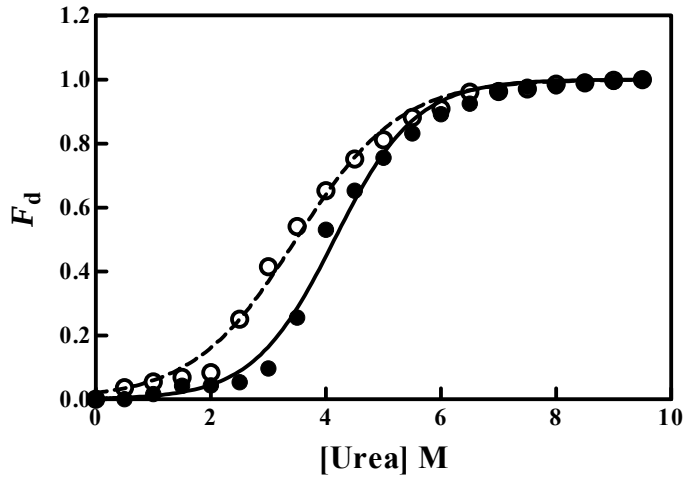


Fig. 5. The urea denaturation transition curves of unmodified **(●)** and modified BSAs **(○)**.

The denaturation processes were then analyzed in terms of a two-state mechanism for each transition [18]. The thermodynamic parameters calculated from the curves in Figure 5 are given in Table 4. The stability of the modified BSA was less than the unmodified protein indicating increased structural flexibility of BSA upon its modification. This is in agreement with a PSH data (Table 4) and shows induction of some degree of looseness in the protein structure upon modification. The most probable explanation may be exposing unfavorable destabilizing effect of hydrophobic residues in aqueous solution [30].

Table 4. The thermodynamic parameters of unmodified and modified BSA urea denaturation in the absence and presence of furosemide.

BSA	m (kJ.mol^{-1}.M^{-1})	$d_{1/2}$ (M)	$\Delta G^{\circ}_{H_2O}$ (kJ.mol^{-1})
Unmodified	2.534±0.076	4.06±0.122	10.31±0.31
Unmodified+Furosemide	13.083±0.262	6.46±0.129	84.55±1.69
Modified	1.528±0.023	3.66±0.055	5.60±0.084
Modified+Furosemide	3.6501±0.128	6.93±0.243	25.29±0.89

Moreover, the dramatic enhancement of conformational stability observed for both the unmodified and oxidized proteins, in the presence of furosemide (8-fold and 5-fold respectively) are in accordance with PSH results. This may be due to remarkable reduction of water penetration into protein structure and induction of local and global destabilization.
In brief decreased conformational stability of the oxidized albumin could affect its half-life in different manner such as rapid clearance, elimination, decomposition by liver and enzymatic proteolysis.

Conclusion

Addition of NaOCl led to an extensive oxidation of susceptible side chains of albumin, as evidenced by the loss of Trp fluorescence and formation of carbonyl groups. Protein retained its binding capacity up to 6:1 molar ratio of NaOCl/protein. Furthermore, the binding properties (such as binding mode and

number of binding sites) remained almost unmodified. Although, the extent (and rate) of albumin oxidative modifications, *in vivo*, is difficult to assess, these observations could be of relevance regarding protein capacity to fulfill its role in transportation even after suffering a progressive oxidation along its lifetime.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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