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Metal-catalyzed oxidation of human serum albumin does not alter the interactive binding to the two principal drug binding sites

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ABSTRACT

It is well known that various physiological factors such as pH, endogenous substances or post-translational modifications can affect the conformational state of human serum albumin (HSA). In a previous study, we reported that both pH- and long chain fatty acid-induced conformational changes can alter the interactive binding of ligands to the two principal binding sites of HSA, namely, site I and site II. In the present study, the effect of metal-catalyzed oxidation (MCO) caused by ascorbate/oxygen/trace metals on HSA structure and the interactive binding between dansyl-L-asparagine (DNSA; a site I ligand) and ibuprofen (a site II ligand) at pH 6.5 was investigated. MCO was accompanied by a time-dependent increase in carbonyl content in HSA, suggesting that the HSA was being oxidized. In addition, The MCO of HSA was accompanied by a change in net charge to a more negative charge and a decrease in thermal stability. SDS-PAGE patterns and α-helical contents of the oxidized HSAs were similar to those of native HSA, indicating that the HSA had not been extensively structurally modified by MCO. MCO also caused a selective decrease in ibuprofen binding. In spite of the changes in the HSA structure and ligand that bind to site II, no change in the interactive binding between DNSA and ibuprofen was observed. These data indicated that amino acid residues in site II are preferentially oxidized by MCO, whereas the spatial relationship between sites I and II (e.g. the distance between sites), the flexibility or space of each binding site are not altered. The present findings provide insights into the structural characteristics of oxidized HSA, and drug binding and drug-drug interactions on oxidized HSA.

1. Introduction

Human serum albumin (HSA) is the most abundant protein in plasma and functions as regulator of colloidal osmotic pressure, as an antioxidant in human plasma, and as a transporter of endogenous compounds such as fatty acids, hormones, toxic metabolites (e.g. bilirubin), bile acids, amino acids, and metals [1,2]. A wide variety of drugs also bind to HSA [1–3], and therefore HSA has a significant impact on the pharmacokinetics and pharmacological effects of these drugs [4]. The high affinity binding of drugs to HSA predominantly occurs at two specific sites on the HSA molecule, namely, site I and site II [4–7]. X-ray crystallographic data clearly indicate that HSA contains three structurally similar α-helical domains, i.e., domains I–III, which can be further divided into subdomains A and B [8]. Sites I and II are separated from one another and are located in subdomains IIA and IIIA, respectively [1,8,9]. In spite of such differences in the locations of these sites, interactions between ligands that bind to sites I and II have been reported [10–14].

In solution, HSA is an assembly of flexible and resilient parts, and can change its conformation through the opening and closing of crevices between domains or subdomains [2]. Physiological factors such as pH, endogenous substances or post-translational modification could influence the conformational state of HSA [2,5,15]. We previously reported that competitive-like allosteric interactions between a site I ligand (dansyl-L-asparagine) and a site II ligand (ibuprofen or diazepam) at pH 6.5 changed to nearly independent binding with increasing pH or in the presence of long chain fatty acids [16,17]. Such a change in interaction mode is thought to be due to extending the distance between ligands and/or changes in the flexibility or the size of the ligand binding site on the HSA molecule when the pH is increased or in the

Abbreviations: HSA, human serum albumin; MCO, metal-catalyzed oxidation; DNSA, dansyl-L-asparagine

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case of fatty acid binding. The post-translational modification of HSA is another physiological factor that influences the structure and functions of HSA [15,18–20]. The oxidation of HSA has been one of the widely studied post-translational modifications because HSA is generally thought to be a major antioxidant in the plasma and extracellular compartments [19,21,22]. It has been suggested that a conformational change in HSA via oxidation results in a decrease in its drug binding capacity and antioxidant activity [19,21,23]. However, the issue of whether a conformational change of HSA due to oxidation could alter the interaction between ligands that bind to sites I and II remains unclear.

In the present work, we report on an examination of the effect of the oxidation of HSA on the interactive binding between DNSA (a site I ligand) and ibuprofen (a site II ligand). Metal-catalyzed oxidation (MCO) was used to oxidize HSA, since, under *in vivo* conditions, trace metal ions would be expected to generate radicals that could oxidize a protein such as HSA [24]. We discuss the effect of HSA oxidation on interactive binding between sites I and II on the basis of the structural features of the oxidized HSA.

2. Materials and methods

2.1. Materials

HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). Prior to use, HSA was defatted with activated charcoal in an aqueous solution that had been adjusted to pH 3 with H_2SO_4 at 0 °C, dialyzed against de-ionized water and then freeze-dried, as originally described by Chen [25]. The molecular mass of HSA was assumed to be 66,500 Da. The HSA used in this study gave only one band in SDS-PAGE. Dansyl-L-asparagine (DNSA) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ibuprofen, diethylenetriaminepentaacetic acid (DTPA) and 2, 4-dinitrophenylhydrazine (DNPH) were obtained from Wako Chemical Co. (Osaka, Japan). All other chemicals were of analytical grade. All ligand molecules were first dissolved in methanol and the final concentration of methanol was less than 1% (v/v).

2.2. Metal-catalyzed oxidation of HSA

HSA was oxidized according to the method of Meucci et al. (ascorbate/oxygen/trace metals system) [26] with minor modifications. Briefly, 0.3 mM HSA and 100 mM ascorbic acid were incubated in 50 mM Tris buffer (pH 7.4) prepared with highly purified water (resistivity: 18 MΩ· cm). Tris buffer was used because it can effectively inhibit excessive protein modification, and the concentrations of cationic metals, iron and copper in the buffer were determined to be 0.91 and < 0.47 μM, respectively by Metallo Assay LS (Funakoshi Co., Ltd., Tokyo, Japan). Samples without ascorbic acid or with 1 mM DTPA as a chelating agent were also prepared. Sodium azide (0.02 w/v) was added to these samples as an antibacterial agent. Aliquots were withdrawn after different incubation times (6, 12, 24 and 48 h), and the oxidative process was terminated by adding 1 mM DTPA. Each sample was dialyzed against 50 mM Tris buffer (pH 7.4) and then de-ionized water. After ascorbic acid was not detected in the dialysate, the samples were freeze-dried and stored at − 20 °C. The prepared samples gave only one band at the position of native HSA in SDS-PAGE.

2.3. Characterization of oxidized HSA

The structures of the native and oxidized HSAs were characterized by carbonyl content, secondary structures, net charges and the thermal stabilities of these proteins. Carbonyl contents were determined according to the method of Levine et al. [27,28]. Briefly, the carbonyl groups were derivatized by treatment with DNPH and their concentration calculated using the extinction coefficients of DNPH at 370 nm ($\varepsilon_{370\,nm}$ = 22,000 M^{-1} cm^{-1}) using a UV/VIS spectrometer (Ubest-35; JASCO Co., Tokyo, Japan). The contents of secondary structure were determined using the K2D3 web server based on the far-UV CD spectra (190–240 nm) of native and oxidized HSAs (2 μM), measured by a circular dichroism spectrometer (J-720; JASCO Co., Tokyo, Japan) using a 10-mm path length cell [29]. Changes in the net charge of albumin were evaluated by capillary electrophoresis [30]. One mL of sample (2 μM native or oxidized HSAs) was run in 100 mM borate buffer (pH 8.5 and 20 °C), and the migration time was determined by means of a CE990/990-10 type capillary electrophoresis system from Jasco Co. (Tokyo, Japan). For the thermodynamic evaluation of native and oxidized HSAs, differential scanning calorimetry (DSC) was carried out on a MicroCal MC-2 ultrasensitive DSC (microCal 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 (T_m), calorimetric enthalpy (ΔH_{cal}) and van't Hoff enthalpy (ΔH_{vH}), from the temperature dependence of excess molar heat capacity, C_p, using the Origin™ scientific plotting software.

2.4. Equilibrium dialysis

Equilibrium dialysis experiments were performed using 2 mL multiwell plastic dialysis cells (Cosmo Bio Co., Ltd., Tokyo, Japan). The buffer used for binding experiments was prepared with sodium phosphate dibasic and sodium phosphate monobasic salts. The two cell compartments were separated by Visking cellulose membranes. Aliquots (1.5 mL) of samples were dialyzed at 25 °C for 12 h against the same volume of buffer solution. After reaching equilibrium, the concentration of free ligand (C_f) in the buffer compartment was determined by HPLC. The adsorption of ligands to the membrane and/or the dialysis apparatus was negligible, since no adsorption was detected in equilibrium dialysis experiments in the absence of HSA. The volume shift after equilibrium dialysis was corrected according to the method of Giacomini et al. [31].

2.5. HPLC conditions

The HPLC system used in this study consisted of a Hitachi 655A-11 pump, Hitachi 655 A variable wavelength UV monitor and a Hitachi F1000 variable fluorescence monitor. The stationary phase was a LiChrosorb RP-18 column (Cica Merck, Tokyo, Japan) and was maintained at 40 °C. The mobile phase consisted of 5 mM phosphate buffer (pH 7.7)-acetonitrile (77:23 v/v) for DNSA and ibuprofen assays. A fluorescence monitor was used for DNSA detection (excitation at 330 nm and emission at 550 nm). Ibuprofen was detected at a fixed wavelength, 220 nm using a UV monitor.

2.6. Data analysis of ligand binding and ligand-ligand interaction

Binding parameters of ligands to protein were determined by fitting the experimental data to the following equation using GraphPad PRISM® Version 7 (GraphPad Software, Inc, CA, U.S.A.).

$$r = \frac{nKC_f}{1 + KC_f} \quad (1)$$

where r is the number of moles of ligand bound per mole of protein. C_f is the unbound ligand concentration determined by equilibrium dialysis. K and n are the association constant and the number of binding sites for the high-affinity binding site, respectively. All experiments and analyses were performed using the condition, r < 0.5, to minimize the binding of the ligand to any low-affinity binding sites.

In order to estimate the interaction between two ligands, A and B that bind to each primary binding site of the protein, the binding data for one ligand in the presence of another ligand were analyzed using