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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

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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 ($\varepsilon_{370\,\mathrm{nm}} = 22{,}000\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$) 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.).

$$r = \frac{nKC_f}{1 + KC_f} \tag{1}$$

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 following equations described by Kragh-Hansen [3,32].

$$r_A = \frac{K_A A_f + \chi K_A K_B A_f B_f}{1 + K_A A_f + K_B B_f + \chi K_A K_B A_f B_f} \tag{2}$$

$$r_B = \frac{K_B B_f + \chi K_B K_A A_f B_f}{1 + K_B B_f + K_A A_f + \chi K_B K_A A_f B_f} \tag{3}$$

where r_A and r_B are the number of moles of ligands A and B bound per mole protein, respectively. K_A and K_B are the binding constants of ligands A and B, respectively. A_f and B_f are the free concentrations of ligands A and B, respectively. χ is the coupling constant. The independent binding of the two ligands is characterized by $\chi = 1$, while a competitive interaction results in $\chi = 0$. $\chi > 1$ and $0 < \chi < 1$ express cooperative and anti-cooperative interaction between ligands A and B on the protein, respectively.

2.7. Statistical analysis

The data were analyzed statistically using one-way analysis of variance (ANOVA), followed by Tukey multiple comparisons to evaluate differences between the means. All statistical procedures were performed with GraphPad PRISM® Version 7 (GraphPad Software, Inc, CA, U.S.A.). Differences with $P > 0.05$ were deemed to be statistically non-significant.

3. Results

3.1. Structure of oxidized HSA

To evaluate changes in the structure of HSA caused by MCO, the carbonyl contents, secondary structures, net charges and thermal stabilities of the oxidized HSA were compared to those of native HSA (Table 1). The carbonyl content of the HSA was increased during the treatment by ascorbate/oxygen/trace metals system. The α-helical content of the oxidized form were not different from that of native HSA. In addition, changes in migration time on capillary electrophoresis and in the thermodynamic data on DSC for the oxidized HSAs indicated that the MCO treatment changed the net charges and thermal stability of the HSA molecule. Meanwhile, the structural characteristics of the samples prepared without ascorbic acid or with DTPA were similar to those for native HSA. These findings strongly suggest that ascorbic acid and metal ions (even in trace amounts) contribute to the oxidative modification of HSA as the result of the MCO treatment.

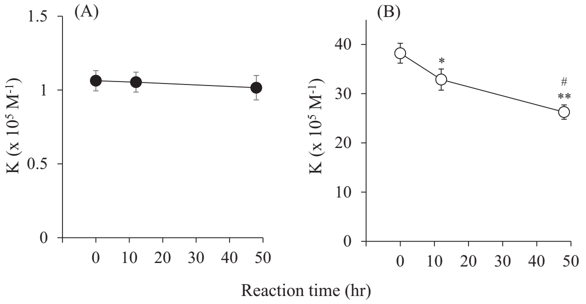

Fig. 1. Effects of MCO reaction time on the primary association constants (K) of DNSA (A, Closed circles) and ibuprofen (B; Open circles) at pH 6.5 and 25 °C. The number of primary binding sites (n) was 1 for each system. The concentration of HSA was 40 μM. The results are the mean ± S.D. for three observations. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared to native HSA (0 h). $^{\#}P < 0.05$ as compared to HSA oxidized for 12 h.

3.2. Binding of DNSA and ibuprofen to oxidized HSA

The primary association constants for the bindings of DNSA and ibuprofen to oxidized HSA at pH 6.5 were determined by equilibrium dialysis (Fig. 1). The affinity of ibuprofen to the oxidized HSA decreased significantly with increasing oxidation time, whereas the affinity of DNSA was not affected.

3.3. Mode of interaction between DNSA and ibuprofen bound to oxidized HSA

The mutual binding between DNSA and ibuprofen to oxidized HSA was investigated at pH 6.5. The binding isotherm of DNSA to native HSA in the presence of ibuprofen was fairly close to a theoretical curve assuming anti-cooperative interaction (Fig. 2A, $\chi = 0.116$). The binding isotherm for ibuprofen in the presence of DNSA was also close to the curve assuming anti-cooperative interaction (Fig. 3A, $\chi = 0.257$). The oxidation of HSA had no effect on these findings (Figs. 2A, 2B, 3A and 3B), and in all cases, the coupling constants (χ) was sufficiently low to conclude that the interaction mode was a competitive-like allosteric interaction (Fig. 4)

4. Discussion

The structure of albumin can be influenced by a number of factors, including pH, calcium ions, its redox state, chloride ions, free fatty acids or the concentration of albumin in solution [2,11,33]. Such structural

Table 1
Structural characteristics of native and oxidized HSA.

Incubation time	Carbonyl contents (nmol/ mg HSA)	Content of α-helix (%)[a]	Migration time (h)[b]	Thermodynamic data[c]			
				T_m (°C)	ΔH_{cal} (× 10^5) (cal/ mol)	ΔH_{vH} (× 10^5) (cal/ mol)	$\Delta H_{vH}/\Delta H_{cal}$
0 h	2.29 ± 0.19	67.9 ± 2.3	8.23 ± 0.04	59.63 ± 0.15	1.66 ± 0.04	1.14 ± 0.02	0.69 ± 0.03
6 h	3.31 ± 0.41*	69.9 ± 1.9	8.63 ± 0.06**	59.53 ± 0.12	1.23 ± 0.03**	0.80 ± 0.03**	0.65 ± 0.04
12 h	4.00 ± 0.28**	68.3 ± 2.6	8.71 ± 0.06**	59.50 ± 0.10	1.18 ± 0.02**	0.75 ± 0.03**	0.64 ± 0.02
24 h	4.84 ± 0.35**	69.7 ± 2.4	8.81 ± 0.10**	58.86 ± 0.06**	1.38 ± 0.13**	0.55 ± 0.03**	0.40 ± 0.05**
48 h	5.56 ± 0.21**	68.5 ± 1.5	9.02 ± 0.10**	58.93 ± 0.15**	1.15 ± 0.05**	0.51 ± 0.03**	0.44 ± 0.02**
48 h (without ascorbic acid)	2.46 ± 0.27	66.2 ± 2.1	8.32 ± 0.09	59.73 ± 0.21	1.65 ± 0.05	1.13 ± 0.02	0.68 ± 0.03
48 h (with DTPA)	2.32 ± 0.33	67.7 ± 1.9	8.24 ± 0.13	59.57 ± 0.23	1.63 ± 0.03	1.14 ± 0.04	0.70 ± 0.03

The results are means ± S.D. (n = 3).

* $P < 0.05$.

** $P < 0.01$ as compared to native HSA (0 h).

[a] Data from circular dichroism.

[b] Data from capillary electrophoresis.

[c] Data from DSC.

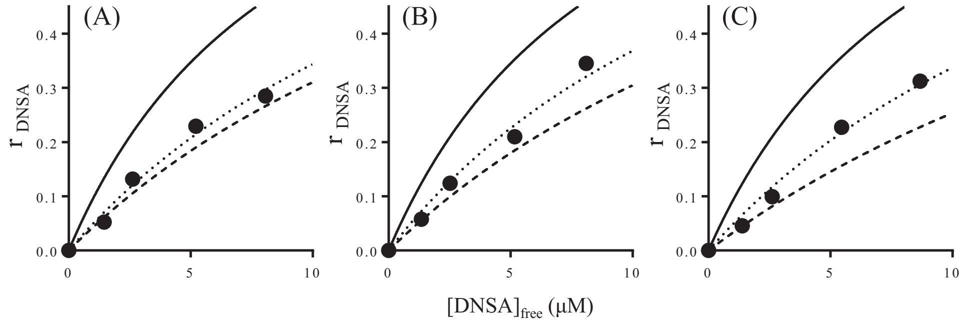

Fig. 2. Effects of MCO treatment time on the binding of DNSA (5–40 μM) to HSA (40 μM) in the presence of ibuprofen (20 μM) at pH 6.5 and 25 °C. Treatment times were 0 h (A), 12 h (B) and 48 h (C). Closed circles denote the experimental values for DNSA binding in the presence of ibuprofen. Solid line represents theoretical curves assuming the independent binding of the two ligands. Broken line represents theoretical curves assuming competitive binding between DNSA and ibuprofen. Dotted line represents theoretical curves assuming anti-cooperative (allosteric) interactions between DNSA and ibuprofen. All theoretical curves were constructed using the association constant for each ligand (A; DNSA 1.06×10^5 M^{-1}, ibuprofen 38.2×10^5 M-1, B; DNSA 1.05×10^5 M^{-1}, ibuprofen 32.9×10^5 M^{-1}, C; DNSA 1.02×10^5 M^{-1}, ibuprofen 24.8×10^5 M^{-1}).

changes may play an important role in its functions, which include the transport of endogenous and exogenous substances [34,35]. We previously reported that pH- and long chain fatty acid-induced conformational changes in HSA modify not only ligand binding, but also the mode of interaction between ligands that bind to different sites, i.e., sites I and II [14,17].

In the present study, the effects of altering the structure of HSA by MCO on competitive-like allosteric interactions between DNSA and ibuprofen that bind to site I and site II, respectively, at pH 6.5 was investigated. The oxidation of proteins is usually accompanied by an increase in the content of carbonyl groups in the protein, which results from the carbonylation of amino acid residues [36]. A time-dependent increase in the carbonyl group content of oxidized HSAs was observed, but this increase was not accompanied by a significant change in α-helical content (Table 1) or the SDS-PAGE pattern (data not shown), indicating that the condition used for the MCO in this study (i.e. ascorbate, oxygen and trace metal system) was relatively mild. The MCO of HSA was accompanied by a change in net charge to a more negative charge than native HSA, as indicated by an increase in the migration time of bands in capillary electrophoresis (Table 1). Furthermore, the DSC data indicated that oxidized HSAs are more easily denatured than native HSA, since the T_m, ΔH_{cal} and ΔH_{vH} values were decreased as the result of the MCO treatment (Table 1). It is generally thought that ΔH_{cal} reflects the hydration of hydrophobic regions buried in the protein structure during the unfolding process. Therefore, hydrophobic regions in oxidized HSA may be more easily exposed than those in native HSA. The ratio of ΔH_{vH} and ΔH_{cal} ($\Delta H_{vH}/\Delta H_{cal}$) is an index of the transition process to the denatured state during thermal denaturation [37]. The $\Delta H_{vH}/\Delta H_{cal}$ values for native and oxidized HSAs were less than 1, suggesting that the denaturation process involves intermediary steps. Furthermore, the decreased values of $\Delta H_{vH}/\Delta H_{cal}$ especially in the case of HSA that had been oxidized for 24 and 48 h as compared to that in native HSA may indicate the increased number of intermediary steps. Such structural characteristics of HSA oxidized by MCO were nearly the same as those of an oxidized HSA prepared in our previous study, although condition used for the MCO in this study was milder [38,39].

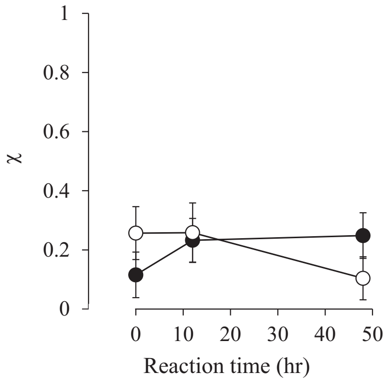

Fig. 4. Effects of MCO reaction time on the coupling constant (χ) for interactions between DNSA and ibuprofen. Closed circles denote coupling constants calculated from the binding of DNSA in the presence of ibuprofen. Open circles are coupling constants calculated from the binding of ibuprofen in the presence of DNSA. The results are the mean ± S.D. for three determinations.

The binding of individual ligands to these oxidized HSAs was also investigated (Fig. 1). Time-dependent decreases in the binding of ibuprofen to site II of the oxidized HSA were detected, and the association constants for ibuprofen were negatively correlated with overall carbonyl content (Fig. 2 and Table 1). Meanwhile, the binding of DNSA to site I was not affected by oxidation. Therefore, the extent of structural change of HSA due to MCO in this study may not be drastic, but appears to be enough to locally influence ligand binding to site II. Crystallographic data for HSA shows that ibuprofen interacts with Tyr411, Arg410 and Ser489 through salt-bridge and hydrogen-bonding [40]. Annibal et al. indicated that the cysteine, methionine, tryptophan and tyrosine residues of HSA are the most sensitive to oxidation of HSA

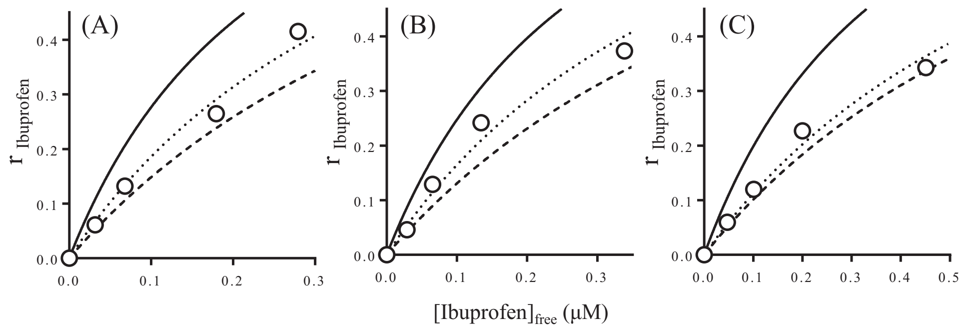

Fig. 3. Effects of MCO treatment time on the binding of ibuprofen (2.5–20 μM) to HSA (40 μM) in the presence of DNSA (40 μM) at pH 6.5 and 25 °C. Treatment times were 0 h (A), 12 h (B) and 48 h (C). Open circles are the experimental values for ibuprofen binding in the presence of DNSA. Solid line represents theoretical curves assuming the independent binding of the two ligands. Broken line represents theoretical curves assuming competitive binding between ibuprofen and DNSA. Dotted line represents theoretical curves assuming anti-cooperative (allosteric) interactions between DNSA and ibuprofen. All theoretical curves were constructed using the association constant for each ligand (A; DNSA 1.06×10^5 M^{-1}, ibuprofen 38.2×10^5 M^{-1}, B; DNSA 1.05×10^5 M^{-1}, ibuprofen 32.9×10^5 M^{-1}, C; DNSA 1.02×10^5 M^{-1}, ibuprofen 24.8×10^5 M^{-1}).

[41]. Furthermore, MCO typically results in the formation of carbonyl groups at arginine, lysine, threonine, proline, cysteine or histidine residues [42]. Therefore, the oxidation of Tyr411 and/or Arg410 would be expected to result in a reduction in the binding of ibuprofen to site II. Ryan et al. reported that Lys199, Arg222 and Ala291 within site I are involved in hydrogen bonding interactions with DNSA [43]. Thus, it is possible that Lys199 and Arg222 are oxidized by MCO. However, DNSA binding to oxidized HSA was not different from that to native HSA. Hence, Tyr411 and/or Arg410 in site II may be preferentially oxidized, and Lys199 and Arg222 in site I may be structurally protected from MCO.

Despite these structural changes of HSA by MCO, the mode of interaction between ibuprofen and DNSA at pH 6.5, a “competitive-like” allosteric interaction, was not affected by MCO as indicated by the finding that there was no significant difference in coupling constants (χ) for all of the oxidized HSAs (Figs. 3 and 4). We previously reported that the allosteric displacement of ibuprofen by DNSA can be attributed to long-distance electric repulsion between ligands in sites I and II [17]. This interaction disappeared when the pH was increased to pH 8 and upon the binding of sodium oleate [16,17]. Therefore, we speculated that the conformation of HSA at pH 6.5 does not possess sufficient structural flexibility to permit the repulsive force between ligands to be eliminated, and results in a decreased binding of each ligand. The conformational change induced by an increase in pH or oleate binding may cause the distance between sites I and II to be increased, thus leading to the elimination of allosteric interactions between ligands, or an increase in the flexibility or the space of binding pocket which would be expected to abolish repulsive forces. Taking these speculations into account, structural changes caused by MCO might not be accompanied by a change in the distance between sites I and II, or the flexibility or the space of each binding site.

5. Conclusions

In this study, we attempted to clarify the effect of the oxidation of HSA by MCO on the ‘competitive-like’ allosteric interactions between DNSA and ibuprofen, which bind to two different sites, site I and site II of HSA, respectively, at pH 6.5. The structural change by oxidation evoked by an ascorbate/oxygen/trace metals system led to the selective reduction in ligand binding to site II, whereas the mode of interaction between the ligands that bind to sites I and II was not altered through MCO. These data suggest that amino acid residues of site II are preferentially oxidized by MCO, but the spatial relationship between sites I and II (e.g. distance between sites), the flexibility or the 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 for oxidized HSA.

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Transparency document. Supplementary material

Supplementary material associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2018.05.002.

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