Characterization of Region Ic in Site I on Human Serum Albumin. Microenvironmental Analysis Using Fluorescence Spectroscopy

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Characteristics of region Ic among at least three overlapping binding regions (regions Ia, Ib and Ic) in site I on human serum albumin (HSA) were analysed using *n*-alkyl *p*-aminobenzoates (*n*-alkyl *p*-ABEs), all of which are specific fluorescent probes for region Ic. In the interaction processes between *n*-alkyl *p*-ABEs and HSA, hydrophobic interaction, van der Waals interaction and local structural changes in region Ic were found to be involved based on the results obtained by analyses of the fluorescence spectra, structure-activity relationships and thermodynamic parameters. In addition, comparison of the fluorescence spectra of *n*-alkyl *p*-ABEs in HSA and detergents indicated that the possibility of a hydrophobic region Ic around which an amino acid with cationic charge locates could not be denied because of the similarity of fluorescence spectra between *n*-alkyl *p*-ABEs in HSA and in neutral and cationic detergents. The deviation of *n*-alkyl *p*-ABEs with long alkyl chains (C₉–C₁₂) in the relationships between association constants and physicochemical properties of a series of *n*-alkyl *p*-ABEs (C₁–C₁₂) suggested that region Ic possess an optimal depth. A conformational change of HSA with increasing pH (pH 6–9) generated an increase in hydrophobicity and adaptability of the binding region and made interaction easy, with an increase in adaptability of the binding region Ic; consequently, it enhanced the binding of *n*-alkyl *p*-ABEs to HSA.

Keywords human serum albumin; drug binding site; drug binding region; fluorescent probe; *n*-alkyl *p*-aminobenzoate

It is well known that many drugs bind to one of the specific high affinity binding sites on human serum albumin (HSA).^{1,2)} Sudlow *et al.*^{3,4)} have termed the best characterized two-drug binding sites as site I (warfarin binding site) and site II (benzodiazepine binding site). Furthermore, Sjöholm indicated one more binding site, namely site III (digitoxin binding site).⁵⁾ Moreover, Fehske *et al.*⁶⁾ suggested two binding regions in site I, the warfarin and azapropazone binding regions. Since then, it has been necessary to separately study site I as two regions. However, most researchers have continued to investigate site I as one region as usual because of the difficulties involved in separate study, as follows. Firstly, these two regions overlap in Site I. Secondly, no ligands which bind selectively to each binding region have been found. Indeed, most drugs which were classified as drugs for each binding region did compete with each other.^{6–10)} Very recently, fortunately, using a homologous series of specific fluorescent probes, *n*-alkyl *p*-ABEs, we found a novel binding region (region Ic) in site I in addition to these other two regions and proposed a new concept that site I consists of at least three regions (regions Ia, Ib and Ic) which overlap each other.¹¹⁾ Regions Ia, Ib and Ic correspond to the warfarin, azapropazone and the third novel binding regions, respectively.

So, in these continuing investigations, we are studying the microenvironment of the new binding region, region Ic, including the hydrophobicity and depth of the binding region.

MATERIALS AND METHODS

Materials HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). It was defatted with activated charcoal in solution at 0 °C,

acidified with H₂SO₄ to pH 3 and then freeze-dried. The molecular mass of HSA was assumed to be 66500 Da. HSA gave only one band in SDS-PAGE. *n*-Alkyl *p*-aminobenzoates (*n*-alkyl *p*-ABEs) were synthesized by alkylation of *p*-aminobenzoic acid according to a conventional method.¹²⁾ The structures of the compounds were confirmed on the basis of NMR and MS spectroscopy. The purities of the compounds were checked by HPLC. Cetyltrimethylammonium bromide (CTAB), sodium lauryl sulfate (SLS) and Brij35 were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CTAB and SLS were gently recrystallized twice by CCl₄ and an ethanol-ether mixture, respectively. Polyoxyethylene lauryl ether (PLE) was purified from commercially available Brij35 according to the procedure of Ikeda *et al.*¹³⁾

All other chemicals were of analytical grade. All the buffers used were prepared with sodium phosphate dibasic and sodium phosphate monobasic.

Apparatus and Method Fluorescence measurements were carried out using a Jasco FP-770 fluorometer (Tokyo, Japan).

The Binding Parameters: The binding parameters were estimated by the fluorescence enhancement method. High (80 μM) and low (2 μM) concentrations of HSA were titrated by the successive addition of a probe stock solution prepared by methanol (to give a final *n*-alkyl *p*-ABEs concentration of 0.1–10 μM). Final concentrations of methanol in all the samples were less than 1 % (v/v). Fluorescence excitation and emission wavelengths were 310:346 nm for all *n*-alkyl *p*-ABEs. The binding parameters by the fluorescence enhancement method were calculated according to the method of Daniel and Weber.¹⁴⁾

The bound fraction (*f_b*) of the ligand was calculated by:

$$f_b = \frac{F_p - F_o}{F_b - F_o} \quad (1)$$

where F_p and F_o are the fluorescence intensities of a given concentration of probes in a solution of low HSA concentration and in a solution without HSA, respectively, and F_b is the fluorescence of the same concentration of fully bound probe. The bound concentration (D_b) and free concentration (D_f) of the ligand are estimated by the following equations:

$$D_b = D_t \times f_b \quad (2)$$

$$D_f = D_t - D_b \quad (3)$$

where D_t is the total ligand concentration.

By plotting these results according to the Scatchard equation,¹⁵⁾ binding parameters were calculated:

$$r/D_t = nK - rK \quad (4)$$

where r is the mole of bound ligand per mol of protein, n is the number of binding sites per protein molecule, and K is the association constant.

Job's Plots: The same temperature, fluorometer and fluorescence excitation and emission wavelengths as described in the estimation of binding parameters were used for these experiments. Job's plots were constructed by measuring the fluorescence intensity of the compounds bound to HSA at the constant total concentration ($10 \mu\text{M}$) of n -alkyl p -ABEs plus HSA.

Quantum Yield: The relative fluorescence quantum yields were calculated using the expression given by Gennis *et al.*¹⁶⁾:

$$\frac{F_s}{F_r} = \frac{\phi_s}{\phi_r} \times \frac{A_r}{A_s} \quad (5)$$

where F is the integrated area of the fluorescence emission spectrum, ϕ is the relative quantum yield, A is the absorbance at the excitation wavelength and the subscripts s and r refer to the sample and reference solution, respectively. The quantum yield (ϕ_r) of quinine bisulfate in 1 N H_2SO_4 was taken as 0.70.

Thermodynamic Parameters: Thermodynamic parameters were calculated based on the temperature dependence of association constants for probe-HSA binding. The temperatures used were 10, 25 and 40 °C. The enthalpy change (ΔH) is calculated from the slope of van't Hoff plots according to following equation:

$$\ln K = -\frac{\Delta H}{RT} + \text{constant} \quad (6)$$

where, R and T are the gas constant and the absolute temperature, respectively. The Gibbs free energy change (ΔG) is estimated from the following relationship:

$$\Delta G = -RT \ln K \quad (7)$$

Finally, the entropy change (ΔS) is obtained by using the parameters calculated above and the following equation:

$$\Delta G = \Delta H - T\Delta S \quad (8)$$

RESULTS

Physicochemical Properties The chemical structures and some physicochemical properties of n -alkyl p -ABEs used in this study are summarized in Table I.

Binding of Fluorescent Probes to HSA The fluorescence intensities of n -alkyl p -ABEs were greatly enhanced by binding to HSA, and their fluorescence emission maximum wavelengths shifted to a shorter wavelength (data not shown). Using the fluorescence enhancement characteristics of n -alkyl p -ABEs bound to HSA, Job's plots were constructed by n -alkyl p -ABEs bound to HSA while maintaining the total concentration of n -alkyl p -ABEs plus HSA at a constant value of $10 \mu\text{M}$ in order to check the maximum number of binding sites. As representative examples, the Job's plot for n -butyl, n -hexyl and n -octyl p -ABE-HSA systems are shown in Fig. 1. In all n -alkyl p -ABEs, the inflection point of Job's plot gives a value of

TABLE I. Chemical Structures and Physicochemical Properties of n -Alkyl p -ABEs

Compounds	R	M.L. (Å) ^{a)}	n -Alkyl p -ABE	
			$PC^b)$	$V_w^c)$ ($\times 10^2 \text{ Å}^3$)
Methyl p -ABE	CH_3	10.8	0.3	1.307
Ethyl p -ABE	C_2H_5	12.0	1.0	1.461
n -Propyl p -ABE	C_3H_7	13.2	3.9	1.615
n -Butyl p -ABE	C_4H_9	14.4	18.8	1.769
n -Amyl p -ABE	C_5H_{11}	15.6	83.3	1.923
n -Hexyl p -ABE	C_6H_{13}	16.8	256.5	2.077
n -Heptyl p -ABE	C_7H_{15}	18.0	642.0	2.231
n -Octyl p -ABE	C_8H_{17}	19.2	2023.3	2.385
n -Nonyl p -ABE	C_9H_{19}	20.4	8368.0	2.539
n -Decyl p -ABE	$\text{C}_{10}\text{H}_{21}$	21.6	31303.2	2.693
n -Undecyl p -ABE	$\text{C}_{11}\text{H}_{23}$	22.8	— ^{d)}	2.847
n -Dodecyl p -ABE	$\text{C}_{12}\text{H}_{25}$	24.0	— ^{d)}	3.001

a) M.L. (molecular length) was estimated by the Corey-Pauling-Koltun model (CPK model), which refers to a space filling model. b) PC (partition coefficient) values were calculated based on the partition between phosphate buffer (pH 7.4) and n -hexane saturated with buffer. c) V_w (van der Waals Volume) was analyzed by the method of Moriguchi *et al.*¹⁷⁾ d) Could not be estimated because the hydrophobicity (large PC) was too strong to be estimated.

Fig. 1. Job's Plots of Relative Fluorescence Intensities as Functions of the Mole Fraction of n -Alkyl p -ABEs

n -Alkyl p -ABEs used were n -butyl ($\bullet$), n -hexyl ($\circ$) and n -octyl esters ($\blacktriangle$). The total concentration ($[n\text{-alkyl } p\text{-ABE}] + [\text{HSA}]$) was kept constant at $10 \mu\text{M}$.

0.5, which corresponds to the number of binding sites, $n=1$. The binding parameters for the interaction of n -alkyl p -ABEs with HSA were also estimated by fluorescence enhancement methods (Table II).

Fluorescent Probe Displacement In an attempt to find out the binding site to which n -alkyl p -ABEs bind on HSA, the fluorescent probe displacement by several drugs whose binding site on HSA is already known was investigated by fluorescence according to the method of Sudlow *et*

TABLE II. Binding Parameters of n -Alkyl p -ABEs by Fluorescence Enhancement Method

Compounds	n	K (M^{-1})
Methyl p -ABE	1.0	2.6×10^3
Ethyl p -ABE	1.0	6.0×10^3
n -Propyl p -ABE	1.0	1.5×10^4
n -Butyl p -ABE	1.0	3.4×10^4
n -Amyl p -ABE	1.0	8.8×10^4
n -Hexyl p -ABE	1.0	2.1×10^5
n -Heptyl p -ABE	1.0	5.0×10^5
n -Octyl p -ABE	1.0	1.4×10^6
n -Nonyl p -ABE	1.0	2.4×10^6
n -Decyl p -ABE	1.0	4.7×10^6
n -Undecyl p -ABE	1.0	8.7×10^6
n -Dodecyl p -ABE	1.0	5.1×10^6

Fig. 2. Effects of Displacer Drugs on the Fluorescence Intensity of n -Hexyl p -ABE Bound to HSA

The numbers represent the displacer drugs used in this experiment: 1, acenocoumarol; 2, dicoumarol; 3, phenylbutazone; 4, glibenclamide; 5, chlorpropamide; 6, iodipamide; 7, iophenoxic acid; 8, diazepam; 9, ibuprofen. Following concentrations were used: HSA, $20 \mu M$; n -hexyl p -ABE, $2 \mu M$.

Fig. 3. Mutual Relationships between Affinity Constants of n -Alkyl p -ABEs-HSA Complexes (K), Partition Coefficients (PC) and van der Waals Volumes (V_w) of n -Alkyl p -ABEs

(A), Double-logarithmic relationship between K and PC ; (B), Semi-logarithmic relationship between K and V_w ; (C), Semi-logarithmic relationship between PC and V_w . C_1 - C_{12} represent the numbers of carbon atoms in the alkyl side chain of n -alkyl p -ABEs.

al.^{3,4}) (Fig. 2). The fluorescence intensity of n -hexyl p -ABE bound to HSA was decreased by some site I bound drugs, acenocoumarol, dicoumarol, iodipamide and iophenoxic acid but not by other site I bound drugs, phenylbutazone, glibenclamide and chlorpropamide. Furthermore, site II drugs, diazepam and ibuprofen, did not decrease. These findings may be observed for other n -alkyl p -ABEs.

Structure-Activity Relationship In light of the structure-activity relationship, quantitative relationships between physicochemical properties and the binding of drugs to protein can be established by multiple regression analysis. Relationships between association constants and partition coefficients or van der Waals volume of n -alkyl p -ABEs by the data of Tables I and II are shown in Figs. 3A and 3B. In both cases, good correlations were observed up to the octyl esters. Furthermore, the partition coefficients correlated well with the van der Waals volume for the all n -alkyl p -ABEs, as illustrated in Fig. 3C.

Fluorescence Spectra in HSA and Detergent Micelles The fluorescence of n -alkyl p -ABEs were enhanced by interaction with HSA. The fluorescence spectra of n -hexyl p -ABE observed during interaction with micelles of either cationic (CTAB), neutral (PLE), or anionic (SLS) detergents instead of HSA were illustrated as a delegate of n -hexyl p -ABE in Fig. 4, compared with HSA. The spectra with the other n -alkyl p -ABEs were in good agreement with those obtained with n -hexyl ester. All studies were performed above the critical micelle concentrations of various detergents. The fluorescence enhancements of n -alkyl p -ABEs during the interaction with HSA were also observed at the interaction with the micelles of positive or neutral detergents.

Thermodynamic Analysis From the temperature dependence of association constants for n -alkyl p -ABEs-HSA bindings, the values for thermodynamic functions involved in the binding process could be determined. The association constants of n -alkyl p -ABEs to HSA estimated at three different temperatures, 10, 25 and $40^\circ C$, were plotted according to the van't Hoff equation (Fig. 5). The thermodynamic parameters are summarized in Table III. For simplicity, only the thermodynamic parameters for n -amyl to n -nonyl p -ABEs were exemplified. The thermodynamic parameters obtained here were evaluated only at the primary binding

site. In addition, the relationship between the entropy or enthalpy changes and number of aliphatic carbon substituents was constructed (Fig. 6).

Effect of pH on the Ligand Binding The effects of pH on the fluorescence intensities of *n*-alkyl *p*-ABEs in the presence and absence of HSA were monitored (Fig. 7A). The fluorescence intensity of *n*-hexyl ester in the presence of HSA increased with pH, in sharp contrast to the ligand alone. Both fluorescence quantum yield and the affinity constant of *n*-hexyl ester in the presence of HSA increased

with pH, similarly to the increase in fluorescence intensity (Fig. 7B). As illustrated in Fig. 7C, the fluorescence emission maximum wavelength of *n*-hexyl ester in the presence of HSA shifted to a shorter wavelength at a higher pH, while that in the absence of HSA was not affected by pH. Similar findings were also observed in the other *n*-alkyl *p*-ABEs.

DISCUSSION

In the preceding paper,¹¹⁾ we proposed the new concept that site I on HSA consists of at least three overlapping regions, namely region Ia, Ib and Ic. Regions Ia and Ib correspond to the warfarin and azapropazone regions reported by Fehske *et al.*,⁶⁾ respectively, and region Ic was first found at these laboratories. This finding has also

Fig. 4. Fluorescence Spectra of *n*-Hexyl *p*-ABE Bound to HSA and Detergents at pH 7.4 and 25°C

(A), *n*-hexyl *p*-ABE-HSA system; (B), *n*-hexyl *p*-ABE-SLS system; (C), *n*-hexyl *p*-ABE-CTAB; (D), *n*-hexyl *p*-ABE-PLE system. Following concentrations were used: *n*-hexyl *p*-ABE, 2 μ M; HSA, 20 μ M; detergents, 2 mM.

Fig. 5. van't Hoff Plots for Binding of *n*-Alkyl *p*-ABEs to HSA

n-Alkyl *p*-ABEs used were *n*-amyl (■), *n*-hexyl (○), *n*-heptyl (△), *n*-octyl (▲) and *n*-nonyl (□) esters.

Fig. 6. Relationships between the Thermodynamic Parameters and the Number of Carbon Atoms in the Alkyl Side Chain of *n*-Alkyl *p*-ABEs

Thermodynamic parameters were: ○, enthalpy change (ΔH); ▲, entropy change (ΔS). C₁–C₁₂ represent the numbers of carbon atoms in the alkyl side chain of *n*-alkyl *p*-ABEs.

Fig. 7. Effect of pH on the Interaction between *n*-Hexyl *p*-ABE and HSA

(A), effect on fluorescence intensity; (B), effect on quantum yield (Φ)(●) and association constants (K)(▲); (C), effect on fluorescence emission maximum wavelength ($\lambda_{\max}$). Following concentrations were used: HSA, 20 μ M; *n*-hexyl *p*-ABE, 2 μ M for (A) and (C).

shown that region Ia overlaps with the other regions, as if region Ia would play a bridging role between regions Ib and Ic. That is, regions Ib and Ic might be located in completely different parts because of the independent binding of ligands for each of these regions. Thus, this study was performed to analyze the microenvironment of a new binding region, region Ic, in detail using fluorescent probes, a series of *n*-alkyl *p*-ABEs.

Possibility as Fluorescent Probes for Region Ic in Site I

We have recently reported the utility of *n*-butyl *p*-ABE as a fluorescent probe for region Ic in site I.¹¹⁾ In order to estimate the possibility of the other *n*-alkyl *p*-ABEs as fluorescent probes, some fluorescent and binding characteristics were investigated. The fluorescence of all *n*-alkyl *p*-ABEs were enhanced by being occupied on only one high affinity binding site on HSA, as shown in Fig. 1 and Table II. Furthermore, the displacement of a fluorescent probe by other drugs was monitored by the decrease in fluorescence intensity in order to identify these probe binding sites (Fig. 2). Fluorescence intensities of all *n*-alkyl *p*-ABEs were selectively decreased by the same drugs as *n*-hexyl *p*-ABE. The fluorescence of *n*-alkyl *p*-ABEs bound to HSA were not decreased by site II (ibuprofen and diazepam) or region Ib bound drugs (phenylbutazone, glibenclamide and chlorpropamide), while the other drugs (region Ia or Ic bound drugs) specifically decreased this fluorescence. Unfortunately, we could not confirm these phenomena by ultrafiltration or equilibrium dialysis because of the marked adsorption of *n*-alkyl *p*-ABEs (more than *n*-hexyl ester) onto the apparatus and membranes. However, taking into consideration the previous findings using fluorescence and ultrafiltration techniques¹¹⁾ that the decreases in fluorescence intensity of *n*-butyl *p*-ABE bound to HSA by acenocoumarol and dicumarol which bind to region Ia are not due to displacement but to fluorescence quenching in contrast to the displacement by iodipamide and iophenoxic acid which bind to the overlapping part between regions Ia and Ic; the decreases in fluorescence of other *n*-alkyl *p*-ABEs seem to be caused by same mechanism. Indeed, by the addition of acenocoumarol and dicumarol, the fluorescence of *n*-alkyl *p*-ABEs in methanol were decreased in the absence of HSA. Accordingly, the similarity of the changes in fluorescence of *n*-alkyl *p*-ABEs bound to HSA by site I drugs might indicate that all these probes may bind specifically to the non-overlapping part of region Ic in site I. This clearly suggests that *n*-alkyl *p*-ABEs must be useful fluorescent probes for the selective analysis of region Ic.

Binding Mode and Microenvironment of Region Ic For the non-covalent interactions, such as reversible protein-ligand interaction, hydrogen bonds, van der Waals forces, hydrophobic bonds and electrostatic interaction play essential roles in the binding.^{18,19)} A good correlation between affinity constants and partition coefficients was obtained until octyl ester (Fig. 3A). Affinity constants also correlated well with van der Waals volumes (Fig. 3B), as expected from the good correlation between partition coefficients and van der Waals volumes (Fig. 3C). The deviations of *n*-nonyl, *n*-decyl, *n*-undecyl and *n*-dodecyl *p*-ABEs from the linearity of log *K*-van der Waals volumes (Fig. 3B) suggest that these molecules are too large to fully

penetrate into region Ic. Possibly, region Ic possesses the optimal depth which accepts a size only up to that comparable to *n*-octyl ester. This may cause the deviations from the linear relationship, suggesting that the first point of deviation represents the region size. From the molecular size of octyl ester, the depth of region Ic is estimated to be 19 Å. The comparison of the size of site II, 16 Å, estimated by Wanwimolruk *et al.*,²⁰⁾ suggested that region Ic is deeper than site II. That is, site I is considered to form a significantly larger region, compared with site II.

As shown in Fig. 4, the fluorescence of *n*-hexyl *p*-ABE was enhanced for the interaction with HSA and neutral (PLE) or positively charged detergent (CTAB) micelles. Furthermore, the other *n*-alkyl *p*-ABEs indicated the same tendency (data not shown). This suggests that the fluorescence enhancement of a ligand is ascribed such that the ligand molecule is located in a hydrophobic area. That is, the chromophore of *p*-ABE molecules is located in the hydrocarbon center of the micelle, away from the water; in other words, the environment of the interaction part in the neutral or positively charged micelles might be similar to region Ic on HSA. Since *n*-alkyl *p*-ABEs are non-charged molecules at pH 7.4, positive charges are unlikely to play a dominant role in the interaction with *n*-alkyl *p*-ABEs. However, the fluorescence enhancement by the interaction with cationic detergent micelles might indicate that the existence of the positive charge in the interaction with HSA could not be ignored. Although the only amino acid residue clearly involved in region Ia is tryptophan, a lysine residue is thought to possibly be located in the close neighborhood of region Ia.²¹⁾ This lysine may be involved in the interaction with *n*-alkyl *p*-ABEs. Regardless, the comparison of the *n*-alkyl *p*-ABEs-HSA interaction with *n*-alkyl *p*-ABEs-detergent micelle indicated that region Ic might be either a hydrophobic region or a hydrophobic region with a cation charge.

Further, investigation of the binding mode was carried out by determination of the thermodynamic parameters for the binding of *n*-alkyl *p*-ABEs to HSA. In these experiments, *n*-alkyl *p*-ABEs-HSA complexes were accompanied by negative enthalpy changes (ΔH) and positive entropy changes (ΔS) for all systems (Table III), which indicates that all binding processes are entropically as well as enthalpically driven. Thus, the binding process was always spontaneous, as evidenced by the negative ΔG value, and was also exothermic as indicated by negative sign of ΔH . Based on the characteristic signs of the thermodynamic parameters at the various interactions,²²⁾ both positive ΔS and ΔH values generally represent hydrophobic interactions. On the other hand, for van der Waals interactions, which are involved in the hydrophobic effect, both ΔH and ΔS indicate negative values. Moreover, a specific electrostatic interaction between ionic species in an aqueous solution is characterized by a positive ΔS value and negative or small positive ΔH value. In addition, the structural change which may make a binding site more flexible gives a large positive ΔS value due to an endothermic effect, as can be observed in protein unfolding. Therefore, the thermodynamic parameters for the interaction of *n*-alkyl *p*-ABEs and HSA can

be explained on the basis of plural intermolecular forces, such as hydrophobic, electrostatic and van der Waals interactions, rather than a single intermolecular force. However, ionic interactions for the binding force can be excluded because of a lack of charge in physiological pH. Consequently, the hydrophobic interaction may be an important binding force for *n*-alkyl *p*-ABEs-HSA complexes. In ligand-macromolecule interaction, the enthalpy change (ΔH) is usually compensated for by a variation of entropy changes (ΔS), which indicates that a single mechanism predominates in the binding process.²³⁻²⁵ The lack of compensation between ΔH and ΔS in Fig. 6 supports that the interaction mode may be due to several intermolecular forces. The region in which such local conformational changes is caused might correspond to the "conformationally labile preformed binding site" suggested by Walji *et al.*²⁶

The fluorescence emission wavelength was also estimated for the binding mode (data not shown) since the wavelength strongly depends on the microenvironment, especially the hydrophobicity around probes. The shift of wavelength toward a shorter, blue shift, upon binding to HSA also indicates the importance of a hydrophobic interaction for binding.

Consequently, hydrophobic interaction, van der Waals interaction and local structural change in region Ic might be involved in the binding process.

Effect of Conformational Change In the pH range between 6–9, HSA changes the conformation form, the form at neutral pH (pH 6–7), the N form, and the form at higher pH (around pH 9), the B form. Thus, the conformational change between the N form and the B form is called the N–B transition.²⁷⁻²⁹ Histidine residues in domain I in HSA are thought to play an important role in the N–B transition.^{30,31} Binding to site I is influenced by domain I, in contrast to a lack of effect on site II binding. These might explain the findings that drug binding to site I seemed to be affected by N–B transition rather than that to site II.^{29,32} Recent reports have shown the possibility of the involvement of N–B transition in the transport and cellular uptake mechanisms of endogenous and exogenous substances.^{30,33,34} In order to investigate the effect of N–B transition on the drug binding site in more detail, region Ic in site I was examined spectroscopically.

With increasing pH, the fluorescence intensity of *n*-alkyl *p*-ABEs increased because of conformational change in HSA (Fig. 7A). The fluorescence enhancement may be due to the two factors related to fluorescence intensity, the increment of the concentration of fluorophore and the quantum yield (Fig. 7B). This indicated that N–B transition may change the microenvironment of region Ic in which *n*-alkyl *p*-ABEs could bind more stably and smoothly. Indeed, the shift of the fluorescence emission maximum wavelength to shorter with an increase in pH (Fig. 7C) suggests that region Ic became more hydrophobic and caused structural change in which the mobility of the probes was restricted by tight binding, as shown in the increase of quantum yield (Fig. 7B). However, the depth might be unchanged with N–B transition, considering the same saturation patterns of *n*-alkyl *p*-ABEs binding as

that shown in Figs. 3A and 3B (data not shown). These findings strongly suggest that N–B transition causes microenvironmental changes (for example, an increase in adaptability, or an increase in hydrophobicity) by its structural fluctuations, and such changes might result in increased binding. Thus, the microenvironment of region Ic might be affected by a pH-dependent conformational change of HSA, the N–B transition. This finding is very similar to the case of warfarin binding,³⁵ which might support that region Ic is located around region Ia in site I.

In conclusion, region Ic was located independently from the other two regions (regions Ia and Ib) in site I. From analysis using a series of *p*-ABEs, it is possible that region Ic is a hydrophobic region which possesses optimal size. These structural and microenvironmental characteristics might be important factors in the construction of the specific binding region. The data presented in this study is therefore useful not only for the prediction of pharmacokinetics and pharmacology of the drugs, but also for biochemical study, such as knowledge of the structure and function of HSA.

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