Classification of the Binding Modes in Bovine Serum Albumin Using Terminally Substituted Alkane Analogues

Kô Takehara,[*†] **Yuki Morinaga,*** **Shinya Nakashima,*** **Shiro Matsuoka,****
Hiroshi Kamaya,*** **and Issaku Ueda*****

*Department of Chemistry, Faculty of Sciences, Kyushu University, Ropponmatsu, Chuo, Fukuoka 810–8560, Japan
**Department of Environmental Science, Faculty of Science, Niigata University, Ikarashi, Niigata 950–2181, Japan
***DVA Medical Center and School of Medicine, University of Utah, Salt Lake City, UT 84148, USA

With the fluorescence probe of 8-anilino-1-naphthalenesulfonate (ANS), the binding modes of terminally substituted alkane analogues (C_nX; X = COOH, OH, CHO, NH_3, $CONH_2$) to bovine serum albumin (BSA) were investigated using a competitive binding technique.   The Scatchard plot of the fluorometric titration of BSA with ANS showed that the maximum binding number of ANS, n_{max}, was 3.81, with the binding constant, K_{bnd}, of 1.42×10^6 mol^{-1} dm^3.   The binding modes of C_nX to BSA were analyzed based on the fluorometric titration of the ANS and BSA mixture with C_nX. C_nCOOH completely displaced the ANS bound to BSA, whereas C_nOH and C_nCHO displaced only about 40% of the ANS bound to BSA.   In contrast, C_nNH_2 and C_nCONH_2 displaced very little bound ANS.   By comparing these results, we classified the binding modes of C_nX to BSA into three types.   Two of them are detectable with the ANS fluorescence and the remaining one is not detectable with the fluorescence.

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Introduction

8-Anilino-1-naphthalenesulfonate (ANS) fluoresces strongly in hydrophobic solvents but is almost nonfluorescent in water.[1] The fluorescence intensities of the ANS ions bound to proteins are nearly equal to those of ANS ions dissolved in organic solvents.   The large increase of the fluorescence intensity of ANS ions, when bound to BSA in water, is attributed to binding to the hydrophobic domains of a protein.[2-4]   We denote these sites as the fluorescence-active sites.   Despite extensive spectrofluorometric studies with ANS, clear understanding of the properties of the binding site in BSA is lacking.   Though it is established that there are at least two types of ANS binding sites in a BSA molecule, uncertainties still remain as to the properties of each binding site.[5-8]

Inhalation anesthetics and alcohols are hydrophobic molecules and are considered to act by interacting with a number of proteins by hydrophobic interaction.[9-11]   Therefore, it is important to make clear the binding properties of the hydrophobic molecules to proteins to elucidate the anesthetic action on proteins.   This study used spectrofluorometry of the ANS to analyze the binding of five types of alkane analogues, C_nX, that is, alkylalcohols (C_nOH, n = 8, 10, 12), alkylaldehyde (C_nCHO, n = 7, 9, 11), alkylacids (C_nCOOH, n = 5, 7, 9, 11), alkylamines (C_nNH_2, n = 8, 10, 12), and alkylamides (C_nCONH_2, n = 7, 9, 11). C_nCOOH are in anionic form in the aqueous solution of neutral pH, whereas C_nNH_2 and C_nCONH_2 are in cationic form in the neutral pH.[12] C_nOH and C_nCHO have no net charges in the usual pH range.   We reported previously that these analogues acted on the protein function in a different

manner.[13,14]   We report here the classification of three types of binding sites in a BSA molecule, estimated by the ANS fluorescence.   Two of these types are fluorescence-active for the ANS ions bound to BSA, whereas the remaining one is fluorescence-inactive and cannot be detected by the ANS fluorescence.   This report will show that the terminal charge of the C_nX critically affects the displacement reaction of the ANS ions bound to the fluorescence-active sites in BSA with C_nX.[15-17]   Hydrophobicity of the C_nX is also suggested to be another key factor to differentiate the binding sites in BSA.   It is demonstrated that the three types of the binding sites in BSA can be classified by the competitive binding measurements of the ANS ions and alkane analogues.[18-20]

Experimental

Materials and sample preparation

Essentially fatty acid free bovine serum albumin (Cat. No. A7511) was purchased from Sigma Chemical Co. (St. Louis, USA) and used as supplied.   An ammonium salt of 8-anilino-1-naphthalenesulfonic acid (ANS·NH_4) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan).   Terminally substituted alkane analogues were purchased from Tokyo Kasei Kogyo, Aldrich Chemical Co. (Milwaukee, USA) or Acros Organics (New Jersey, USA) and were used without further purification. Sample solutions were prepared using the water purified by a MiliQ-SP reagent water system.   Stock solutions of 5 mg cm^{-3} BSA and 2 mmol dm^{-3} ANS were prepared by dissolving BSA and ANS·NH_4, respectively, into purified water containing 0.1 mol dm^{-3} glycylglycine buffer of pH 7.4.   The concentrations of BSA and ANS were calibrated by UV/VIS absorbance using the molar absorption coefficient at 280 nm of 4.36×10^4 cm^{-1} mol^{-1} dm^3 and the molecular weight 65500 for BSA and the molar

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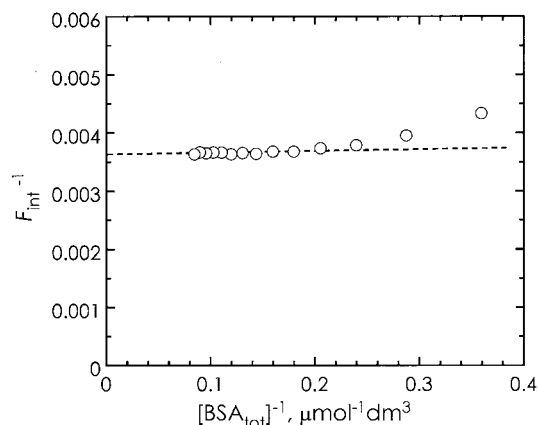† To whom correspondence should be addressed.
E-mail: kou@rc.kyushu-u.ac.jp

Fig. 1   Double reciprocal plot of the fluorescence intensity of ANS, F_{int}, *versus* the total concentration of BSA, [BSA$_{tot}$]. ANS (10.3 μmol dm^{-3}) was titrated with BSA to obtain the fluorescence intensity of the ANS bound to BSA (ANS-BSA). The quantum yield of the ANS-BSA obtained from the extrapolation to the infinite BSA concentration was 0.46, based on quinine as the standard.

Fig. 2   Scatchard plot of the fluorescence titration of BSA with ANS. BSA (0.74 μmol dm^{-3}) was titrated with ANS. The maximum binding number, n_{max}, and the binding constant, K_{bnd}, of the ANS to BSA were estimated from the slope and X-axis intercept and were 3.81 and 1.42×10^6, respectively.

absorption coefficient at 350 nm of 5.83×10^3 cm^{-1} mol^{-1} dm^3 for ANS. The absorbance was measured with a Shimadzu RF-2200 UV/VIS spectrophotometer.

The pH values of all sample solutions were adjusted to 7.4 with 0.1 mol dm^{-3} glycylglycine buffer. For fluorescence measurements, an aliquot of BSA and ANS stock solutions were mixed, diluted to desired concentration and kept standing for at least 15 min at 25°C to equilibrate the binding reaction before measurements.

Fluorescence measurements

Fluorescence was measured using a Shimadzu RF-5000 fluorescence spectrophotometer equipped with a temperature-controlled cell holder. The cell temperature was controlled at 25 ± 0.2°C by a NESLAB RTE-8 circulation water bath. The excitation wavelength was 352 nm and emission spectra were recorded between 460 nm and 480 nm. The peak wavelengths of the emission spectra were observed at around 471 nm.

To avoid the concentration quenching of the fluorophore, we kept the ANS concentrations below 2×10^{-5} mol dm^{-3}. Under this concentration, a linear relationship between the fluorescence intensity and ANS concentration was confirmed by measuring the ANS fluorescence in *n*-propanol.

The fluorescence quantum yield of the ANS bound to BSA, ANS-BSA, was estimated using quinine sulfate as the standard. The absorbance and fluorescence intensities of ANS-BSA solution were compared to those of quinine sulfate solution. The quantum yield of the quinine sulfate in 1.0 mol dm^{-3} H$_2$SO$_4$ was taken as 0.55 at 25°C.

Displacement measurements

The displacement of the ANS on BSA with C$_n$X was estimated by adding an aliquot of C$_n$X solution into the mixture of 0.75 μmol dm^{-3} BSA and 10.0 μmol dm^{-3} ANS. A 0.03-cm^3 volume of the ethanolic solution of C$_n$X was added to 3.0 cm^3 of the mixture of ANS and BSA in 0.1 mol dm^{-3} buffer solution of pH 7.4. The decrease of the fluorescence intensity caused by the addition of C$_n$X was recorded as the measure of the displacement of the ANS on BSA with C$_n$X.
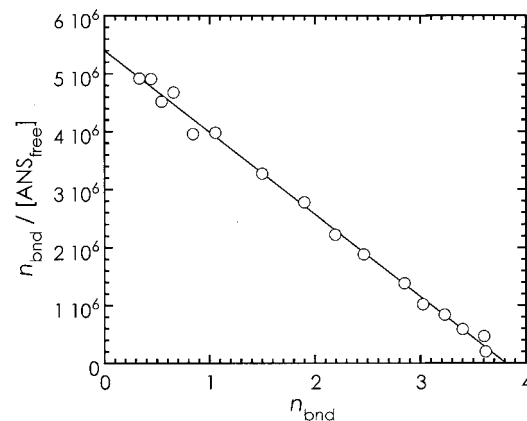
Results and Discussion

Fluorescence intensity of the ANS bound to BSA

The fluorescence intensity of the ANS-BSA was estimated by the following procedure. Under the ANS concentration fixed at 10.3 μmol dm^{-3}, the double reciprocal plot between the fluorescence intensity *versus* the total BSA concentration is shown in Fig. 1. The maximum fluorescence intensity was obtained by extrapolating the linear regression line to infinite BSA concentration. This value is assigned to the fluorescence intensity of the ANS-BSA under the condition that all ANS ions in solution are bound to the BSA molecules. The molar fluorescence intensity of the ANS on BSA was obtained by dividing the maximum fluorescence intensity of the ANS-BSA by the ANS concentration. The molar fluorescence intensity was converted further to the quantum yield using the quantum yield 0.55 of quinine sulfate as the standard.[21] In these procedures, we assumed that the fluorescence intensities of all bound ANS are equivalent and independent of the binding site in BSA. The quantum yield of the ANS-BSA thus obtained was 0.46, which agrees well with the reported values 0.42 – 0.48 for the ANS bound to proteins.[22] This result supports the validity of the assumption of the equivalent fluorescence intensity for all bound ANS ions.

Binding of ANS to BSA

The maximum binding number of ANS to BSA, n_{max}, and the binding constant, K_{bnd}, were estimated by the fluorometric titration of 0.74 μmol dm^{-3} BSA with ANS solution. Figure 2 shows the Scatchard analysis of the titration data. A straight line was obtained with n_{max} 3.81 and K_{bnd} 1.42×10^6 mol^{-1} dm^3. The linearity of the Scatchard plot and the n_{max} value of 3.81 indicate the existence of four binding sites in a BSA molecule with similar affinity to ANS ions. These values agree well with those reported by several authors, in which n_{max} and K_{bnd} values were in the ranges of 3 to 5 and 2.1×10^5 to 2.9×10^6, respectively.[23-25]

Even more ANS binding sites on BSA were reported by Kolb and Weber[26] and Valsami.[27] These sites are identified by equilibrium dialysis and potentiometry but not detectable by fluorescence measurement. Therefore, these sites are considered to be hydrophilic enough that water molecules can
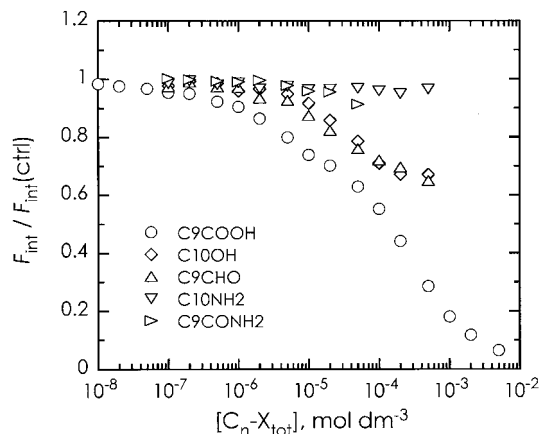
Fig. 3   Dependence of the relative fluorescence intensity of ANS-BSA, F_{int}/F_{int} (ctrl), on the total concentration of decane analogues, $[C_n\text{-}X_{tot}]$, at pH 7.4. The concentrations of ANS and BSA were 10.3 and 0.74 µmol dm^{-3}, respectively. C_9COOH entirely quenched the fluorescence of ANS-BSA. $C_{10}OH$ and C_9CHO decreased the fluorescence intensity about 40% of the initial intensity at their saturated concentrations. $C_{10}NH_2$ and C_9CONH_2 decreased the fluorescence not more than 20% of the initial intensity.

Fig. 4   Dependences of the concentration of free C_nCOOH (n = 5, 7, 9, 11) on the site occupancy, θ, of C_nCOOH in BSA. The concentrations of ANS and BSA were 10.3 and 0.74 µmol dm^{-3}, respectively. All of C_nCOOH entirely displaced the ANS bound to the fluorescence-active sites in BSA, though the concentration required for the entire displacement increased with decreasing the alkyl chain-length. Moreover, the binding curves can be divided in two segments. We classify the C_nCOOH as the group-I analogues.

gain access to the bound ANS ions to quench their fluorescence. Furthermore, the reported binding capacity of these sites is more than ten ANS ions in a BSA molecule, far larger than the capacity of fluorescence-active sites of 3 to 5.

Displacement of the bound ANS with C_nX

Figure 3 shows the fluorescence intensities of the ANS-BSA when C_9COOH, $C_{10}OH$, C_9CHO, $C_{10}NH_2$, or C_9CONH_2 is added to the aqueous solution of 10.3 µmol dm^{-3} ANS and 0.74 µmol dm^{-3} BSA mixture at pH 7.4. In Fig. 3, the relative fluorescence intensity was taken as the fluorescence intensity of the ANS in the presence of C_nX divided by the fluorescence intensity in the absence of C_nX. These alkane analogues are classified in the three different types depending on the inhibition effect on the ANS fluorescence. C_9COOH (group-I*) inhibited the fluorescence intensity most effectively. It almost entirely quenched the fluorescence intensity of the ANS-BSA in a dose-dependent manner. In addition, the plot showed two distinguishable segments with the inflection point at the C_9COOH concentration about 10^{-5} mol dm^{-3}. $C_{10}OH$ and C_9CHO (group-II) inhibited only about 40% of the fluorescence at their highest soluble concentrations. There are two types of the fluorescence-active ANS binding sites in a BSA: one is competitive to group-II alkane analogues, and the other is noncompetitive. $C_{10}NH_2$ and C_9CONH_2 (group-III) inhibited the fluorescence intensity very little even at their highest soluble concentrations.

Assuming that all the fluorescence-active sites in BSA are occupied by either of ANS or C_nX, we estimated the amount of C_nX bound to BSA from the decrease of the ANS fluorescence. Figures 4 to 8 show the site occupancies of C_nX thus obtained as a function of free C_nX concentration for C_nCOOH, C_nOH, C_nCHO, C_nNH_2, and C_nCONH_2, respectively. The site occupancy, θ, is defined as the number of the bound C_nX divided by the number of the fluorescence-active binding sites in a BSA. In these figures, the results for the different alkyl chain-length of C_nX are also plotted. For all alkane analogues examined, the displacement began to occur at lower C_nX concentrations with the increase of the alkyl chain-length.
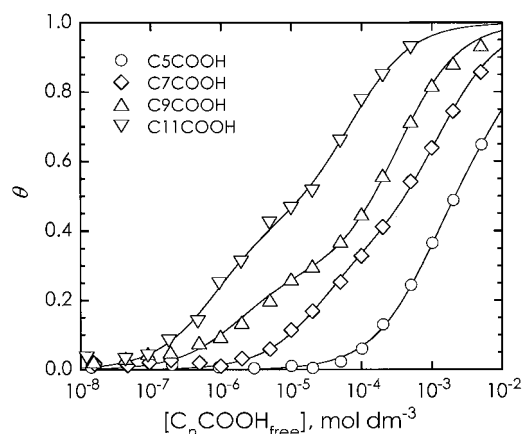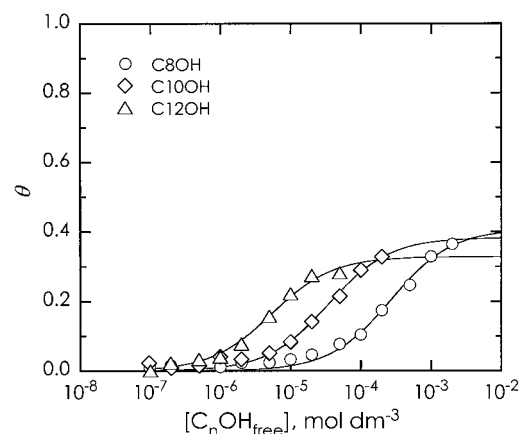

Fig. 5   Dependences of the concentration of free C_nOH (n = 8, 10, 12) on the site occupancy, θ, of C_nOH in BSA. All of C_nOH displaced only about 40% of the ANS bound to the fluorescence-active sites in BSA at their saturated concentrations.

However, the maximum displacement at higher concentration was almost independent of the alkyl chain-length. All group-I analogues almost entirely displaced the ANS on BSA. Moreover, the binding profiles in Fig. 4 show the inflection point at the occupancy of around 40%. This inflection point, however, faded gradually with the decrease of the alkyl chain-length of C_nCOOH. In contrast, all group-II analogues displaced only about 40% of the ANS on BSA, and the remaining 60% of the bound ANS were unaffected. The effects of group-III analogues were not more than 17% of the bound ANS. The results of the group-I and group-II analogues suggest the existence of two different types of the fluorescence-active sites in BSA. These two types of the binding sites in BSA, however, cannot be distinguished by the direct measurement of

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* We categorize the three types of terminally-substituted alkane analogues as group-I (C_nCOOH), group-II (C_nOH and C_nCHO), and group-III (C_nNH_2 and X_nCONH_2).
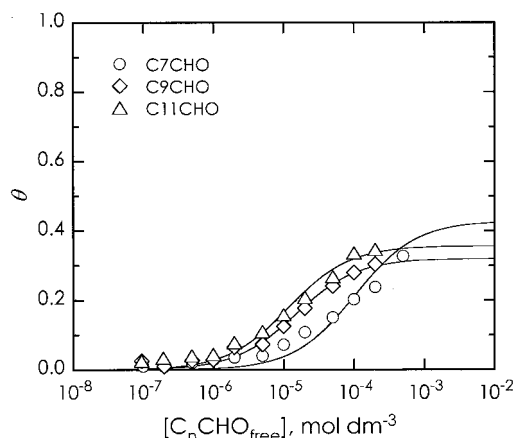
Fig. 6   Dependences of the concentration of free C_nCHO (n = 7, 9, 11) on the site occupancy, θ, of C_nCHO in BSA. All of C_nCHO displaced only about 40% of the ANS bound to the fluorescence-active sites in BSA at their saturated concentrations, similar to the case of C_nOH. C_nOH and C_nCHO are classified as group-II analogues.
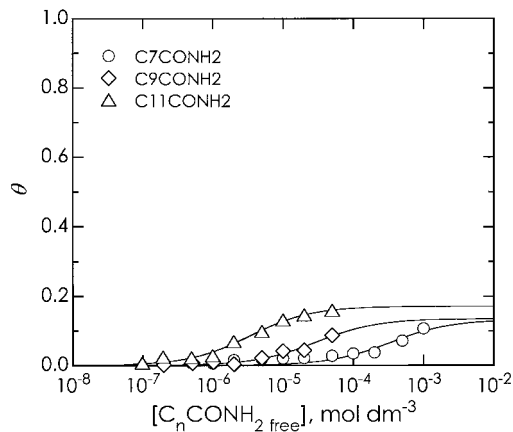

Fig. 8   Dependences of the concentration of free C_nCONH_2 (n = 7, 9, 11) on the site occupancy, θ, of C_nCONH_2 in BSA. Like the C_nNH_2, the C_nCONH_2 also only slightly decreased the fluorescence intensity of the ANS bound to BSA even at their saturated concentrations. C_nNH_2 and C_nCONH_2 are classified as group-III analogues.
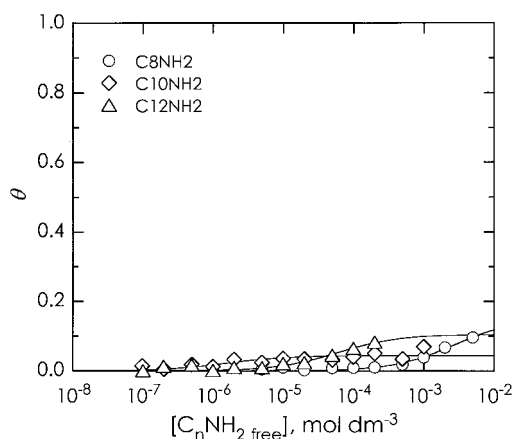

Fig. 7   Dependences of the concentration of free C_nNH_2 (n = 8, 10, 12) on the site occupancy, θ, of C_nNH_2 in BSA. These amines only slightly decreased the fluorescence intensity of the ANS bound to BSA even at their saturated concentrations.

the ANS binding to BSA as shown in Fig. 2.

 The solid lines in Figs. 4 to 8 were obtained by a nonlinear least-square curve-fitting method with the following two assumptions: (1) There are two different types of C_nX binding sites in BSA. One type binds all analogues of group-I to group-III, whereas the other binds only the group-I analogues. (2) The binding of C_nX obeys the Langmuir binding isotherm. With these assumptions, the curve fitting was performed using the following equations. For the binding of group-I analogues, the site occupancy, θ, is expressed as a function of free C_nX concentration, $[C_nX_{free}]$, according to the Langmuir binding isotherm for two types of independent sites,[15]

$$\theta = \frac{\eta_1 K_1 [C_nX_{free}]}{1 + K_1 [C_nX_{free}]} + \frac{\eta_2 K_2 [C_nX_{free}]}{1 + K_2 [C_nX_{free}]} \qquad (1)$$

in which subscripts 1 and 2 signify two types of the binding sites, site-1 and site-2, in BSA, and η and K are the fraction of each site and the binding constant, respectively. By using the experimental values of θ and $[C_nX_{free}]$, we can estimate the

values of η and K. For group-II and group-III, Eq. (1) is reduced to the following form.

$$\theta = \frac{\eta_1 K_1 [C_nX_{free}]}{1 + K_1 [C_nX_{free}]} \qquad (2)$$

 The η and K values obtained from the best fitted curves are listed in Table 1 for group-I and Table 2 for group-II and group-III analogues. From these results, one can conclude that the fractions of 40% and 60% for site-1 and site-2, respectively, are reasonable assignments. The ANS on site-1 can be displaced by both group-I and group-II analogues, whereas the ANS on site-2 can be displaced only by the group-I analogues. Because the number of fluorescence-active binding sites in a BSA was four, as estimated from the Scatchard plot in Fig. 2, the values of site-1 (40%) and site-2 (60%) correspond to the average number of 1.6 and 2.4 sites, respectively, in a BSA. Two different types of the ANS binding sites were also reported for human serum albumin (HSA) by Bagatolli *et al.* using phase modulation fluorescence spectroscopy.[6] The fractions of these two sites in a HSA were 31% and 69%, respectively. They also reported that only the ANS molecules bound to site-1 were displaced by other ligand like a diazepam. More importantly, the difference of the binding affinity between the two sites decreased with the decrease of the alkyl chain-length of the C_nCOOH as shown in Fig. 4 and Table 1. This result indicates that the hydrophobicity is also an important factor to differentiate the binding properties of the two types of the binding sites.

 In the case of the group-III analogues, both the numbers of the binding sites and the binding constants were lower than those of group-I and group-II analogues. It indicates that the group-III analogues scarcely displaced the ANS ions on BSA at all. However, this does not indicate that these analogues are incapable of interaction with BSA, because a variety of cationic detergents bind to BSA with large binding numbers exceeding 100 ions on a BSA.[28] One possible cause of this lack of displacement may be that cationic alkane analogues do not share the fluorescence-active binding site with anionic ANS ions, but they do bind to the fluorescence-inactive sites in BSA. As reported by Kolb and Weber[26] and Valsami,[27] more than ten fluorescence-inactive ANS binding sites were identified by

Table 1   Estimated binding parameters of group-I

Group	C_nX	n	η_1	K_1	η_2	K_2
Group-I	C_nCOOH	5	0.37	8.9×10^1	0.63	1.1×10^3
		7	0.37	7.8×10^2	0.63	3.7×10^4
		9	0.28	3.1×10^3	0.72	5.5×10^5
		11	0.43	1.4×10^4	0.57	1.2×10^6

Table 2   Estimated binding parameters of group-II and group-III

Group	C_nX	n	η_1	K_1
Group-II	C_nOH	8	0.41	3.7×10^3
		10	0.38	2.9×10^4
		12	0.33	1.9×10^5
	C_nCHO	7	0.43	9.7×10^3
		9	0.32	6.7×10^4
		11	0.36	8.6×10^4
Group-III	C_nNH_2	8	0.15	3.6×10^2
		10	0.04	6.5×10^5
		12	0.10	1.7×10^4
	C_nCONH_2	7	0.13	3.0×10^3
		9	0.14	6.6×10^3
		11	0.17	3.0×10^3

equilibrium dialysis and potentiometry. These sites can be assigned to the binding sites of group-III analogues labeled as site-3.

There are three types of ANS binding sites in a BSA molecule, as demonstrated by the competitive binding of three different types of alkane analogues in connection with the results of nonfluorometric techniques. The ANS ions bound to the site-2 of BSA can be displaced only by group-I analogues, whereas the ANS ions on the site-1 can be displaced by the both group-I and group-II analogues but not by group-III analogues. Only the ANS ions bound to the site-3 of BSA can be displaced by the group-III analogues, though such displacement cannot be detected by the ANS fluorescence.

At the present experimental condition of pH 7.4, both ANS and group-I analogues C_nCOOH are negatively charged, and group-III analogues C_nNH_2 and C_nCONH_2 are positively charged. The group-II analogues C_nOH and C_nCHO have no net charge. Therefore, one would expect that the electrostatic interaction is one of the key factors to differentiate the three types of the binding sites in BSA. Site-2 has a specific affinity to the negative charges. The ANS ions bound to this site can only be displaced by the negatively charged group-I analogues. In contrast, site-1 has higher affinity to hydrophobicity than to negative charge. The ANS ions bound to this site can be displaced by either group-I or group-II analogues but not by the positively charged group-III analogues. As for the site-3 locations, these sites are considered to be the ionizable side chains of the amino acid residues located at the protein–water interface. Although there are hydrophobic interactions between these sites and ANS ions, the interactions are not as strong as for site-1 and site-2. Therefore, water molecules are easily accessible to the ANS ions bound to the site-3 in BSA, enough to quench the fluorescence. These sites constitute the third binding sites for hydrophobic ligands. This conclusion suggests that the hydrophobic bindings to these sites are not specific. Nonspecific interactions at multiple uncharged and charged sites are candidates for the site-3 in BSA.

As shown in the Scatchard plot in Fig. 2, the site-1 and site-2 in BSA cannot be differentiated by the binding of ANS to BSA. On the other hand, the differences of the binding interactions in two sites are most explicit at C_9COOH and decreased with decreasing the alkyl chain-length, as shown in Fig. 4. The hydrophobicity of C_nCOOH also decreases with decreasing the alkyl chain-length. ANS is more soluble in water than the C_nCOOH examined. Therefore, we can conclude that suitable hydrophobicity is required to differentiate the site-1 and site-2 in BSA.

In conclusion, the competitive binding technique using terminally-substituted alkane analogues provides an easy and useful way to classify the binding modes of the hydrophobic small molecules or ions to proteins.

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