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Two Distinguishable Fluorescent Modes of 1-Anilino-8-Naphthalenesulfonate Bound to Human Albumin

Luis A. Bagatolli,[1] Silvia C. Kivatinitz,[1] Felipe Aguilar,[2] Marco A. Soto,[2] Patricio Sotomayor,[2] and Gerardo D. Fidelio[1,3]

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We study the interaction of 1-anilino-8-naphthalenesulfonate (ANS) with human (HSA) and bovine serum albumin (BSA) by phase and modulation fluorescence spectroscopy. We determined that both HSA and BSA show one or two distinguishable fluorescent sites, depending of the ANS/serum albumin ratio. At above a 1:1 ANS/HSA molar ratio, the steady-state emission spectra for ANS can be resolved in two components: component 1, emitting with a lifetime (τ_1) of 16 ns and a λ_{1max} of 478 nm, with a quantum yield (ϕ_{f1}) of 0.67, and component 2, with a lifetime (τ_2) of 2–4 ns and a λ_{2max} of 483 nm, with an average quantum yield (ϕ_{f2}) of about 0.11. Considering these findings, the binding analysis is fitted with a model of two independent sites. Site 1 has an association constant $K_{as1} = 0.87 \times 10^6\ M^{-1}$ and a capacity of 1.04 mol of ANS/mol of HSA, and site 2 a $K_{as2} = 0.079 \times 10^6\ M^{-1}$ and a capacity of 2.34 mol of ANS/mol of HSA. Analysis of fluorescence lifetime distributions shows that the rigidity of the fluorophore environment at site 1 changes when site 2 is occupied. These findings suggest an interconnection between the two sites and that ligands can stabilize the protein's globular structure. To assess the identity of the ANS binding sites we used diazepam as a marker of the site located at the IIIA HSA subdomain and aspirin as a marker of sites located at the IIIA and IIA HSA subdomains. Both ligands displace ANS only from site 1, suggesting that it corresponds to the binding site located at the IIIA subdomain of the protein. We determined that the K_{as} values for diazepam and aspirin are 0.113×10^6 and $0.021 \times 10^6\ M^{-1}$, respectively.

KEY WORDS: Human serum albumin; 1-anilino-8-naphthalenesulfonate binding; diazepam; time-resolved fluorescence; fluorescence lifetime.

INTRODUCTION

Human serum albumin (HSA) is the major plasma protein involved in the transportation of endogenous and exogenous ligands. HSA, a protein of M_r 65 kDa, consists of 585 residues[1] and the complete three-dimensional structure has recently been determined by X-ray crystallography.[2] It comprises three homologous domains (denoted I, II, and III) that assemble to form a heart-shaped molecule.[2] Each domain is formed by two subdomains (denoted A and B) that possess common structural motifs. The main regions of ligand interaction are located at subdomains IIA and IIIA, IIIA having the highest affinity.[2] According to reported binding data, the more accurate denomination of the binding site located at the IIA and IIIA subdomains should be equiv-

[1] Departamento de Química Biológica-CIQUIBIC, Universidad Nacional de Córdoba, Córdoba, Argentina.
[2] Instituto de Química, Facultad de Ciencias Básicas y Matemáticas, Universidad Católica de Valparaíso, Valparaíso, Chile.
[3] To whom correspondence should be addressed at Departamento de Química Biológica-CIQUIBIC, Facultad de Ciencias Químicas, Ciudad Universitaria, Casilla de Correo 61, 5016 Córdoba, Argentina.

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alent to the earlier nomenclature of sites I and II, respectively.[3–5]. These binding sites can accommodate a wide range of small amphiphilic molecules including fatty acids, hydrophobic amino acids, many small carboxylic acids, and numerous pharmaceuticals such as diazepam, AZT, digitoxin, aspirin, and others compounds.[2,6] Fluorescent probes are of great aid in the study of interactions between drugs and serum albumins and in revealing fine features of structure and dynamics. The fluorescent probe 1-anilino-8-naphthalenesulfonate (ANS) was used to monitor the binding sites of bovine serum albumin.[7,8] At that time the main relevant conclusion arrived at by the authors was the presence of cooperativity in a nonoligomeric protein. The assumption in those papers and in others[9,10] was to consider a single fluorescence quantum yield for the bound ligand at all ANS/albumin ratios. On the other hand, the binding extent taken by direct fluorescence titration was calculated graphically. Recently, a new strategy using a nonlinear weighted least-squares fitting program was employed to overcome the lack of precision of graphical methods[11] but one unique fluorescent environment for the emitting ANS molecule was assumed.

Using phase-modulation fluorescence measurements and performing fluorescence lifetime distributions, we were able to detect that ANS binds to both HSA and bovine serum albumin (BSA) in two different fluorescent environments, depending on the ANS/serum albumin ratio. This is the first time, to our knowledge, that the binding analysis has been performed individually for each fluorescent component in the ANS/HSA system because they can be discerned by their different fluorescence lifetimes. We used a nonlinear weighted least-squares fitting program[12] to perform a binding analysis for the ANS/HSA system in a rigorous manner. We also performed competition analysis with aspirin and diazepam to speculate on the identity of ANS binding sites.

MATERIALS AND METHODS

HSA fatty acid free; BSA fatty acid free; ANS, and aspirin were purchased from Sigma Chemical Co. (St. Louis, MO). ANS stock solution was dissolved in methanol. Diazepam was a gift from Hoffman–La Roche, Argentina. The protein stock solutions were prepared in 10 m*M* Tris–HCl, 145 m*M* NaCl, pH 7, and all assays were performed in it as well. Fluorescence phase-shift and modulation for lifetime determinations, fluorescence phase and modulation spectra, and fluorescence steady-state spectra were recorded in a SLM 4800C fluorometer using a xenon arc lamp as light source and in a Greg 200 (ISS) multifrequency phase-shift and modulation fluorometer with a xenon arc lamp and He/Cd Liconix laser as light source. For the fluorescence phase, modulation, and steady-state spectra, excitation was performed at 390 nm and emission fluorescence monitored in the 410- to 580-nm range. We used the magic-angle method to eliminate polarization effects in the dynamic measurements.[13] All the fluorescence steady-state spectra were corrected by inner filter and dilution effects. The fluorescence phase shift and modulation for lifetime determinations were measured at 6 and 18 MHz with the SLM 4800C equipment and at 12 frequencies in a 2- to 100-MHz range with the ISS fluorometer. The fluorescence was observed using a cutoff filter (420 nm) to eliminate scattering radiation from the light source. In the reference cell a solution of $(Me)_2POPOP$ in ethanol was used to correct possible color effects.[13] Data analyses were made according to software packages of SLM 4800C and Greg 200 (ISS), respectively. Data were collected until the standard deviation from each measurement of phase and modulation was, at most, 0.2° and 0.004, respectively. The standard deviations introduced in the lifetime measurements were determined with at least three similar experiments for each sample and condition and were below ± 0.4 ns. The overall error in the estimation of the quantum yield was below 3%. The chi-square values for the discrete analysis were slightly higher than those obtained with the Lorentzian distribution analysis for ANS bound to albumin. In Table I the data and chi-square values for the Lorentzian distributions are given.

A mixture of emitting fluorophores differing in lifetimes and emission spectra (or, in our case, the same fluorophore emitting with two distinguishable lifetimes) will have phase and modulation spectra which vary according to their relative individual components at a given wavelength. If the single lifetime of both components can be independently determined, the phase or modulation spectra can be used to calculate the individual steady-state spectra of the mixture. This was performed using the software package of our instruments. The theory and equations for such procedure were described by Jameson *et al.*.[23] So the term deconvolution used here is intended only to describe the procedure indicated above. The λ_{max} values for the first and second emitting environments were calculated by the first derivative of each deconvoluted spectra and the standard deviation was ± 0.5 nm from three identical experiments.

The treatment described under Theory section allows us to compute the fluorophore bound concentration at each site if the lifetimes are distinguishable and if we

Table I. Analysis of the Fluorescence Emission Decay of ANS Bound to Serum Albumins, Assuming a Lorentzian Distribution of Lifetimes[a]

Ratio	C_1 (τ_1)	$FWHM_1$	f_1	C_2 (τ_2)	$FWHM_2$	f_2	χ^2
ANS in methanol	6.18	0.05	1.00	—	—	—	0.75
ANS/HSA							
1:1	16.24	1.41	1.00	—	—	—	0.81
20:1	15.53	0.56	0.88	2.04	2.53	0.12	0.95
50:1	16.00	0.05	0.79	4.46	4.66	0.21	0.87
Plus diazepam (ANS/HSA/diazepam), 50:1:50	15.56	0.05	0.73	4.16	2.84	0.27	1.02
ANS/BSA							
1:1	16.30	3.62	1.00	—	—	—	1.07
50:1	14.48	2.28	0.85	1.84	4.67	0.15	0.99

[a] C_1 and C_2 — center of distribution of τ_1 and τ_2 (ns). FWHM — full width at half-maximum of distribution (ns). f_1 and f_2 — fractional intensities. The values shown are for one of three similar experiments. The standard deviations in the FWHM were below 4%.

know the deconvoluted individual fluorescence steady-state spectrum of each environment from the sum spectra. The association constants for diazepam and aspirin were calculated using Eq. (8) (Theory), assuming that loss of fluorescence was due to displacement of ANS by the drug. The binding data were calculated by nonlinear weighted least-squares fitting using the built-in equations for one site from the commercial GraFit computer program, Version 2.0.[12]

The fluorescence properties of ANS in methanol with diazepam or aspirin showed that neither a static nor a collisional quenching effect is observed even at high concentrations of these drugs.

All the experiments were carried out at room temperature (25°C). The absorbance measurements were made in a Gilford Response II UV–VIS spectrophotometer.

Theory

The fluorescence quantum yield of a fluorophore F is calculated[13] as

$$\phi_f = \frac{\tau}{\tau_0} \tag{1}$$

where τ_0 denotes the intrinsic (natural) fluorescence lifetime of the chromophore (interpreted as the fluorescence lifetime in the absence of nonradiative transitions) and τ is the fluorescence lifetime of F. When F binds to a macromolecule in more than one site (each one modifies its fluorescence lifetimes differently), the ϕ_f for each binding site can be calculated using Eq. (1). ANS has a very low ϕ_f in water ($\phi_f = 0.0032$)[14] and its contribution to the total fluorescence intensity is negligible. Therefore the amount of bound fluorophore $[F_b]$ can be obtained from Eq. (2):

$$[F_b] = \frac{I_{f\ \text{sample}}}{\alpha \phi_{f\ \text{sample}}} \tag{2}$$

where α is a proportional factor determined as $\alpha = I_{f\ ref}/[F]_{ref}\ \phi_{f\ ref}$ taken in homogeneous media of a known lifetime (methanol) at the same excitation (390 nm) and emission (477 nm) wavelength and identical experimental and instrumental conditions as the sample. The maximum of ANS in methanol (reference) is centered at 475 nm. As the emission spectra of ANS either in methanol or bound to HSA are rather broad, with similar emission maxima, the possible errors by choosing the same wavelength to determine the constant α parameter of Eq. (2) is below 5%.

Competition Analysis

The equilibrium constant when ANS (denoted F) and a nonfluorescent ligand (denoted L) compete for the same single site of the protein (denoted P) in the presence of a known total concentration of nonfluorescent ligand (denoted L_T) is

$$F + P \leftrightarrow FP, \qquad K_{asF} = \frac{[FP]}{[F][P]} \tag{3}$$

$$L + P \leftrightarrow LP, \qquad K_{asL} = \frac{[LP]}{[L][P]} \tag{4}$$

The mass balance for the single protein site is

$$[P] = F_{bmax} - F_b - L_b$$

where F_{bmax} is the concentration of F that saturates P in the absence of L, $F_b = [FP]$, and $L_b = [LP]$. From Eqs.

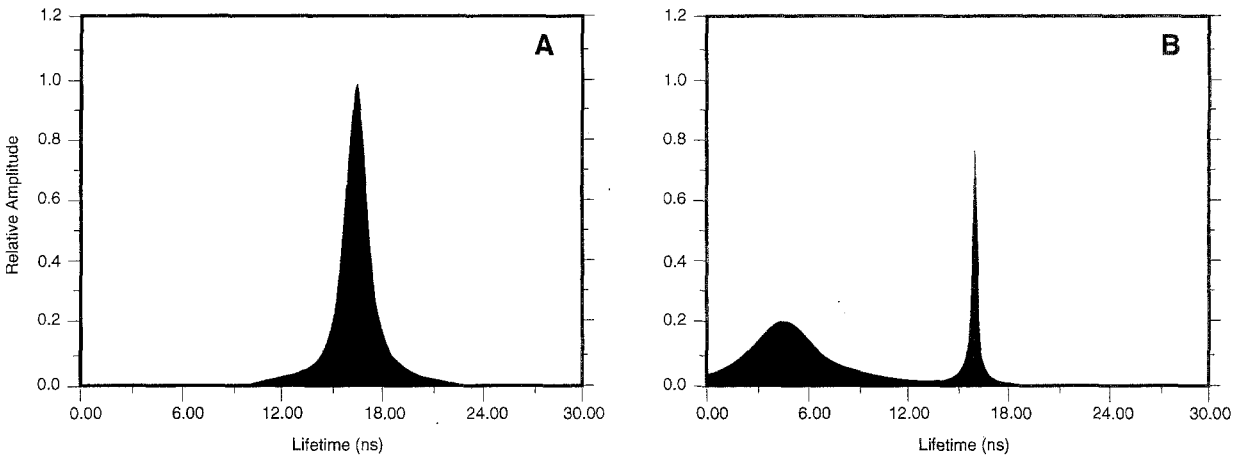

Fig. 1. Lifetime distribution of (A) ANS/HSA, 1:1 molar ratio, and (B) ANS/HSA, 50:1 molar ratio. HSA concentration, 2.5 μ*M*. Data correspond to one of three experiments.

(3) and (4)

$$K_{asL} = \frac{L_b F_f}{L_f F_b} K_{asF} \tag{5}$$

where $F_f = [F]$ and $L_f = [L]$. The subscripts f and b mean the concentrations of free and bound F and L at equilibrium. The amount of bound and free ligand can be determined from

$$L_b = F_{bmax} - F_b - [P] \tag{6}$$

$$L_f = L_T - L_b \tag{7}$$

When the concentrations of fluorophore and ligand are high enough to occupy almost all the sites of the protein, the free protein concentration [P] is negligible and replacing Eqs. (6) and (7) in Eq. (5), the association constant of the ligand K_{asL} can be evaluated as

$$K_{asL} = \frac{(F_{bmax} - F_b)}{X(L_T - (F_{bmax} - F_b))} K_{asF} \tag{8}$$

where $X = F_b/F_f$.

RESULTS

Fluorescence Properties of ANS Bound to Human Albumin

The amphiphilic fluorophore 1-anilino-8-naphthalenesulfonate (ANS) is completely quenched by water with a quantum yield of about $\phi_{water} = 0.0032$ and an emission maximum at 550 nm.[15] The contribution of the fluorescence signal of ANS in water is negligible under our experimental conditions. ANS in a more hydrophobic medium such as methanol displays a single exponential decay with a τ of 6 ns (see Table I). From Eq. (1) and considering a $\tau_0 = 28$ ns,[14] we calculate $\phi_{methanol} = 0.21$, which agrees with a previous report.[14] Fluorescence phase-shift and modulation measurements were done with two instruments (see Materials and Methods). After applying discrete exponential analysis, they showed one lifetime value of 16 ns for an ANS/HSA molar ratio from 1:6 to 1:1 and two lifetime values of 16 and 3.5 ns from a 1:1 to a 50:1 ratio range, whatever instrument was employed. With the multifrequency data we also performed lifetime distribution analysis,[16] obtaining one or two Lorentzian lifetime distributions of the emitting ANS, also, depending on ANS/HSA ratios (Figs. 1A and B). When the range of ANS/HSA ratio was from 1:6 to 1:1 the results displayed a single broad lifetime distribution centered at 16 ns (site 1). When the ANS/HSA ratio is greater than 1:1, the second component (site 2) shows a broader lifetime distribution, centered at 2–4.6 ns (Fig. 1B, Table I). The value of the full width of the half-maximum of the distribution (FWHM) decreases on the first site when site 2 is occupied (compare Figs. 1A and B and see Table I).

A similar behavior is found for ANS bound to BSA. With the multifrequency data we calculated from discrete exponential analysis one lifetime value of 16.31 ns for the ANS/BSA ratio of 1:1 and two lifetime values of 14.48 and 1.84 ns for the 50:1 ANS/BSA ratio. Performing the fluorescence lifetime distribution analysis the same effect was found for the ANS/HSA system. The FWHM of the site 1 lifetime distribution decreased when the shortest lifetime distribution (site 2) began to appear (see Table I). The quantum yield for

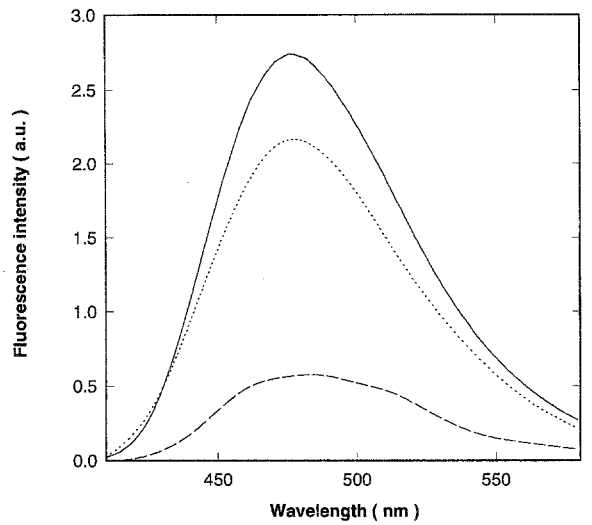

Fig. 2. Deconvolution of the two emission components of ANS bound to HSA. Fluorescence steady-state emission spectrum (—); component 1, of higher affinity (···), component 2, of lower affinity (– –). ANS/HSA, 50:1; HSA concentration, 2.5 μ*M*. Data correspond to one of three experiments.

ANS at ANS/BSA ratio of 1:1 (site 1) was 0.65, similar to the value of 0.67 calculated for the ANS/HSA system.

From the experimental evidence that two distinguishable lifetimes are observed at a high fluorophore/protein molar ratio, we concluded that ANS is bound in at least (from a fluorescent point of view) two sites. If two fluorescence lifetimes are experimentally distinguishable and that can also be independently determined, it is possible to separate the steady-state emission spectra in their components even when their maximum wavelengths are very close (Fig. 2; see Materials and Methods). The λ_{max} values for the first and the second emitting environments (sites 1 and 2) were centered at 478 and 483 nm, respectively.

Once the two components are deconvoluted the fluorescence intensity at a given wavelength is used to calculated the bound ANS fraction according to Eqs. (1) and (2) using a known concentration of ANS in methanol as reference. The value of the natural lifetime τ_0 in HSA of 24 ns was estimated for site 1 from the absorption spectrum[17] at an ANS/HSA ratio below 1:1, compared with a reference absorption spectrum (ANS in methanol; data not shown). On the contrary, direct calculation of τ_0 for site 2 from its own absorption spectrum is not possible since the fluorescence coming from site 2 begins to appear when site 1 is practically saturated. It is known that τ_0 depends on the fluorophore environment;[14] then we assumed a τ_0 of 28 ns for ANS in site 2 equal to methanol because of its close lifetime value. However, it is possible to introduce a systematic error in the calculated amount of ANS bound to site 2 and therefore in its capacity (see Discussion).

Once the amounts of bound and free ANS were estimated, we calculated, for each component, the binding curve by nonlinear weighted least-squares fitting analysis (Fig. 3). Considering two independent binding sites for HSA we calculated an association constant K_{as1} for the higher-affinity site (site 1) of 0.87 × 10^6 M^{-1}, with a capacity of 1.04 mol ANS/mol HSA, and a K_{as2} of 0.079 × 10^6 M^{-1}, with a capacity of 2.340 mol ANS/mol HSA, for the second site (Table II).

Diazepam and Aspirin Competition

When a solution of ANS/HSA (ratio, 10:1) was titrated with diazepam, successive ANS displacement from the higher-affinity site occurred (Fig. 4). The displacement takes place with changes in the fractional intensities but without altering the lifetime values (τ_1 and τ_2) and the FWHM of the fluorescence lifetime distribution of site 1. A decrease in the FWHM value of the site 2 fluorescence lifetime distribution was observed when ANS was displaced by diazepam (Table I). The total fluorescence intensity of the lower-affinity site remained unchanged whatever the concentration of diazepam present in the medium (Fig. 4). So we conclude that diazepam displaced ANS only from the higher-affinity site. According to Eq. (8) it is possible to assess the association constant of the nonfluorescent ligand. Assuming a stoichiometry of 1 for diazepam and aspirin,[2] the average K_{as} calculated from several diazepam/ANS ratios was 0.113 × 10^6 M^{-1} (Table II), eight times lower than the value of ANS for its high-affinity site. Aspirin displaced ANS in a very similar way, eliciting all the effects described for diazepam (data not shown), the association constant for aspirin being 0.021 × 10^6 M^{-1} (Table II). These values are of the same order as those reported for the association constant of the aspirin and diazepam highest-affinity site.[18–20]

DISCUSSION

We found that ANS binds to serum albumin in at least two modes, distinguishable by their fluorescence properties: lifetime (τ) and quantum yields (ϕ). To our knowledge this is the first time that the interaction of an extrinsic fluorophore bound to a macromolecule can be resolved in two modes. ANS bound to HSA displays two fluorescent microenvironments; the highest-affinity site

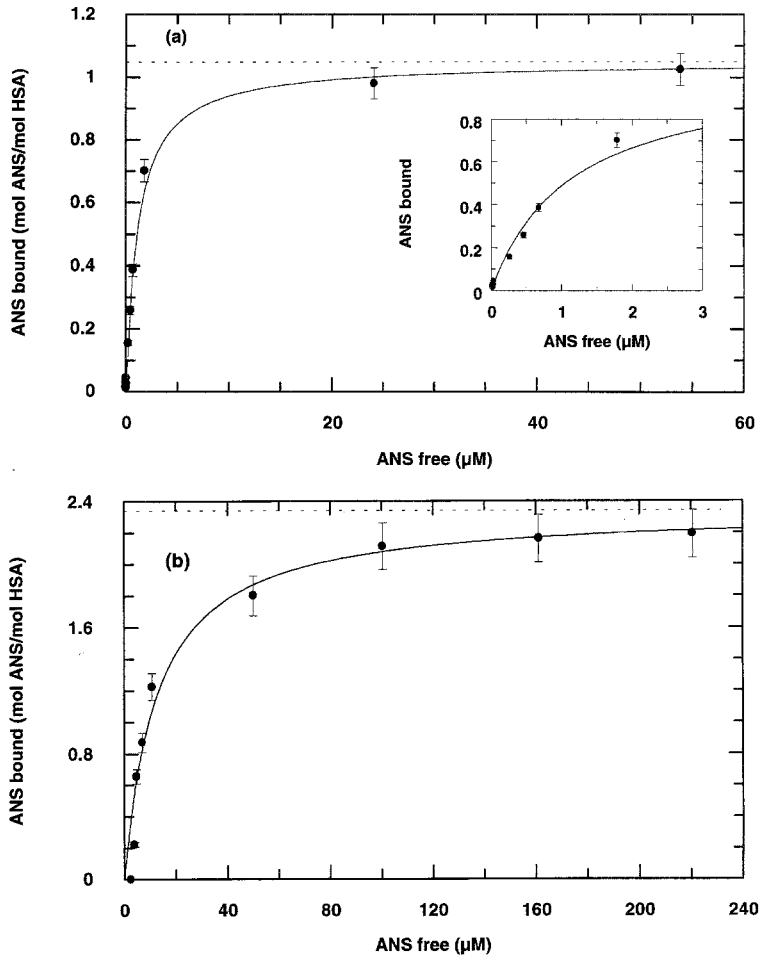

Fig. 3. Binding plot for the ANS/HSA interaction. (a) Binding curve for component 1, higher affinity (inset shows the same graphics with an expanded scale in order to show the first eight values), and (b) for component 2, lower affinity. The dashed line indicates the capacity fitted by the computer program. The HSA concentration was 6 μ*M*. Data points are the means ± standard deviations from three similar experiments. For sites 1 and 2 the reduced χ^2 values of the fitting analyses were 0.001 and 0.004, respectively.

(site 1) has a lifetime of 16 ns and $\phi_f = 0.67$ (Fig. 1A) and the lower-affinity site (site 2) has a lifetime centered at 2–4.6 ns (see Table I). A lifetime value of 16 ns for the defined "inner site" of BSA has already been reported in previous work from Weber's group.[9] These authors also indicate the existence of a second class of site ("outer site") that was detected by equilibrium dialysis, with noncontribution to the total fluorescence. Probably, this site agrees with our second component bound to BSA, emitting with a fluorescence lifetime of 1.8 ns and with a low quantum yield. The similarity in the behavior of HSA and BSA in fluorescence lifetime distribution experiments agrees with the high homology found in their structure.[6,21]

From the deconvolution of individual fluorescence steady-state spectra, we calculated the ANS bound concentration in each environment per mole of HSA and therefore we calculated the binding curve and capacity directly for each site with a nonlinear least-squares analysis (Fig. 2 and Table II). This approach is a more reliable method than the previous double-reciprocal or nonlinear weighted least-squares estimations assuming a single quantum yield for all the fluorophore species bound to albumin,[7,9–11,22] Even when the assumption made in the natural lifetime for ANS bound to site 2 may be introducing a systematic error in the real capacity of this site, the lower-affinity site is contributing up to 20% of the emitting fluorescence at a saturating ratio. Taking into account the new evidence reported here, one single fluorescent environment for ANS bound to albumin can no longer be sustained. On the other hand, the Hill coefficients were very close to 1 when the analysis

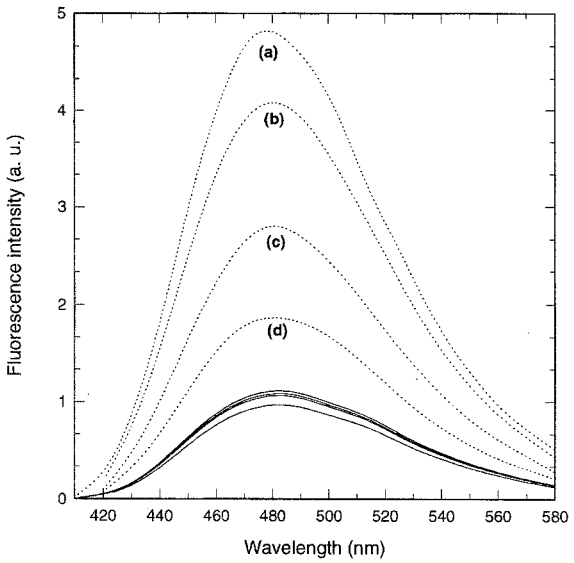

Fig. 4. Displacement of ANS by diazepam. Fluorescence steady-state deconvolution of component 1, of higher affinity (dotted lines) in the absence (a) or the presence of 25 μ*M* (b), 100 μ*M* (c), or 150 μ*M* (d) diazepam. ANS and HSA concentrations were 12.5 and 1.25 μ*M*, respectively. The fluorescence spectra at the bottom (solid lines) correspond to component 2. Data correspond to one of three experiments.

Table II. Binding Parameters for ANS, Diazepam, and Aspirin to HSA

	K_{as} ($\times 10^{-6}\ M^{-1}$)	Capacity (mol ligand/mol HSA)
ANS site 1	0.87 ± 0.08	1.04 ± 0.02
ANS site 2	0.079 ± 0.007	2.340 ± 0.008
Diazepam site 1	0.113 ± 0.008	1[a]
Aspirin site 1	0.021 ± 0.001	1[a]

[a] Assumed from He and Carter [2].

for each binding datum shown in Fig. 3 was performed individually. These findings undoubtedly suggest a noncooperative mode of binding.

The main and most active binding site on HSA is located in subdomain IIIA of the protein. This domain has recently been characterized by X-ray crystallography and many ligands were found to bind preferentially to this site.[2] The binding site is a hydrophobic pocket formed by the spatial combination of several nonpolar residues in subdomain IIIA and many pharmacological ligands such as ibuprofen, diazepam, and aspirin are known to bind to this site.[2] The other important binding region of HSA is located at subdomain IIA. This site is also hydrophobic and is located close to the IIIA site. It is claimed that both sites interact with aspirin but diazepam binds only to the IIIA site.[2] To assess the identity of the HSA binding sites occupied by ANS, we used diazepam as a marker of the site located at the IIIA subdomain and aspirin as a marker of the sites located at the IIIA and IIA subdomains. Both ligands displace ANS only from site 1, suggesting that it corresponds to the binding site located at the IIIA HSA subdomain. The displacement of ANS from the highest-affinity site of HSA observed in the presence of diazepam indicates that our site 1 corresponds to the main binding pocket of HSA localized in subdomain IIIA because it occurs without affecting the affinity or capacity of the lower-affinity site (site 2). According to X-ray crystallographic data,[2] equilibrium dialysis data,[20] and multidimensional fluorescence measurements,[5] aspirin seems to bind to two sites. However, the affinity constant that we found for aspirin binding to site 1 (IIIA site) is almost 40 times lower than that for ANS, and if we expect an even lower affinity for site 2 (probably the IIA site), any displacement of ANS under the experimental conditions assayed is not possible (Table II). We believe that the lower-affinity site of ANS (site 2) corresponds to that localized at the IIA subdomain. However, to assign the lower-affinity site of ANS to this subdomain safely, further investigations with other known ligands, such as warfarin and bilirrubin, will be necessary.

According to X-ray crystallography data,[2] it is claimed that even when the chemistry of ligand binding is similar for both sites, there are marked differences in the location of bound ligand in the pocket. These differences may be influencing the fluorescent properties of the ANS molecules bound to the protein sites and may explain why ANS is emitting at two close emission maxima with particularly different quantum yields and lifetimes. In this connection, Slavik[14] has emphasized that simple and direct comparison of hydrophobicity of the sites from the lifetime, quantum yield, and emission maximum of bound ANS to proteins with respect to the same fluorophore in homogeneous solvent of different polarity is difficult to achieve. There is experimental evidence that the fluorescence properties of ANS bound to the protein surface may be influenced by the rigidity of the fluorophore environment.[14] Therefore, further experiments (such as the degree of polarization and dynamic quenching at different ANS/HSA molar ratios) will be necessary to clarify this point.

When the ANS/HSA ratio was between 1:1 and 50:1 a decline in the FWHM value in the lifetime distribution of site 1 was observed consistently (Figs. 1A and B, Table I). The microenvironment sensed by ANS at site 1 becomes more homogeneous as site 2 begins to appear. A similar effect is observed with BSA. On the

other hand, when the two sites of HSA were occupied with ANS and diazepam was added, displacing ANS from the high-affinity site, a narrowing of the lifetime distribution of the shorter component was observed (site 2) (Table I). These findings suggest that both binding environments interact because they are close neighbors in the protein.[2] Therefore the entry of a second ANS molecule induces an increase in the rigidity on the high-affinity site. A similar effect was also observed at ANS site 2 when diazepam displaces ANS from site 1 (Table I). Taking into account the above facts and the small shift of τ_2 to higher values at a saturating ratio of ANS, it can be postulated that the ligands elicit a conformational change of the protein to a more stabilized structure (Figs. 1A and B, Table I). Conformational changes of albumin upon binding of ligands have already been reported by several authors.[2,6] Intriguingly enough is the fact that all these conformational changes do not seem to affect the affinity of ANS for both sites.

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