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Binding Sites of Fluorescent Probes on Human Serum Albumin

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Abstract—Human serum albumin is known to have two major and selective drug binding sites, termed sites I and II. The fluorescent probes, dansylamide and dansylsarcosine selectively interact with sites I and II, respectively. However, the binding site of the fluorescent probe dansylglycine on human serum albumin is not clear from the literature. This study investigated whether dansylglycine interacts tightly with site I or II. Spectrofluorimetric titrations (quenching and complex) and circular dichroism measurements were performed to determine the binding characteristics of dansylglycine to human serum albumin. Modification in probe fluorescence was described by fluorescence titrations to be a result of competitive displacement by ligands. The pattern of displacement of this probe by several ligands whose primary binding sites are exactly known, enabled the identification of its specific binding site. The fluorescence of dansylglycine is only extensively changed when ligands of site II are added, suggesting that it strongly interacts with the benzodiazepine/indole binding site on human serum albumin.

The knowledge of drug binding sites is of interest to deduce possible interactions for protein binding sites. This identification requires the use of probes specific for binding sites of human serum albumin (HSA). The binding sites of the fluorescent probes dansylamide and dansylsarcosine on the HSA molecule are termed site I and II, respectively. However, the binding of dansylglycine is in question, since each site has been described as its primary binding site (Sudlow et al 1976; Kasai et al 1987).

The aim of this study was to propose an unambiguous primary binding site of dansylglycine and to compare the binding parameters of the fluorescent probes dansylamide, dansylsarcosine and dansylglycine.

Materials and Methods

Materials

Albumin (mol. wt 69 kDa) was purchased from Sigma (Saint Quentin Fallavier, France; reference A 1887, lot 118F-9311) as essentially fatty-acid free (below 0·005%), prepared from fraction V of human serum albumin (HSA). A 14·5 μM solution of albumin was prepared in a Sørensen's phosphate buffer 0·067 M, pH 7·4.

The fluorescent probes, digitoxine, dicoumarol and salicylate were purchased from Sigma (Saint Quentin Fallavier, France) and prepared at different concentrations in methanol, except for salicylate which was prepared in double-distilled water.

Phenylbutazone was a gift from Ciba-Geigy (Rueil-Malmaison, France), warfarin from Merrell-Dow (Levallois-Perret, France), diazepam from Roche (Neuilly sur Seine, France) and ibuprofen from Boots-Pharma (Carbevoie, France). All these products were prepared in methanol at different concentrations.

All other products used in the study were of analytical grade (Merck, Nogent sur Marne, France).

Spectrofluorimetry

The spectroscopic measurements were carried out using a fluorescence spectrophotometer Hitachi Lou-Perkin Elmer 240 (Saint Quentin en Yvelines, France). Excitation and emission slit widths of 20 nm were used for all fluorescence measures. The fluorescence sample cell had a pathlength of 1 cm. The quenching of the single tryptophan residue was achieved by each fluorescent probe (λ_{ex} 290 nm; λ_{em} 340 nm).

The fluorescence value of tryptophan in the presence of a ligand was corrected for the inner filter effect. The fluorescence quenching curve was analysed according to the method of Steiner et al (1966), using the tangent line constructed with the fluorescence values at lowest concentrations, to determine the concentration of ligand bound near to the tryptophan.

The binding of each probe to HSA was estimated by measuring the resultant increase in fluorescence intensity of the complex. Measurements were made at 480 nm using an activating wavelength of 340 nm for dansylamide and of 350 nm for the other two probes. For all experiments, the molar ratio, probe/HSA, varied from 0·025 to 5.

The concentration of bound ligand was calculated using the initial slope of the plot of the fluorescence intensity of the complex against ligand concentration. The ability of several ligands to displace the fluorescence of each complex probe-HSA was observed at the same wavelengths, as described previously. The probe was present at a molar probe/protein ratio of 1. The different ligands were then added and the corresponding molar ratio of ligand/protein varied from 0·025 to 5. It was confirmed that these ligands emitted no fluorescence at the wavelengths used for the displacement experiments.

Circular dichroism

The circular dichroism analyses were performed with a Dichrograph Jobin Yvon IV (France). Cotton effects of the site-specific probes and drugs were studied over a range of wavelengths (200–500 nm).

Upon the addition of dansylamide, dansylsarcosine and dansylglycine, a large, positive ellipticity band appeared at 335, 345 and 340 nm, respectively. A negative ellipticity band was also observed at 320 nm when diazepam was bound to

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Table 1. Binding parameters (±s.e.) of several site-specific ligands to HSA using the Scatchard model (1 or 2 sites) determined by spectrofluorimetry (SF) or circular dichroism (CD).

Site	Ligand	n_1	k_1 (μM^{-1})	n_2	k_2 (μM^{-1})	Methods	n	Reference
I	Dansylamide	0·328±0·027	0·1871±0·046	2·898±0·391	0·0102±0·0023	SF-quenching	12	This study
		0·509±0·056	0·4925±0·1128	2·610±0·3234	0·0072±0·0019	SF-complex	16	This study
		1·606±0·0116	0·1282±0·0027	—	—	CD	6	This study
		0·5	0·1790	1·7	0·007	SF-complex	—	Sudlow et al (1975)
		0·555	0·110	4	0·0045	SF-complex	—	Kasai et al (1987)
	Warfarin	0·880±0·0027	0·4730±0·0053	—	—	SF-quenching	17	This study
		0·9	0·25	—	—	SF-complex	—	Sudlow et al (1975)
		0·7	0·47	—	—	SF-complex	—	Kasai et al (1987)
II	Dansylsarcosine	0·791±0·0054	0·3521±0·0056	—	—	SF-quenching	12	This study
		0·706±0·0023	0·5079±0·0092	—	—	SF-complex	16	This study
		0·766±0·0028	0·5090±0·01025	—	—	CD	6	This study
		1	0·170	—	—	SF-complex	—	Sudlow et al (1975)
		0·8	0·2	—	—	SF-complex	—	Kasai et al (1987)
	Diazepam	0·821±0·0075	5·4119±0·4659	1·132±0·0328	0·0110±0·00075	SF-quencing	14	This study
		0·662±0·00315	1·0250±0·0266	—	—	CD	8	This study
		1·28	0·860	8·8	0·012	SF-complex	—	Kasai et al (1987)
?	Dansylglycine	1·033±0·010	0·3108±0·0116	—	—	SF-quenching	11	This study
		0·892±0·003	0·487±0·0087	—	—	SF-complex	12	This study
		0·818±0·0068	0·584±0·024	—	—	CD	16	This study
		1	0·588	1	0·05	SF-complex	—	Sudlow et al (1975)
		0·87	0·12	—	—	SF-complex	—	Kasai et al (1987)
		1	0·46	—	—	SF-complex	—	Chignell (1969a)

n_1 and n_2 are respectively the number of sites of the first and second classes.
k_1 and k_2 define the affinity constants of each class n is the number of experiments.

HSA, but no extrinsic Cotton effect was observed, resulting from the interaction of warfarin with the protein.

The different probes were added at a molar probe/protein ratio varying from 0·025 to 10. They showed no intrinsic Cotton effect at the same wavelength as the complex ligand/HSA.

All the circular dichroism measurements were performed using a 14·5 μM HSA solution at room temperature (21°C). The ellipticity vs ligand concentration curve was analysed according to the method proposed by Rosen (1970), to determine the bound concentration at the site of highest affinity.

Mathematical analysis

The experimental data of binding, obtained by circular dichroism and fluorescence methods, were plotted by the model of Scatchard (1949). The binding parameters were calculated by linear regression when the Scatchard plot was a straight line. When the Scatchard plot was curved, the binding parameters were obtained by iteration using the following expression:

$$\frac{B}{P}=\frac{n_1k_1F}{1+k_1F}+\frac{n_2k_2F}{1+k_2F}$$

where F and B are the concentrations of free and bound drug, respectively, P is the albumin concentration, n_1 and n_2 the respective number of binding sites in classes 1 and 2, and k_1 and k_2 are the association constants for these sites.

The binding parameters (n_1, n_2, k_1 or k_2) were determined using an optimization method by quadratic polynomial interpolation with the least-squares criterion using the SIPHAR software (Version, 3.3, Créteil, France) installed on an IBM AT microcomputer.

Results and Discussion

Spectrofluorimetric techniques can be performed with very low protein concentration media (Muller et al 1991). An excellent correlation between tryptophan fluorescence intensity (λ_{ex} 290 nm; λ_{em} 340 nm) and HSA concentration was observed for HSA solutions varying from 0·1 (1·45 μM) to 2 g L^{-1} (29 μM) (r=0·999) (data not shown). Above this value, a deviation from the linearity was observed. This may be attributed either to saturation of detection of the apparatus or to an enhancement in self-association of HSA molecules with increasing concentrations (Geddes & White 1979; Blatt et al 1986). Indeed, according to Zini et al (1981), the higher the HSA concentration, the more the monomer proportion decreases. Nevertheless, for HSA concentration up to 2 g L^{-1} (29 μM), the degree of HSA polymerization was negligible; only 8% is present as dimer. Therefore, using HSA concentrations up to 29 μM, the dependence of quenching on protein concentration could be considered regardless of self-association of the protein. Thus, all spectrofluorimetric measurements used 14·5 μM HSA.

Binding parameters of site specific drugs and probes

Spectrofluorimetric titrations (quenching and complex) and circular dichroism measurements were performed to determine the concentrations of each bound drug and probe. The data were analysed using a Scatchard plot (1949) (Fig. 1). Our results and those found in the literature are shown in Table 1. A good consistency between all the results can be noted. Small discrepancies could be attributed either to the difficulties in applying the Scatchard model (Monot et al 1983) or to the lack of accuracy of the initial slope in the construction of the Steiner et al (1966) and Rosen (1970) diagrams.

For dansylglycine, an excellent agreement was observed between the binding parameters calculated by the three

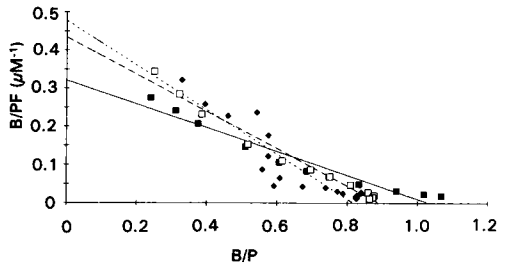

FIG. 1. Scatchard plot of the binding of dansylglycine to HSA. Spectrofluorimetric titrations (quenching ■ and complex □) and circular dichroism measurements (◆) and optimization fitting -, ——, – –, respectively. F and B are the concentration of free and bound drug, respectively, and P is the albumin concentration.

techniques and those reported in the literature (Table 1). Comparison of the values of k obtained by spectrofluorimetry and circular dichroism with the literature values shows that the binding constants are of the same order of magnitude. Our investigations indicate that circular dichroism detected a single binding site for dansylglycine on HSA. In addition, the complex dansylglycine/HSA studied by spectrofluorimetry reveals one binding site of high affinity for dansylglycine on HSA, suggesting that the site identified by circular dichroism and spectrofluorimetry is the same and can be assumed to be the primary binding site for dansylglycine. This result confirms that the displacement experiments will effectively concern the primary binding site located near to the tryptophan residue of the HSA molecule, according to the data obtained by the quenching experiments.

Displacement experiments

The displacement of the fluorescent site probes by nonfluorescent ligand was monitored by measuring the resultant evolution in the site-specific probe fluorescence intensity. The quantum yield of fluorescence of dansylglycine bound to HSA was measured before and after the addition of the antagonists. The measured fluorescence value corresponds to a relative fluorescence intensity expressed as a percentage of the initial fluorescence.

A decrease in the fluorescence intensity of the complex (probe/HSA) can be interpreted as a displacement of the probe from its binding site by the added ligand, probably through a competitive mechanism (Chignell 1969c). The displacer and the fluorescent probe may be bound to the same site. Therefore, this technique is often employed to characterize the binding site of several ligands. The comparison of the well-known profiles of the dansylamide and dansylsarcosine/HSA fluorescence obtained in the presence of several ligands, with that monitored in the case of dansylglycine, may be a useful tool to define the strongest binding site for dansylglycine.

In the literature, dansylamide is described as bound to site I of the HSA molecule (Sudlow et al 1976). The primary binding sites of these ligands are well defined (Table 2).

An important decrease in the fluorescence of the complex dansylamide/HSA was observed when competitors primarily bound to site I are added, such as salicylate, phenylbutazone, warfarin and dicoumarol (data not shown). Conversely, the addition of digitoxin, which is bound to HSA in a region far from the warfarin site, did not affect the fluorescence of the complex (Sudlow et al 1975; Fehske et al 1981). In the case of ligands bound primarily to site II, such as diazepam and ibuprofen, the fluorescence intensity of the complex dansylamide/HSA is only decreased when these ligands are added at molar ratio ligand/protein above 1, suggesting that their secondary binding sites are involved in this phenomenon.

Table 2. Binding of ligands to human serum albumin.

Warfarin binding site (Site I)	Reference	Diazepam binding site (Site II)	Reference
Acenocoumarin	Fehske et al (1979)	Benoxaprofen	Sudlow et al (1976)
Azapropazone	Maruyama et al (1984)	Bromazepam	Müller & Wollert (1973)
Chlorothiazide	Sjöholm et al (1979)	Clofibric acid	Zini & Tillement (1989)
Chlorpropamide	Brown & Crooks (1976)	Clonazepam	Müller & Wollert (1973)
Clofibric acid	Spector (1975)	Cloxacilline	Sjöholm et al (1979)
Clorazepate	Sjöholm et al (1979)	Diazepam	Sjöholm et al (1979)
Dicoumarol[1]	Sjöholm et al (1979)	Dicoumarol[2]	Sjöholm et al (1979)
Diflunisal	Sjöholm et al (1979)	Fenbufen	Sudlow et al (1976)
Flurbiprofen[2]	Sudlow et al (1976)	Flufenamic acid	Bree et al (1989a)
Furosemide	Sjöholm et al (1979)	Flurazepam	Müller & Wollert (1973)
Glibenclamide	Sjöholm et al (1979)	Flurbiprofen[1]	Sudlow et al (1976)
Indomethacin	Sjöholm et al (1979)	Glibenclamide	Sjöholm et al (1979)
Isoxicam[1]	Bree et al (1989a)	Ibuprofen[1]	Sjöholm et al (1979)
Ketoprofen[2]	Sjöholm et al (1979)	Indomethacin	Sjöholm et al (1979)
Naproxen[2]	Sudlow et al (1976)	Isoxicam[2]	Bree et al (1989a)
Oxyphenbutazone	Sjöholm et al (1979)	Ketoprofen[1]	Sjöholm et al (1979)
Phenprocoumon	Maruyama et al (1984)	Lorazepam	Müller & Wollert (1973)
Phenylbutazone	Sjöholm et al (1979)	Naproxen[1]	Sjöholm et al (1979)
Phenytoin	Sjöholm et al (1979)	Nitrazepam	Müller & Wollert (1973)
Pindolol	Sjöholm et al (1979)	Oxazepam	Müller & Wollert (1973)
Piroxicam	Bree et al (1989a)	Piroxicam	Bree et al (1989a)
Salicylamide	Sjöholm et al (1979)	Probenecid	Sjöholm et al (1979)
Salicylic acid	Maruyama et al (1984)	Tamoxifen	Sjöholm et al (1979)
Sulphinpyrazone	Sjöholm et al (1979)	Tenoxicam[2]	Bree et al (1989a)
Tamoxifen	Sjöholm et al (1979)	Tolbutamide	Sjöholm et al (1979)
Tenoxicam[1]	Bree et al (1989a)	Tryptophan	Sjöholm et al (1979)
Tolbutamide	Sjöholm et al (1979)		
Warfarin	Sjöholm et al (1979)		

[1]Primary binding site, [2]secondary binding site.

Similarly, the variation of fluorescence intensity of the complex dansylsarcosine/HSA was analysed after the introduction of the same ligands. The fluorescence value of the complex dansylsarcosine/HSA was only slightly affected by the addition of site I ligands such as salicylate, dicoumarol and phenylbutazone up to a molar ratio ligand/protein of 1 (data not shown). Above this value, only dicoumarol decreased the fluorescence to a great extent. Digitoxin and dansylsarcosine are bound to distant sites (Sudlow et al 1975; Fehske et al 1981). This was confirmed by these experiments since no modification of the fluorescence intensity occurred, suggesting digitoxin would not displace dansylsarcosine from its binding site on HSA. The introduction of ligands such as ibuprofen and diazepam, sharing the same primary binding site as dansylsarcosine, induced an important decrease in the fluorescence of the complex dansylsarcosine/HSA.

Dansylglycine was present at a molar probe/protein of 1, as proposed by Bree et al (1989a, b). The different ligands (phenylbutazone, dicoumarol, warfarin, salicylate, diazepam and ibuprofen) were then added and the corresponding molar ratio of ligand/protein varied from 0·025 to 5. However, in order to investigate accurately the possibility of displacement of dansylglycine from its primary binding site by some drugs, the same experiments were performed at molar dansylglycine/protein ratios of 0·05 and 0·5 for several ratios of competitor to protein (data not shown). The displacement profiles obtained with both ratios were identical to that defined with a probe/protein ratio of 1. The fluorescence of the complex dansylglycine/HSA after the addition of competitors was analysed comparatively with the results obtained in the case of dansylamide and dansylsarcosine/HSA complexes (Fig. 2).

For each ligand added, the resultant evolution in the danysylglycine/HSA fluorescence showed the same profile as that observed with dansylsarcosine: a large decrease with ligands of site II (diazepam and ibuprofen), no modification with warfarin and digitoxin, and an important decrease with phenylbutazone, dicoumarol and salicylate when introduced at a molar ligand/protein above 1.

In contrast to dansylamide and dansylsarcosine, for which binding locations on HSA agree in the relevant literature, there is disagreement concerning the primary binding site for dansylglycine from one author to the other.

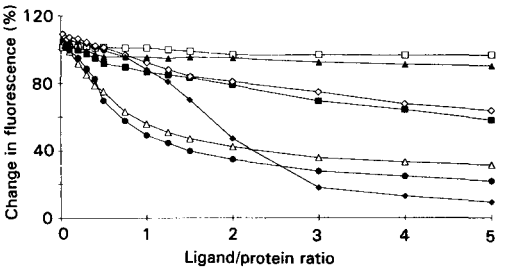

FIG. 2. Drug-induced changes in fluorescence of dansylglycine bound to HSA. Solution containing HSA (14·5 μM) and dansylglycine (14·5 μM) was titrated with phenylbutazone (■), digitoxin (□), dicoumarol (◆), salicylate (◇), warfarin (▲), diazepam (△) or ibuprofen (●), added at molar ligand/protein ratios varying from 0·025 to 5. The fluorescence was measured at 480 nm, with excitation at 340 nm.

According to Sudlow et al (1976), dansylglycine binds strongly to site I. However, Chignell (1969c) concluded that dansylglycine and warfarin must be bound to different sites because of the distance between bound dansylglycine and the lone tryptophan residue of HSA. This was confirmed by experiments showing that warfarin would not displace dansylglycine from its binding site on HSA (Chignell 1970). Therefore, if dansylglycine is bound to site I of HSA, this could only be on the azapropazone part and not on the warfarin area. This assumption was confirmed by Chignell (1969a) who suggested that phenylbutazone and dansylglycine shared a common binding site on HSA.

Chignell (1969a, b, 1970) observed competitive binding between flufenamic acid, dicoumarol and dansylglycine. Dicoumarol was described as being primarily bound to site I (Chignell 1970). Spectrofluorimetric measurements showed a decrease in the fluorescence of the complex dansylsarcosine/HSA and dansyl L-proline/HSA in the presence of flufenamic acid, suggesting they shared the same binding site, in this case site II (Sudlow et al 1975). Due to the competition observed between flufenamic acid and dansylglycine (Chignell 1969b), it is logical to suppose they share the same binding site, in this case site II. An ambiguity seems to result from these data: dansylglycine binds competitively with a ligand of site I (dicoumarol) and with a ligand of site II (flufenamic acid). Furthermore, according to Kasai et al (1987), dansylglycine and flufenamic acid were bound to site II. In this study, to characterize the drug binding sites on HSA, determination was made of the distance of sites I and II from the unique tryptophan residue in HSA, when these sites were each occupied by a ligand. This effect was studied at the saturated conditions of primary binding sites. The asymptotic distance values ranged from ~22 to 23Å for site I ligands and from ~16·1 to 17·5Å for site II ligands, when added at a concentration corresponding to nearly complete saturation of primary binding sites. The value defined for dansylglycine (17·5Å) was included in those obtained for ligands specific for site II, suggesting its binding to the indole/benzodiazepine site.

Despite discrepancies found in the literature, our results show convincingly that dansylglycine is primarily selective for the benzodiazepine/indole binding site (site II).

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