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Binding of Fluorescent 7-Amino-4-nitrobenzoxadiazole Derivatives to Bovine Serum Albumin

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The quantum yield and binding to bovine serum albumin (BSA) were investigated with various fluorescent 7-alkylamino (bearing 2—12 carbon atoms), 7-benzylamino, and 7-*p*-methoxybenzylamino derivatives of 4-nitrobenzoxadiazole (NBD). Among them, alkylamino derivatives gave high quantum yield in both ethanol and BSA solution, and greater affinity to BSA was found with those having a longer alkyl chain. In particular, those with C_8–C_{12} alkyl chains may be useful in research on hydrophobic regions as fluorescent probes. In the binding of these probes to BSA, the logarithm of the primary association constant increased linearly with increase in the length of the side chain; a hydrophobic cleft similar to that on the human serum albumin molecule was suggested to exist also on the BSA molecule. In the investigation of the binding displacement of 7-amino-NBD derivatives based on Sudlow's classification, almost no displacement was observed with site II drugs, ibuprofen and flufenamic acid. However, diazepam (a site II drug) showed different behavior from other site II drugs. It was particularly interesting that the difference of the displacing behavior between diazepam and other site II drugs increases with increase of the side chain length of the alkylamino-NBD probes.

Keywords——protein binding; bovine serum albumin; binding site; 7-amino-4-nitrobenzoxadiazole derivative; warfarin; ibuprofen; diazepam; fluorescence quantum yield

Solutions of some fluorescent substances such as 1-anilinonaphthalene-8-sulfonate (ANS)[2,3] and 1-dimethylaminonaphthalene-5-sulfonate (DNS)[4-6] generally show an increase in the quantum yield and a shift of the emission wavelength to shorter regions (blue shift) when the polarity of the solvent is decreased. These changes are also seen in the interaction between fluorescent substances and bovine serum albumin (BSA).[5,7,8] Therefore, fluorescent substances are considered to bind to hydrophobic areas of protein. Using such changes in fluorescence as an index, fluorescent substances have been applied widely as sensitive probes or reporters for detection of local structure changes at binding sites and to examine the state of hydrophobic areas on protein molecules. Ghosh and Whitehouse[9] reported that the 7-chloro derivative of 4-nitrobenzoxadiazole (NBD) reacts with amino groups to form stable, highly fluorescent probes. Then, Kenner and Aboderin[10] reported the use of two closely related derivatives of 7-Cl-NBD, 7-benzylamino-NBD and 7-(*p*-methoxybenzylamino)-NBD, as sensitive fluorescent reporters of nonpolar areas and conformational changes on ribosomes and trypsin.

Sudlow *et al.*[11] have suggested that there are two specific binding sites on human serum albumin (HSA), site I and site II, for anionic drugs. Sjöholm *et al.*[12] have suggested that HSA has a third high affinity site for drugs in addition to site I and site II.

In this paper, with the aim of developing useful fluorescent probes for studies of protein binding, 7-amino-NBD derivatives which are almost neutral were synthesized, and their fluorescence character and binding behavior to BSA were examined. Furthermore, the binding specificity of these probes was examined in connection with Sudlow's classification[11] of the binding sites.

Experimental

Materials——BSA (fraction V, Lot. No. X86103) was purchased from Armour Pharmaceutical Co., Kankakee, and was used without further purification. The molecular weight was assumed to be 67000,[13] and the concentration was determined by measuring the absorbance at 280 nm using $E_{1\,cm}^{1\%}$=6.67.[13] 7-Cl-NBD (Dojin Chemical Co., Kumamoto) was used without further purification. Ethyl-, cyclohexyl-, and undecylamine, and *o*- and *p*-toluidine were purchased from Tokyo Kasei Co., Tokyo. Propyl-, butyl-, hexyl-, heptyl-, octyl-, nonyl-, decyl-, and laurylamine, and morpholine and aniline were purchased from Wako Pure Chemical Industries, Ltd., Osaka. Phenylbutazone was kindly provided by Nihon Ciba-Geigy Co., warfarin by Eisai Co., diazepam by Yamanouchi Seiyaku Co., ibuprofen by Kaken Kagaku Co., and flufenamic acid by Sankyo Co. All other reagents used were commercial products of special grade. All final solutions were made with 0.15 M Tris–HCl buffer, pH 7.0. All the experiments were performed at 25 °C.

Synthesis of 7-Amino-NBD Derivatives——7-Amino-NBD derivatives were synthesized according to a procedure outlined by Kenner and Aboderin.[10] 7-Cl-NBD (4.0 mmol) was treated with 4.3 mmol of amines each in 80 ml of ethyl acetate at room temperature. After 2 h, 15 ml of water was added to remove excess amines. The ethyl acetate layer was dried with anhydrous $MgSO_4$ and then evaporated to dryness. Derivatives were recrystallized three times from absolute ethanol. Elemental analysis gave the expected values for the 7-amino-NBD derivatives (Table I). All melting points are uncorrected (Table I).

TABLE I. Elemental Analysis and Melting Points of 7-Amino-NBD Derivatives

7-Substituent	Calcd (%)			Found (%)			mp (°C)
	C	H	N	C	H	N	
Ethylamino	46.16	3.87	26.91	46.43	3.92	27.17	165—168
Propylamino	53.66	4.09	22.75	53.39	4.01	23.01	95— 97
Butylamino	50.85	5.12	23.71	50.81	5.11	23.72	96— 98
Hexylamino	54.54	6.10	21.19	54.80	6.08	21.16	95— 98
Cyclohexylamino	54.96	5.38	21.36	54.81	5.39	21.49	141—143
Heptylamino	56.11	6.52	20.12	55.85	6.55	20.16	100—103
Octylamino	57.42	6.90	19.16	57.26	6.91	19.08	100—102.5
Nonylamino	58.81	7.24	18.28	58.52	7.20	18.10	100—103
Decylamino	59.99	7.55	17.48	59.47	7.38	17.31	105—108
Undecylamino	61.17	7.83	16.77	60.94	7.87	16.61	105—108.5
Laurylamino	62.05	8.10	16.07	61.82	8.14	15.91	106—108
Morpholino	48.00	4.03	22.39	47.89	3.99	23.05	220—223

Fluorescence Quantum Yield (ϕ)——The quantum yields of 7-amino-NBD derivatives (2.0×10^{-6} M) in ethanol (ϕEtOH), distilled water (ϕH_2O), and BSA solution (10 mg/ml) (ϕBSA) were determined. The values in BSA solution are apparent ones. The calculation of ϕ was made according to the equation described by Parker and Rees[14] and quinine sulfate in 1 N H_2SO_4 was used as the standard. Fluorescence spectra and intensity were measured with a Hitachi MPF-4 fluorescence spectrophotometer. Absorption was measured with a Hitachi 200-20 spectrophotometer.

Calculation——The data obtained by fluorometry were analyzed by using Scatchard's plot. The binding parameters were calculated by means of the Scatchard equation.[15] The characteristics of the binding affinity were examined quantitatively by the use of multiple regression analysis. The binding parameters and correlations were calculated on a JEOL digital computer, model JEC-7E.

Side Chain Length——The approximate lengths (L) between the amino N atom and the terminal C atom of 7-substituents of NBD derivatives were estimated as fully extended structures by the use of the HGS Biochemistry Molecular Model (Maruzen Co., Tokyo).

Partition Coefficient——The values of the logarithm of the octanol–water partition coefficients ($\log P$) for ethylamine, cyclohexylamine, benzylamine, and morpholine were obtained from reference 16. The $\log P$ values for other amines were estimated by the method of Hansch and Leo.[16]

Results and Discussion

Fluorescence Characteristics of 7-Amino-NBD Derivatives

The excitation and emission spectra were measured and the quantum yield was

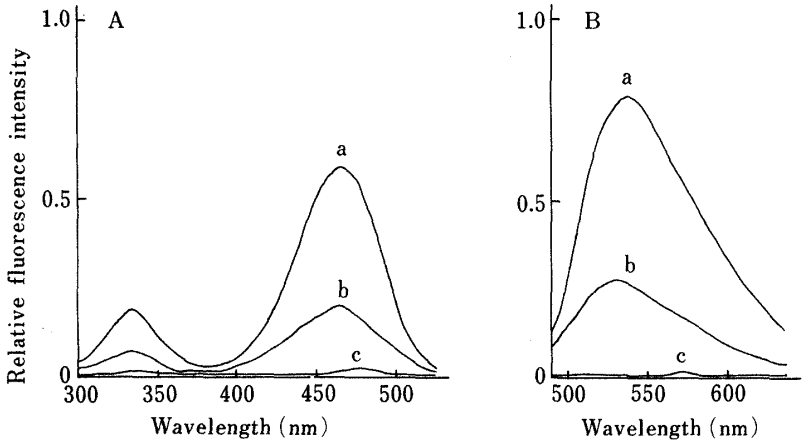

Fig. 1. Excitation and Emission Spectra of 2.0×10^{-6} M 7-Laurylamino-NBD in Ethanol (Curve a), 1.0×10^{-5} M BSA Solution (Curve b) and 0.15 M Tris–HCl Buffer (Curve c)

A: excitation spectra (emission wavelength was at 540 nm). B: emission spectra (excitation wavelength was at 460 nm).

TABLE II. Quantum Yields (ϕ) of 7-Amino-NBD

7-Substituent	ϕEtOH	ϕH_2O	ϕBSA	ϕEtOH/ϕH_2O	ϕBSA/ϕH_2O	EtOH Em_{max} (nm)	H_2O Em_{max} (nm)	BSA Em_{max} (nm)
Ethylamino	0.299	0.031	0.029	9.65	1.32	540	563	540
Propylamino	0.316	0.038	0.056	8.32	1.47	540	563	540
Butylamino	0.307	0.034	0.064	9.03	1.88	540	563	540
Hexylamino	0.305	0.033	0.093	9.24	2.82	540	565	538
Cyclohexylamino	0.317	0.038	0.071	8.34	1.87	545	567	540
Heptylamino	0.305	0.030	0.128	10.17	4.27	540	566	537
Octylamino	0.322	0.018	0.186	17.89	10.33	540	566	538
Nonylamino	0.316	0.018	0.209	17.57	11.61	540	570	538
Decylamino	0.312	0.003	0.199	104.00	66.33	540	570	538
Undecylamino	0.322	0.004	0.161	80.50	40.25	540	570	538
Laurylamino	0.353	0.004	0.149	88.25	37.25	540	570	535
Benzylamino	0.377	0.041	0.038	9.20	0.93	537	562	535
p-Methoxybenzylamino	0.323	0.004	0.025	80.75	6.25	537	562	535
Morpholino	0.011	0.003	0.010	3.67	3.33	547	580	543
Anilino	0.016	0.001	0.002	16.00	2.00	538	580	543
o-Toluidinyl	0.000	0.000	0.000	0.00	0.00	—	—	—
p-Toluidinyl	0.000	0.000	0.000	0.00	0.00	—	—	—
α-Naphthylamino	0.000	0.000	0.000	0.00	0.00	—	—	—

determined with 18 7-amino-NBD derivatives. Figure 1 shows the excitation and emission spectra of 7-laurylamino-NBD in 0.15 M Tris–HCl (pH 7.0), ethanol and BSA (1.0×10^{-5} M) solution. The excitation spectra show peaks near 340 and 460 nm in ethanol and BSA solution and near 340 and 480 nm in buffer solution. The emission spectra show a peak near 540 nm in ethanol and BSA solution and near 560 nm in buffer solution. Similar patterns were observed with other derivatives. Table II lists the values of quantum yield and wavelength of the maximum fluorescence emission for 7-amino-NBD derivatives in ethanol, distilled water, and BSA (10 mg/ml) solution. It is noteworthy that alkylamino-NBD compounds bearing C_8–C_{12} *N*-alkyl chains show greater ϕBSA values, whereas they give almost the same ϕEtOH values as other congeners. Benzylamino-NBD and *p*-methoxybenzylamino-NBD show lower ϕBSA;

this may reflect their lower affinity for BSA as compared with the alkylamino-NBD congeners. The ratios of quantum yield, $\phi EtOH/\phi H_2O$ and $\phi BSA/\phi H_2O$ are also listed in Table II. These values are considered to represent the degree of response of probes when they are moved from water to a hydrophobic environment. Therefore, probes with higher ratios report environment changes more sensitively. 7-Decylamino-, 7-undecylamino-, 7-laurylamino- and 7-*p*-methoxybenzylamino-NBD gave rather high $\phi EtOH/\phi H_2O$ values. The same tendency was found in the case of $\phi BSA/\phi H_2O$, except with *p*-methoxybenzylamino-NBD. Derivatives with a longer alkyl chain appear to be well fitted to the hydrophobic area on the BSA molecule. 7-Benzylamino-NBD and 7-*p*-methoxybenzylamino-NBD, developed by Kenner and Aboderin,[10] have low $\phi BSA/\phi H_2O$ values of 0.93 and 6.25, respectively, which do not seem appropriate for researches on BSA binding. It is also suggested that 7-lower-alkylamino, 7-cyclohexylamino, 7-morpholino, and other 7-aromatic amino-NBD derivatives prepared for the present study are not applicable to binding studies.

Binding of 7-Amino-NBD Derivatives to BSA

The parameters for the binding to BSA were determined by fluorescence measurement for 14 derivatives (excluding aromatic amine derivatives) on the basis of the values of quantum yield. The Scatchard plot (r/C *vs.* r) for the binding of these probes to BSA is shown in Fig. 2. The linearity of the Scatchard plots indicates that the probes bind to only one class of sites on BSA. Table III shows the binding parameters thus obtained. Derivatives with alkylamino chain lengths of 12.7 to 15.2 Å (C_{10}–C_{12}) gave an inwardly curved Scatchard plot at higher probe concentrations, as seen in Fig. 2b. This seems to be caused by low solubility of the probes, but this is not a practical probelm since the probes are sufficiently sensitive at much lower concentrations. Derivatives with side chain lengths of 2.6 to 7.6 Å (C_2–C_6) gave rather small n values (0.10 to 0.24); the reason for this is not clear.

It appears that $\log nK$ increases with increasing length of the alkylamino chain. Thus, the $\log nK$ values for 14 derivatives were examined by the use of multiple regression analysis. In the analysis, the side chain length (L) and $\log P$ values listed in Table III were used as the predictor variables. Eqs. 1 and 2 were obtained

$$\log nK = 0.19(\pm 0.03)L + 3.65(\pm 0.27) \quad (1)$$

$$n = 14 \qquad r = 0.968 \qquad s = 0.212$$

$$\log nK = 0.40(\pm 0.09)\log P + 4.28(\pm 0.26) \quad (2)$$

$$n = 14 \qquad r = 0.939 \qquad s = 0.292$$

where the figures in parentheses are the 95 percent confidence intervals, n is the number of data points, r is the correlation coefficient, and s is the standard deviation. Equation 1 shows a very good linear correlation between side chain length and binding affinity ($\log nK$). The correlation between $\log P$ and binding affinity is also significant. However, the two-parameter equation using both L and $\log P$ as predictor variables was not significant at the $p < 0.05$ level. Since the intercorrelation between L and $\log P$ is very high ($r = 0.972$), it seems likely that the side chain length is the major factor affecting the binding to BSA. It was shown that the derivatives with side chain length of about 10 to 15 Å have high affinity for the BSA molecule. Recently, Wanwimolruk *et al.*[17] reported that a hydrophobic cleft about 12—16 Å deep and about 8 Å wide exists on the HSA molecule. A similar cleft may also exist on the BSA molecule.

Identification of the Binding Site for 7-Amino-NBD Derivatives

To investigate the class of binding site for the NBD derivatives, the relative fluorescence intensities of 7-amino-NBD derivatives (2.0×10^{-6} M) with 2.0×10^{-5} M of BSA were mea-

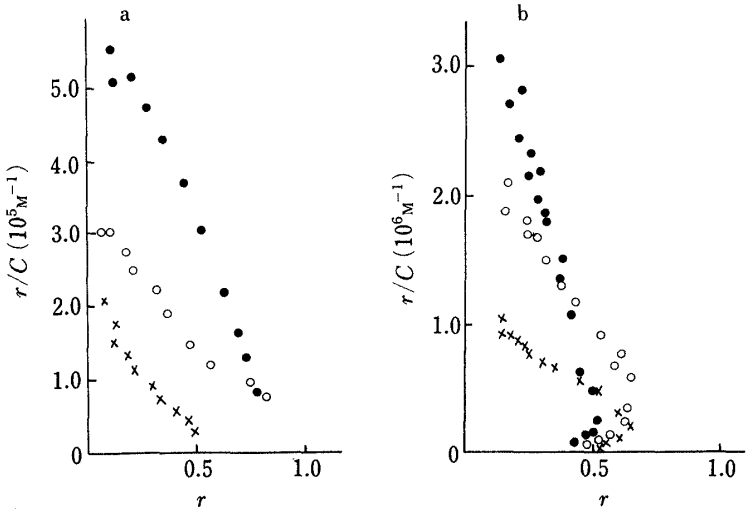

Fig. 2. Scatchard Plots for the Binding of 7-Amino-NBD Derivatives (0.4—6.0×10^{-6} M) to BSA (2.0×10^{-6} M)

Each data point represents the average of two experiments.
a: (×), heptylamino-; (○), octylamino-; (●), nonylamino-NBD. b: (×), decylamino-; (○), undecylamino-; (●), laurylamino-NBD.

TABLE III. Binding Parameters and Molecular Characteristics of 7-Amino-NBD Derivatives

7-Substituent	n	K (10^5 M^{-1})	log nK	$L^{a)}$	log $P^{b)}$
Ethylamino-	0.10	2.56	4.39	2.6	−0.13
Propylamino-	0.05	5.70	4.48	3.8	0.53
Butylamino-	0.07	3.16	4.32	5.1	1.07
Hexylamino-	0.24	1.73	4.61	7.6	2.15
Cyclohexylamino-	0.09	3.39	4.47	4.3	1.49
Heptylamino-	0.56	3.58	5.30	8.8	2.69
Octylamino-	1.13	2.51	5.46	10.2	3.23
Nonylamino-	0.93	8.37	5.89	11.4	3.77
Decylamino-	0.95	12.83	6.08	12.7	4.31
Undecylamino	0.76	35.10	6.42	13.9	4.85
Laurylamino-	0.55	77.40	6.63	15.2	5.39
Benzylamino-	0.20	2.66	4.73	5.3	1.09
p-Methoxybenzylamino	0.64	0.98	4.80	7.3	1.02
Morpholino	0.08	3.00	4.38	2.8	−1.08

a) For 7-amino substituents. *b*) For amines reacted with 7-Cl-NBD.

sured in the presence of several drugs ($2.0—20.0 \times 10^{-5}$ M), the binding sites of which have been classified according to Sudlow *et al.*[11] The Sudlow classification has also been utilized for BSA bindings[18] as well as HSA bindings. Figure 3 shows the relative fluorescence intensity values for 7-heptylamino- and 7-laurylamino-NBD. Table IV shows the decrease in the fluorescence intensity values for C_7 to C_{12} alkylamino derivatives. It is apparent from Fig. 3 that 7-heptylamino- and 7-laurylamino-NBD gave only 10—20% decrease of the intensity in the presence of site I marker drugs, warfarin and phenylbutazone, but showed a marked decrease in the presence of site II marker drugs, ibuprofen and flufenamic acid. C_7–C_{12} 7-alkylamino-NBD derivatives are thus considered to have the same binding site on BSA as site II drugs such as ibuprofen and flufenamic acid. However, diazepam, one of the site II drugs, caused a much smaller decrease of the intensity than other site II drugs and thus is suggested

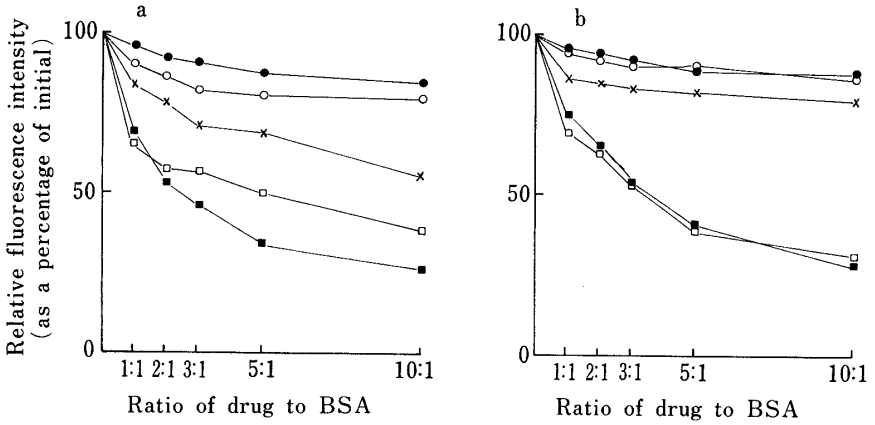

Fig. 3. Effects of Various Drugs on the Fluorescence Intensity of 7-Heptylamino- and 7-Laurylamino-NBD in BSA Solution

a: 7-heptylamino-NBD. b: 7-laurylamino-NBD. (●), warfarin; (○), phenylbutazone; (×), diazepam; (■), ibuprofen; (□), flufenamic acid.
The relative fluorescence intensity of probes (2.0×10^{-6} M) in BSA (2.0×10^{-5} M) solution was measured at the excitation and emission wavelengths given in Table I. Drugs were added to give 1:1, 2:1, 3:1, 5:1, and 10:1 ratios of drugs to BSA.

TABLE IV. Percent Decreases of Fluorescence Intensity of 7-Alkylamino-NBD in BSA Solution on Addition of Drugs[a)]

Drug	Heptylamino		Octylamino		Nonylamino		Decylamino		Undecylamino		Laurylamino	
	1:1	5:1	1:1	5:1	1:1	5:1	1:1	5:1	1:1	5:1	1:1	5:1
Warfarin	3.9	12.4	2.1	9.8	5.2	11.6	4.7	10.4	5.0	11.4	4.9	10.7
Phenybutazone	10.0	19.7	1.6	7.0	8.2	12.3	6.3	11.0	7.3	11.0	6.2	10.0
Diazepam	16.5	31.4	8.5	28.1	15.3	26.2	13.8	19.0	13.0	18.0	13.5	17.8
Ibuprofen	31.7	65.7	26.8	65.5	20.7	60.0	24.5	56.1	26.0	57.1	24.5	59.0
Flufenamic acid	34.5	50.0	32.2	61.3	27.1	52.3	31.9	63.5	32.9	64.0	31.0	61.0

a) The fluorescence intensities of probes (2.0×10^{-6} M) in BSA (2.0×10^{-5} M) were measured at the excitation and emission wavelengths given in Table I before and after the addition of drugs. Drugs were added to give 1:1 and 5:1 ratios of drugs to BSA.

to have a different binding site from that for ibuprofen and flufenamic acid. It is particularly interesting that the difference of the % decrease between diazepam and other site II drugs increases with increase of the side chain length of NBD probes. Thus, 7-alkylamino-NBD derivatives having various lengths of side chain are expected to be useful probes for protein binding studies, especially for the identification of binding sites.

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