Binding of Pyrene-1-butyric Acid to Serum Albumin: Species Differences

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Changes of the fluorescence spectra and quantum yield of pyrene-1-butyric acid (PYB) induced by rat (RSA), horse (HoSA), and pig serum albumins (PSA) and rat serum were compared at pH 7.4. RSA and rat serum caused marked quenching of the PYB fluorescence. On the contrary, HoSA and PSA increased the fluorescence intensity. In RSA and rat serum solutions, the PYB fluorescence intensity due to monomer emission was greatly diminished, and an additional emission due to excimer formation appeared at 480 nm. On the other hand, in the case of HoSA and PSA solutions, monomer emission gave rise to high fluorescence intensity and no excimer formation was observed.

The binding parameters of PYB to RSA, HoSA, and PSA as well as human (HSA), bovine (BSA), rabbit (RbSA) and dog serum albumins (DSA) were compared by equilibrium dialysis method. The primary binding site affinity was in the order $\text{RSA} > \text{HSA} > \text{HoSA} > \text{RbSA} > \text{DSA} \cong \text{BSA} > \text{PSA}$. However, for RSA and HSA, which showed reduced monomer emission and clear excimer fluorescence, the primary binding site number (n_1) was 2.71 and 2.90, respectively, considerably greater than those values for the other serum albumins. These findings suggest that PYB binds to RSA and HSA as a dimer. On the other hand, PYB appears to bind with BSA, RbSA, DSA and HoSA as a monomer. The primary binding site was not clearly recognized in PSA, which showed little enhancement of fluorescence. The similar behavior of RSA and HSA in the PYB binding may be attributed to their resemblance in the hydrophobic environment and flexibility around their binding sites.

Keywords — protein binding; serum albumin; species difference; pyrene-1-butyric acid; fluorescent probe; equilibrium dialysis

Introduction

The binding of drugs and fluorescent probes to human serum albumin (HSA) as well as various animal serum albumins, particularly bovine serum albumin (BSA) has been measured by various methods. For some drugs, certain differences in albumin binding affinity among animal species are known to exist.^{1–5)} However, in general, considerable differences have not been reported among HSA, BSA, and rat serum albumin (RSA) with respect to binding affinity,⁶⁾ molecular shape,⁶⁾ and amino acid sequence.^{7–9)} In the previous paper,¹⁰⁾ we demonstrated marked differences in albumin-induced changes of fluorescence spectra of pyrene-1-butyric acid (PYB) and its quantum yields between HSA and other animal serum albumins (bovine, rabbit, and dog). PYB showed a marked decrease in the fluorescence intensity in the presence of HSA, while it showed an increase in the presence of any of the three animal

albumins.

In the present study, PYB fluorescence spectra and quantum yield were observed with RSA, horse serum albumin (HoSA), and pig serum albumin (PSA) as well as the four above-mentioned serum albumins. Moreover, bindings of PYB with the various serum albumins were measured by the equilibrium dialysis method, and the binding parameters were calculated. Binding affinities were compared among these albumins, and the characteristics of the binding sites were investigated.

Materials and Methods

Materials — The manufacturers and lot numbers of serum albumins used in the present study are listed in Table I. The molecular weight was assumed to be 67 000 for all the albumins. PYB (lot No. 5B) was purchased from Molecular Probes, Inc., Junction City, OR. All other reagents used were commercially available and of

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TABLE I. Serum Albumin

Albumin	Type	Manufacturer	Lot No.
Human (HSA)	Fraction V (Fr. V)	Miles Laboratories	152 & 153
Human (HSA)	Fatty acid free (defat.)	Miles Laboratories	18
Human (HSA)	Crystallized (cryst.)	Miles Laboratories	39
Bovine (BSA)	Fr. V	Armour Pharma.	MGF1131
Bovine (BSA)	Defat.	Miles Laboratories	22
Bovine (BSA)	Cryst.	Miles Laboratories	74
Rabbit (RbSA)	Fr. V	Miles Laboratories	126
Dog (DSA)	Fr. V	Sigma Chemical	56F-9305
Rat (RSA)	Fr. V	ICN	0033
Rat (RSA)	Defat.	Sigma Chemical	97F-93211
Rat (RSA)	Cryst.	Sigma Chemical	57F-9300
Horse (HoSA)	Fr. V	Sigma Chemical	84F-9355
Pig (PSA)	Fr. V	Sigma Chemical	95F-9358

special grade. Unless otherwise specified, all final solutions were prepared with 0.15 M Tris-HCl buffer (pH 7.4), and all the experiments were performed at 25 °C.

Fluorescence Spectrum and Quantum Yield (ϕ) — Fluorescence spectra were recorded at an excitation wavelength of 347 nm and an emission wavelength of 360–500 nm in 0.15 M

Tris-HCl buffer of pH 7.4 and serum albumin solutions. The quantum yields, ϕ , for PYB (1.0×10^{-6} M) were determined in several serum albumin (5.0×10^{-6} M) solutions. They were given as the mean value of two experiments. The calculation of ϕ was made according to the equation described by Melhous,¹¹⁾ and quinine sulfate in 1 N H₂SO₄ was used as the standard.

Fig. 1. Fluorescence Spectra of PYB (4.0×10^{-7} M) with Serum Albumins (Fr. V)

A) 1. 0.15 M Tris-HCl buffer (pH 7.4), 2. HSA, 3. BSA, 4. RSA, 5. RbSA, 6. DSA, 7. HoSA, 8. PSA. Serum albumin concn.: 4.0×10^{-7} M. B) RSA. Serum albumin concn.: 1. 0.0, 2. 2.0, 3. 4.0, 4. 6.0, 5. 8.0, 6. 10.0, 7. 20.0, 8. 30.0, 9. 40.0×10^{-7} M.

Fig. 2. Fluorescence Spectra of PYB (4.0×10^{-7} M) with Serum Albumins (Fr. V)

A) HSA and B) HoSA. Serum albumin concn.: 1. 0.0, 2. 2.0, 3. 4.0, 4. 6.0, 5. 8.0, 6. 10.0, 7. 20.0, 8. 30.0, 9. 40.0×10^{-7} M.

Fluorescence spectra and intensity were measured with a Hitachi MPF-4 fluorescence spectrophotometer. Absorption was measured with a Hitachi 200-20 spectrophotometer.

Equilibrium Dialysis (ED) Method — By using a 3.0 ml dialysis cell devised by Goto *et al.*,¹²⁾ sample solutions (3.0 ml) were shaken for 16 h at 25 °C in a thermostat. Spectrapor 1 dialysis membrane (Spectrum Medical Inc., Los Angeles, CA) was used after being boiled four times in distilled water. The decrease in the unbound PYB content was measured, and the binding parameters were calculated. Adsorption of probes on the membrane was negligible. The concentration of albumins was 5.0×10^{-6} M. Each point in the Scatchard plots¹³⁾ (Fig. 6) represents the mean value of three or four experiments.

Calculation — The binding data obtained by ED method were expressed as the mean value of three or four measurements, and analyzed by using Scatchard plot. The binding parameters were calculated by linear regression when the Scatchard plot was linear. When the plot was curved, the data were fitted to the Karush's equation¹⁴⁾ by a nonlinear least

squares method (modified Marquardt method). The calculation of binding parameters was performed with a NEC PC-9801 personal computer.

Fig. 3. Fluorescence Spectra of PYB with Rat Serum

Serum concn.: 1. 0.0, 2. 4.0, 3. 8.0, 4. 12.0, 5. 16.0×10^{-7} M.

Results and Discussion

Changes in PYB Fluorescence Spectra in Serum Albumin Solutions

Figure 1A shows the fluorescence spectra of PYB (4.0×10^{-7} M) in various animal serum albumin (Fr. V) solutions (4.0×10^{-7} M). Fluorescence intensity in RSA solution is markedly decreased as the case HSA¹⁰⁾ previously reported. On the other hand, in HoSA, PSA, BSA, and rabbit serum albumin (RbSA) solutions, the fluorescence intensity increased, the order of the enhancement being HoSA > dog serum albumin (DSA) > BSA > RbSA > PSA. The fluorescence spectra of PYB with various concentrations of RSA, HSA, and HoSA are shown in Figs. 1B, 2A, and 2B, respectively. It can be seen that RSA caused clear quenching dependent on the albumin concentration, while HoSA, increased fluorescence intensity, on the contrary. These results resemble the contrast in spectral patterns between HSA and BSA previously reported.¹⁰⁾ Thus, in terms of changes in PYB fluorescence, these serum albumins can be divided into two groups: RSA and HSA which quench the fluorescence, and HoSA, DSA, BSA, RbSA, and PSA which enhance it. Moreover, in the case of RSA as shown in Fig. 1B, a new broad peak appears in the neighborhood of 480 nm. This phenomenon occurs also in the

Fig. 4. Elution Pattern of Rat Serum Chromatographed on a Sephadex G-200 Column (2.6×96 cm)

case of HSA (Fig. 2A). This broad peak around 480 nm was not significantly recognized in RSA and HSA solutions without PYB. It is though that the reduction in PYB monomer fluorescence (378, 398, 417 nm) and the appearance of a broad peak around 480 nm in RSA and HSA solutions is attributed to a newly formed excimer.^{15,16)} X-Ray analyses of pyrene have revealed that the long side of the molecular plane is approximately 9 Å,¹⁷⁾ but that this increases to about 11 Å¹⁸⁾ when the excimer is formed.¹³⁾ The excimer assumes a stacking structure with the molecular planes separated by approximately 3 Å.¹⁹⁾ It is likely that clefts for

Fig. 5. Fluorescence Spectra of PYB in the Presence of Fr. 40, 49, 57, and 77

A) Fr. 40, B) Fr. 49, C) Fr. 57, and D) Fr. 77. Protein concn.: 1. 0.0, 2. 4.0 , 3. 8.0×10^{-7} M.

Fig. 6. Fluorescence Spectra of PYB in the Presence of Albumin Fraction on Sephadex G-200 Column of RSA and PSA (Fr. V)

A) RSA, B) PSA. Albumin concn.: 1. 0.0, 2. 8.0, 3. 12.0×10^{-7} M.

binding of PYB on RSA and HSA molecules can contain and stabilize PYB excimer unlike albumin molecules of other species.

PYB fluorescence spectra were determined also in rat serum at its various dilutions, but the results were similar to those observed for RSA (Fig. 3).

Next, albumins were purified from rat serum and commercially available preparations of RSA, HSA, BSA, and PSA by Sephadex G-200

gel filtration, and their influences on PYB fluorescence spectra were examined. Sephadex G-200 gel filtration of rat serum yielded an elution pattern with four peaks shown in Fig. 4. Measurement of the fluorescence spectra of PYB in the presence of any of four fractions (40, 49, 57, and 77) revealed that only fraction 77 (albumin fraction) induced the spectral changes (Fig. 5). Measurement of PYB fluorescence in the presence of RSA (Fr. V) purified by the gel

TABLE II. Apparent Quantum Yield (ϕ) of PYB^{a)} in Various Serum Albumin Solutions^{b)}

Solution	ϕ	Solution	ϕ
Buffer (0.15 M Tris-HCl)	0.340 ^{c)}	RSA (Fr. V)	0.200
HSA (Fr. V)	0.223 ^{c)}	RSA (Defat.)	0.201
HSA (Defat.)	0.224 ^{c)}	RSA (Cryst.)	0.201
HSA (Cryst.)	0.215 ^{c)}	RbSA (Fr. V)	0.422 ^{c)}
BSA (Fr. V)	0.461 ^{c)}	DSA (Fr. V)	0.508 ^{c)}
BSA (Defat.)	0.445 ^{c)}	HoSA (Fr. V)	0.506
BSA (Cryst.)	0.405 ^{c)}	PSA (Fr. V)	0.359

a) PYB concentration was 1.0×10^{-6} M.

b) Albumin concentration was 5.0×10^{-6} M. c) Reference 10.

filtration exhibited a spectral pattern (Fig. 6A) similar to those of unpurified RSA (Fr. V, Fig. 1B), rat serum (Fig. 3), and albumin fraction of rat serum (Fr. 77, Fig. 5). Similar spectral changes were also observed with fatty acid free and crystallized RSA preparations (commercial product). Therefore, one can conclude that the changes in PYB fluorescence are due to albumin, and not to any effect of impurities in the commercial products nor to substances such as fatty acid bound to albumin.

Apparent Quantum Yield of PYB in Serum Albumin Solutions

The apparent quantum yield (ϕ) of PYB in serum albumin solutions was determined. The values are listed in Table II. In the previous paper,¹⁰⁾ we demonstrated PYB quantum yields in organic solvents. In isopropanol-water solutions, ϕ became higher with 10, 20, and 25% isopropanol, showing a maximum at 25%, and then reduced at higher isopropanol concentrations. The ϕ value of PYB with RSA corresponds to that in 50–60% isopropanol ($\phi=0.26-0.18$). However, the values of ϕ with HoSA and PSA are similar to or greater than ϕ value in 20–30% isopropanol solution (0.37–0.40). RSA showed quantum yields approximating those previously reported for HSA, while the values for HoSA and PSA were close to those observed for BSA and RbSA solutions.

The fluorescence lifetime²⁰⁾ and quantum yield¹⁰⁾ of PYB are known to be reduced in organic solvents, and the hydrophobicity of the solvent is known to be concerned with the reduction of fluorescence. It seems likely from this viewpoint that RSA is similar to HSA in hydrophobic environment of PYB binding site. The binding site for PYB on RSA and HSA is supposed to provide a much more hydrophobic environment than that on other serum albumins. The interspecies differences in the quantum yields of PYB-albumin solutions may be attributed to the differences of hydrophobicity and other factors related to the structure around the PYB binding site on the albumin molecules, but the details are unknown.

Binding of PYB to Various Animal Serum Albumins Determined by ED Method

The binding of PYB in various animal serum albumins was determined by the ED method, and the results were compared. The binding data for each albumin were analyzed by means of Scatchard plots. Figure 7 shows plots for binding of PYB to HSA and six animal serum albumins. The Scatchard plots for six kinds of serum albumins excluding PSA are curvilinear, suggesting the presence of at least two types of independent binding sites. In the case of RSA and HSA, which displayed formation of PYB excimers, the initial level of the curves, *i.e.*, the affini-

Fig. 7. Scatchard Plots for the Binding of PYB to Serum Albumin

A) ●, HSA; □, RbSA; ×, DSA; ○, PSA. B) ●, HSA; □, BSA; ×, RSA; ○, HoSA.

TABLE III. Parameters for Binding of PYB ($3.0\text{--}60.0 \times 10^{-6}$ M) with Various Serum Albumins (5.0×10^{-6} M) Determined by Equilibrium Dialysis

Albumin	n_1	K_1 (10^6 M^{-1})	$\log n_1 k_1$	n_2	K_2 (10^4 M^{-1})	$\log n_2 K_2$
HSA (Fr. V)	2.90	1.04	6.48	10.65	3.19	5.53
HSA (Defat.)	2.83	1.02	6.46	24.19	1.56	5.58
HSA (Cryst.)	2.78	0.94	6.42	9.49	4.17	5.60
BSA (Fr. V)	1.27	1.03	6.12	5.52	5.16	5.45
BSA (Defat.)	0.90	1.56	6.15	9.85	5.07	5.70
BSA (Cryst.)	1.04	1.46	6.18	7.95	6.70	5.73
RSA (Fr. V)	2.71	1.77	6.68	10.40	2.43	5.40
RSA (Defat.)	1.95	4.99	6.99	10.93	3.15	5.54
RSA (Cryst.)	2.08	2.89	6.78	12.33	1.64	5.31
RbSA (Fr. V)	1.13	1.72	6.29	6.14	9.13	5.75
DSA (Fr. V)	0.40	3.50	6.15	7.11	10.03	5.85
HoSA (Fr. V)	1.31	1.91	6.40	6.47	4.41	5.46
PSA (Fr. V)	—	—	—	8.01	9.00	5.86

ty of the primary binding site was more pronounced than that of other serum albumins. On the other hand, the plot for PSA, which exhibited relatively small enhancement of PYB fluorescence, showed a marked decrease of binding at lower probe concentrations (Fig. 7). The plot appears to be linear, suggesting a single class of binding site.

The calculated binding parameters are listed in Table III. The apparent numbers of primary binding sites (n_1) on RSA and HSA were 2.71 and 2.90, respectively, which were more than twofold the values for other serum albumins. This supports the formation of excimer. This difference in number of primary binding sites is probably related to the difference in changes of PYB fluorescence intensity. The value of the primary binding affinity ($\log n_1 K_1$) was higher for RSA, followed by the other six kinds of serum albumins in the order HSA > HoSA > RbSA > DSA $\approx$ BSA > PSA (Table III). For RSA, HSA, and BSA, the measurements were performed with commercially produced fatty acid free and crystallized preparations as well as Fr. V. The results were almost the same as those obtained for Fr. V. It seems that the binding site for PYB is different from the binding site for fatty acid.

Concerning the fluorescence changes of pyrene derivatives, Vaughan and Weber²¹⁾

reported that, in the interaction between PYB and poly-L-lysine, the fluorescence lifetime of PYB was prolonged when the content of α -helix of poly-L-lysine increased. Soutar *et al.*¹⁸⁾ reported that increased ability for formation of pyrene excimer was accompanied by increased flexibility of phospholipid vesicles. As regards α -helix content, it is lower in HSA (44–57.8%)^{22,23)} than in BSA (68%).²⁴⁾ The formation of excimer and the lowering of monomer emission in the presence of HSA may indicate that difference in α -helix content, that is, difference in flexibility, of albumin molecules is closely related to the enhancement or quenching of PYB fluorescence by serum albumins.

The degree of homology of the amino acid sequences of HSA and RSA as well as that of HSA and BSA is approximately 80%, while that of these three serum albumins collectively is about 63%, representing a high degree of structure similarity.⁶⁾ The marked interspecific differences in the binding with PYB indicate that the physicochemical character of the binding site is of greater importance for the PYB binding than similarity in primary structures. As previously described, the longer side of PYB molecular plane is 9 Å¹⁷⁾ in the monomer and 11 Å¹⁸⁾ in the dimer (excimer), which assumes a stacking structure with a separation of approximately 3

Å¹⁹⁾ between the planes. From the molecular model (HGS Biochemistry Molecular Model; Maruzen Co., Tokyo), the shorter sides of monomer and dimer are both estimated to be about 7.5 Å. Wanwimolruk *et al.* reported²⁵⁾ that a hydrophobic cleft about 8 Å wide exists on the HSA molecule. A similar cleft may also exist on the RSA. These clefts seem to have sufficient flexibility to permit binding of the stacked PYB excimers. The other five kinds of animal serum albumins are thought to have the cleft of less flexibility.

As regards the identification of the class of PYB binding sites, displacement of albumin-induced PYB fluorescence spectral change is now investigated qualitatively by using several key drugs (warfarin, phenylbutazone, diazepam, ibuprofen, *etc.*) to the Sjöholm classification.²⁶⁾ The details are to be published in the near future.

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