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Location of Drug Binding Sites on Human Serum Albumin

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The location of Site I on the human serum albumin (HSA) molecule was investigated. Naphthol yellow- S (NY-S) was used as a representative ligand for Site I, since the bilirubin-displacing effect of NY-S was similar to that of Site I drugs such as warfarin and phenylbutazone. NY-S was bound to HSA with at least three classes of binding sites. The binding parameters of the primary class were one binding site ($n_1 = 1$) and a binding constant (K_1) of $> 10^7\,\text{M}^{-1}$, and the complex at equimolar ratio of dye to albumin exhibited metachromasy. The intrinsic fluorescence intensity, attributed to the single tryptophan residue (position 214), decreased significantly in this complex. Fragments A and C (residues 299—585 and 124—298, respectively, in the amino acid sequence of HSA) obtained by cyanogen bromide cleavage of HSA showed NY-S-binding ability. The binding of NY-S to fragment A or C caused the same metachromasy as seen with NY-S–HSA complex. Fragment C is markedly hydrophobic and contains the single tryptophan residue. The binding of NY-S to HSA was diminished by the chemical modification of lysine residues with pyridoxal 5′-phosphate (PLP). Further, lysine residues modified by PLP existed in fragment C. These results suggest that the primary binding site of NY-S, which corresponds to Site I, is located in the fragment C region of the HSA molecule.

Keywords——protein binding; binding site; albumin; human serum albumin; naphthol yellow-S; pyridoxal 5′-phosphate

There have been many reports on drug-binding sites on human serum albumin (HSA).[1-3] Although the binding to HSA is generally assumed to be rather nonspecific, very specific binding sites have been demonstrated for some substances. Previous reports in this series have suggested the existence of three specific binding sites for drugs on HSA.[4,5] These three distinct binding sites were designated as Site I, Site II and the Diazepam-site. However, present knowledge does not allow a complete assignment of the high-affinity binding regions for different drugs on albumin.

The aim of the present study was to locate Site I on the HSA molecule. Naphthol yellow-S (NY-S) was used as a representative ligand for Site I. In order to evaluate the characteristics of the primary binding site of NY-S on HSA, chemical modification with pyridoxal 5′-phosphate (PLP) and fragmentation with cyanogen bromide (CNBr) were undertaken.

Experimental

Materials——Crystalline HSA (fraction V), bilirubin, peroxidase (type I) and PLP were purchased from Sigma Chemical Co. The molecular weight of HSA was assumed to be 66250 and the concentration was determined by using an extinction coefficient $E^{0.1\%}_{1\,\text{cm}}$ of 0.531 at 279 nm.[6] NY-S was purchased from Nakarai Chem. Co. All other reagents were of special grade and were used without further purification. Unless otherwise stated, 1/15 M phosphate buffer (pH 7.4) was used.

Modification of HSA with PLP (PLP–HSA)[7]——HSA solution (2%) in distilled water was added to PLP in 1-, 2- and 3-fold excess per mol of albumin, the pH was adjusted to 8.5 with 2 N KOH, and the solution was left for an hour at room temperature. Then the solution was adjusted to pH 4.2 to 4.3 with 4.5 N acetic acid, and a 1% aqueous solution of $NaBH_4$ was immediately injected under the surface at a rate of 1 ml per minute until the yellow color

disappeared, the pH being held between 4.2 and 4.4 by further addition of acetic acid. After being allowed to stand for 1 h at room temperature, the solution was dialyzed, first against 0.05 M sodium acetate, pH 4.2, to remove all unlabeled PLP and then exhaustively against distilled water. A dry product was obtained by lyophilization. The degree of modification was calculated from the absorption at 323 nm, taking the molar absorbancy to be 5800.[7]

Fragmentation of HSA by CNBr——The fragmentation of HSA by CNBr was carried out according to McMenamy *et al.*[8] Three large fragments were separated by means of various column chromatographies and were designated as fragments A, B, and C in order of elution, respectively. Each of the three fragments, A, B and C, moved as a single zone on sodium dodecyl sulfate (SDS)-electrophoresis, and their molecular weights were determined to be approximately 33000, 13000 and 18000, respectively. The amino acid analysis of these fragments was in good agreement with that of McMenamy *et al.*[8] The concentration of fragment A, B, or C in 1/15 M phosphate solution was determined spectrophotometrically at 279 nm using an extinction coefficient of 11845, 3280 or 13750, respectively.[9]

Bilirubin-Displacing Experiment——The experimental method, based on those of Jacobsen[10] and Brodersen,[11] was described in the previous reports.[4,12] Increasing amounts of NY-S were added to bilirubin–HSA solution (1.8×10^{-5} and 3.0×10^{-5} M, respectively). Changes of the equilibrium concentration of unbound bilirubin were measured by a kinetic technique, based upon oxidation of free bilirubin with hydrogen peroxide and horseradish peroxidase. The velocity of oxidation was determined with and without NY-S. The ratio of these velocities was equated with the ratio of free bilirubin concentration. The value of b/b_0 was plotted on the ordinate against the concentration of NY-S on the abscissa. Here b and b_0 are the free bilirubin concentrations in the presence and in the absence of NY-S, respectively.

Equilibrium Dialysis——The general procedure was the same as described in a previous report.[13] The binding data were subjected to Scatchard analysis. The following equation was used;

$$r = \sum \{n_i K_i C_f / (1 + K_i C_f)\}$$

where the subscript i denotes the i-th class of binding sites, C_f is the free concentration of ligand, r is the ratio of bound ligand to protein, K_i is the binding constant of the i-th binding sites and n_i is the number of the i-rh sites.

Results and Discussion

The relative changes of free bilirubin concentration caused by NY-S are depicted in Fig. 1. The b/b_0 values increased with increasing concentration of NY-S. This result is in good agreement with that for Site I drugs such as warfarin and phenylbutazone, but distinct from that for Site II drugs and benzodiazepines.[4,5] This result indicates that the primary binding site of NY-S can be classified into Site I. The slope of the initial linear part ($r < 1.5$) of the curve is equal to the binding constant (K_b) of the ligand for the high-affinity site of bilirubin (bilirubin-site). The calculated K_b value was $2.6 \times 10^4\ \mathrm{M}^{-1}$. This value is twice that of warfarin,[4] and suggests that the primary binding site of NY-S is adjacent to the bilirubin-site.

Figure 2 shows Scatchard plots for the binding data of NY-S to HSA obtained by equilibrium dialysis. The plots are apparently curvilinear, suggesting the presence of at least three types of independent binding sites. Mathematical analysis of the Scatchard plots indicates the presence of one strong binding site. As a matter of fact, it was confirmed that NY-S could not be released from the equimolar complex of NY-S and HSA by dialysis. These results are similar to previous findings on the binding studies of NY-S to bovine serum albumin.[13,14] The binding parameters were calculated according to the previously outlined procedure.[14] The binding parameters of the first binding site class were assumed to be $n_1 = 1$ and $K_1 > 10^7\ \mathrm{M}.^{-1}$ The other binding parameters were obtained by recalculation of the Scatchard plots obtained by subtracting the above values mathematically. They are $n_2 = 2.5$ and $K_2 = 1.5 \times 10^6\ \mathrm{M}^{-1}$ for the secondary binding site class and $n_3 = 5$ and $K_3 = 3.6 \times 10^4\ \mathrm{M}^{-1}$ for the tertiary binding site class (Table I).

Addition of NY-S to HSA solution queched the intrinsic fluorescence intensity of HSA as shown in Fig. 3. A significant decrease of fluorescence intensity was seen until NY-S and HSA were present in equimolar amount, but increase of NY-S over that amount had little effect. The combination of this observation and the binding parameters suggests that NY-S binds stoichiometrically to HSA at the first binding site ($n_1 = 1$). It is known that the HSA

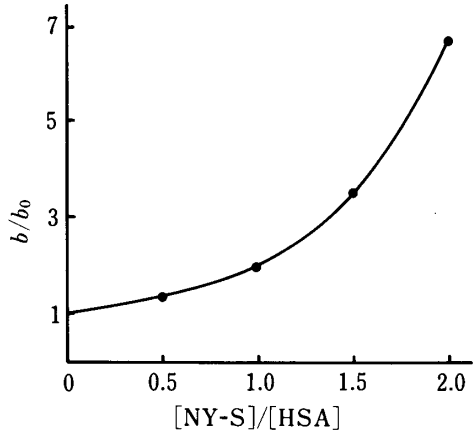

Fig. 1. Relative Concentration of Free Bilirubin in Bilirubin–HSA Complex with and without NY-S as a Function of NY-S Concentration

HSA concentration was 3.0×10^{-5} M. The molar ratio of bilirubin to HSA was 0.6.

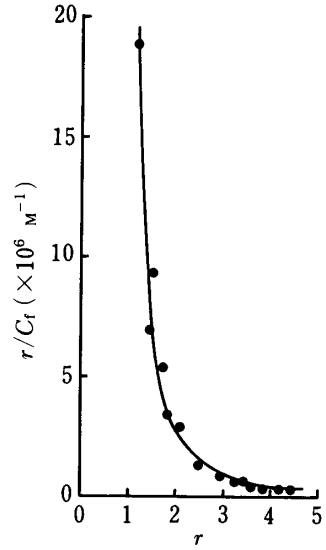

Fig. 2. Scatchard Plot for the Binding of NY-S to HSA

HSA concentration was 2.0×10^{-5} M. Binding parameters from this plot are summarized in Table I.

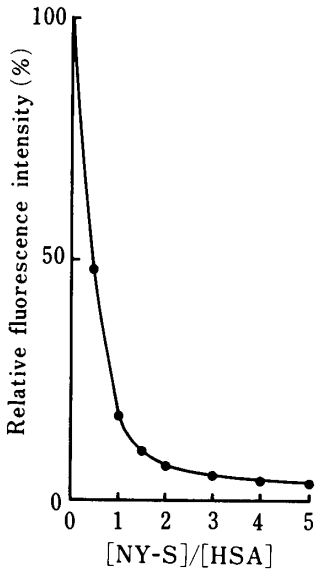

Fig. 3. Quenching of HSA Fluorescence by NY-S

The decrease of fluorescence intensity by NY-S was measured at 345 nm (emission) and 300 nm (excitation). HSA concentration was 2.0×10^{-6} M.

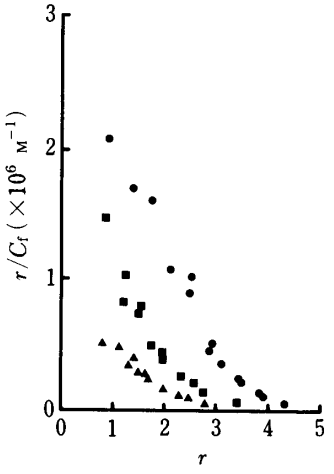

Fig. 4. Scatchard Plots for the Binding of NY-S to PLP–HSA

The amounts of lysine residues modified by PLP in albumin were 0.8 (●), 2.0 (■) and 2.7 (▲) mol per mol of HSA. The PLP–HSA concentration was 2.0×10^{-5} M. The binding parameters are summarized in Table I.

fluorescence (300 nm excitation and 345 nm emission) is attributed to the single tryptophan residue at position 214[15] on the amino acid sequence of HSA. Therefore, the primary binding site of NY-S is in the vicinity of the tryptophan residue.

The influence of lysine-modification on the binding of NY-S to HSA was studied by equilibrium dialysis. As shown in Fig. 4, modification of lysine residues with PLP reduced the binding capacity for NY-S. The degree of reduction depended on the modification ratio of

TABLE I. Binding Parameters of NY-S to PLP–HSA

	Amount of PLP (mol/mol)	Binding parameters					
		n_1	K_1	n_2	K_2	n_3	K_3
					($\times 10^6\,M^{-1}$)		($\times 10^4\,M^{-1}$)
HSA		1	$>10^7\,M^{-1}$	2.5	1.5	5	3.6
PLP–HSA[a]	0.8			2.4	1.2	5	3.6
	2.0			1.8	1.0	3	3.1
	2.7			1.5	0.6	2	2.0

a) The first binding site class for the binding of NY-S to PLP–HSA had disappeared, but the values were entered in the corresponding columns of the native HSA.

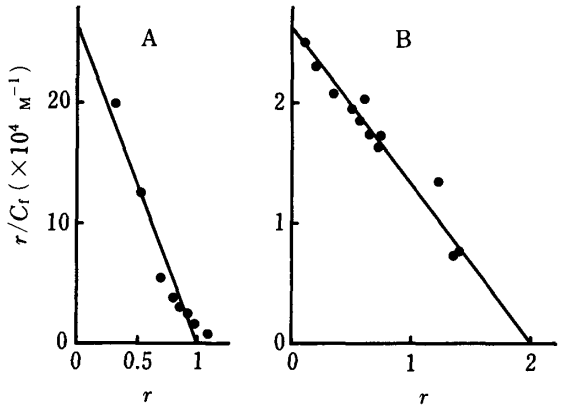

Fig. 5. Scatchard Plots for the Binding of NY-S to HSA Fragments

(A) fragment A; (B) fragment C.
Concentrations of fragments A and C were 2.14×10^{-5} and 1.73×10^{-5} M, respectively. The binding parameters are summarized in Table II.

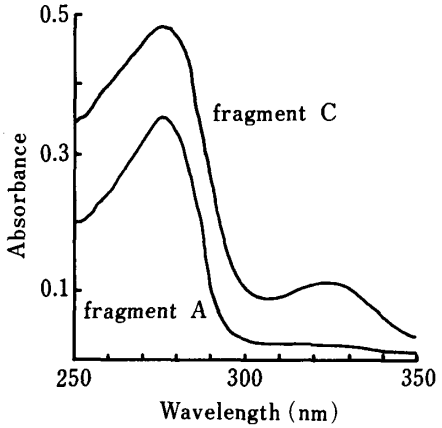

Fig. 6. Absorbance Spectra of PLP–HSA Fragments

The concentration of each fragment was 3.0×10^{-5} M.

TABLE II. Binding Parameters for the Binding of NY-S to HSA Fragments[a]

Fragment	Molecular weight	Binding parameters		Difference spectrum[b]
		n	K (M^{-1})	
A	33000	1	2.6×10^5	Similar to NY-S–HSA complex
B	13000	—	—	None
C	18000	2	1.3×10^4	Similar to NY-S–HSA complex

a) Concentrations of fragments A, B, and C were 2.14×10^{-5}, 1.86×10^{-5} and 1.73×10^{-5} M, respectively. *b*) The difference spectrum of NY-S–fragment complex against free NY-S after equilibration in the dialysis experiment.

lysine residues. The binding parameters are summarized in Table I. When 0.8 mol of lysine residues per mol of HSA was modified, the binding of NY-S to its primary site disappeared, but the secondary and tertiary binding remained.

To study where the primary binding site is located in HSA molecule, fragmentation of HSA and PLP–HSA was carried out by the CNBr-cleavage method. Following the procedures used by McMenamy *et al.*, the three major fragments were isolated by chromatog-

raphic techniques. They were designated as fragments A, B and C in order of elution, as done by McMenamy *et al.*[8] The binding ability of each fragment for NY-S was examined by equilibrium dialysis and light absorption measurement. Scatchard plots of the binding data are shown in Fig. 5, and these results are summarized in Table II. When NY-S is bound to HSA in equimolar ratio, the NY-S–HSA complex showed a new absorption spectrum, clearly different from that of NY-S alone (not shown). The binding capability was found in fragments A and C, but not in fragment B. Further, the absorption spectrum of NY-S–fragment A or –fragment C complex was similar to that of NY-S–HSA complex.

To investigate the labeling position of PLP, PLP–HSA incorporating 0.8 mol of PLP per mol of HSA was subjected to CNBr fragmentation. In the same manner as above, the three major fragments were isolated. As shown in Fig. 6, 0.7 mol of PLP per mol of fragment remained in fragment C. Therefore, PLP in fragment C accounted for about 88% of the total incorporated PLP. The combination of this result and the data of Table I suggests that the primary binding site of NY-S is located in fragment C.

Gambhir and McMenamy[9] reported that fragment C, having a molecular weight of 18000, was situated at position 124 to 298 between fragments A and B. This fragment is markedly hydrophobic, containing five tyrosine residues, six phenylalanine residues and the single tryptophan residue (position 214). Further, this fragment also has five lysine residues, which can be modified with PLP. Thus, fragment C plays an important role in the binding of NY-S to HSA. The present data suggest that Site I is located in the fragment C region, which corresponds to domain 2 in the structure of HSA.

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