[Chem. Pharm. Bull. 33(9)3960—3965(1985)]

An Equation for Ligand Binding to Protein over a Wide Range of Ligand Concentration

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(Received January 18, 1985)

A model with three parameters is proposed for the description of ligand binding behavior to protein over a wide range of ligand concentration. The equation includes a novel parameter, α, which takes account of the increase in the binding capacity of albumin caused by the binding of ligands. The validity of the equation and the implications of the parameters were examined. The equation described well the binding of bovine serum albumin (BSA) with salicylate, 4′-hydroxyazobenzene-2-carboxylate, 2-naphthoate, and benzoates. The binding data of 2-naphthoate and benzoates were analyzed by the use of multiple regression analysis to investigate the characteristics of the parameters in the equation.

Keywords——binding equation; protein binding; bovine serum albumin; binding parameter; ultracentrifugation method; salicylate; substituted benzoate

For the description of nonlinear protein-binding behavior, Karush's four-parameter[1] expression assuming the presence of two types of binding sites has been used extensively. However, the results of experiments performed over a wide range of drug concentration revealed that Karush's equation could not describe the binding of serum albumin (SA) with drugs precisely. Moreover, Karush's equation is inconvenient for use since it contains as many as four parameters.

The interaction between SA and ligands is often accompanied by conformational changes of the protein.[2] Therefore, assuming that an increase in the degree of binding of SA causes some changes in its higher-order structure which give rise to an increase in the capacity for binding, we have derived an equation with three parameters from Langmuir's equation. In the present study, the validity of the new equation was confirmed by using the binding data of salicylate and 4′-hydroxyazobenzene-2-carboxylate (HABCA) over a wide range of concentration to bovine serum albumin (BSA), measured by the ultracentrifugation method.[3] Moreover, the characteristics of the three binding parameters in the equation were examined by the use of multiple regression analysis of BSA binding with 2-naphthoate and 4-substituted benzoates.

Experimental

Materials——Bovine serum albumin (BSA, Fraction V) was purchased from Armour Pharmaceutical Co., Kankakee. The molecular weight was assumed to be 67000[4] and the concentration was determined by measuring the optical absorbance at 280 nm using $E^{1\%}_{1\,cm}=6.67$.[4] 4-Hydroxyazobenzene-2-carboxylate (HABCA, Daiichi Pure Chemicals Co., Tokyo), 2-naphthoate (Tokyo Kasei Co., Tokyo) and 4-dimethylaminobenzoate (Tokyo Kasei Co., Tokyo) were purchased. All other benzoates were obtained from Wako Pure Chemical Industries, Tokyo.

Ultracentrifugation (UC) Method——The general procedures for determination of the amount of bound drugs were the same as those described previously.[3] For the binding study of HABCA, 0.15 M phosphate buffer of pH 5.0 and 6.0, and 0.15 M Tris–HCl buffer of pH 7.0, 8.0, and 9.0 were used. Measurements of other drugs were performed using 0.15 M Tris–HCl buffer at pH 7.0. The concentration of BSA was 2.08×10^{-5} M. The concentrations of ligands

TABLE I. Concentration and Wavelength for Spectrophotometry of Ligands

Ligand	Concn. (10^{-4}M)	λ (nm) for spectrophotometry
Salicylate	0.50—120.0	296
HABCA (pH 5.0)	0.17— 25.0	348
(pH 6.0)	0.10— 25.0	348
(pH 7.0)	0.10— 25.0	348
(pH 8.0)	0.10— 25.0	352
(pH 9.0)	0.10— 25.0	394
2-Naphthoic acid	0.08— 10.0	231
Benzoic acid	1.20— 40.0	225
4-Cl-Benzoic acid	0.10— 50.0	235
4-NO_2-Benzoic acid	0.10— 25.0	272
4-OH-Benzoic acid	0.20— 80.0	246
4-NH_2-Benzoic acid	0.60— 38.0	265
4-CN-Benzoic acid	0.15— 38.0	238
4-CH_3-Benzoic acid	0.12— 15.0	235
4-CH_3O-Benzoic acid	0.10— 25.0	248
4-CH_3CO-Benzoic acid	0.15— 30.0	254
4-$(CH_3)_2N$-Benzoic acid	0.10— 25.0	290

and wavelengths used for spectrophotometry are listed in Table I. All the experiments were performed at 15 °C.

Calculation——The characteristics of the three binding parameters in the new equation were examined quantitatively by the use of multiple regression analysis. The predictor variables included the van der Waals volume (V_W),[5] hydrophilic effect (V_H),[5] hydrophobic constant (π),[6] and pK_a.[7] Correlations, regression equations, and binding parameters were calculated on a JEOL digital computer, model JEC-7E.

Results and Discussion

Equation for the Binding of Drugs to Serum Albumin

Protein binding data are usually plotted according to Scatchard.[8] The binding equation of Langmuir's type (Eq. 1) fits only in cases where the Scatchard plot yields a straight line.

$$r=nKC/(1+KC) \tag{1}$$

where r is the average number of drugs per mol of protein, n is the number of binding sites, K is the binding constant, and C is the unbound drug concentration. In fact, the Scatchard plot often gives a curve (not a straight line) and in such cases, Karush's equation[1] (Eq. 2), assuming the presence of two kinds of binding sites, has been applied.

$$r=n_1K_1C/(1+K_1C)+n_2K_2C/(1+K_2C) \tag{2}$$

However, when the binding of drugs is measured over a wide range of drug concentrations, the plot does not intersect with the abscissa at a constant point (n_2), but the number of sites appears to increase with increase of binding. As an equation for such binding data, we propose the following equation (Eq. 3) obtained by modifying Eq. 1,

$$r=\{(n_0+\alpha C)KC\}/(1+KC) \tag{3}$$

where n_0 is the number of initial binding sites and α is a constant related to the increase in binding sites. Equation 1 can be transformed as follows:

$$r/(n-r)=KC \tag{4}$$

The left term of Eq. 4 represents the ratio of the number of binding sites occupied by drugs to

the number of sites left unbound. We call this ratio "degree of binding," and it is proportional to the drug concentration C (Eq. 4). Assuming that an elevation in the degree of binding of SA causes changes in the higher-order structure which give rise to an increase in the capacity for binding, Eq. 5 is obtained, where α is a constant related to the increase in the number of binding sites, which is proportional to the degree of binding, *i.e.*, proportional to C.

$$n=n_0+\alpha C \tag{5}$$

Such a proportionality may be valid in a certain concentration range. Supposing all sites (whether initial sites or not) possess the same instrinsic affinity for the drugs, substitution of Eq. 5 into Eq. 1 leads to Eq. 3 that we have proposed.

As a general measure of binding in the use of Eq. 3, the association constant $K_{1:1}$ for the binding of drug to BSA in 1 : 1 ratio is given by the following equation.

$$K_{1:1}=n_{1:1}K=(n_0+\alpha C_{1:1})K \tag{6}$$

In Eq. 6, $n_{1:1}$ is the binding capacity (number of sites) in the 1 : 1 binding, and the concentration of unbound drug, $C_{1:1}$ is expressed as follows from Eq. 3.

$$C_{1:1}=\{-n_0+1+\sqrt{(n_0-1)^2+4\alpha/K}\}/2\alpha \tag{7}$$

Goodness of Fit of the New Equation for Binding

The Scatchard plot (r/C *vs.* r) for the binding of salicylate ($0.5—120.0\times10^{-4}$ M) to BSA (2.08×10^{-5} M) determined by the use of the UC method is shown in Fig. 1. Each data point represents the mean of two determinations in our UC experiment. It is apparent that Karush's equation using four parameters does not fit, but Eq. 3 fits well (Fig. 1). Table II shows the binding parameters of this study, along with those in the literature. As can be seen from Table II, n_2 of Karush's equation varies greatly from 3.5 to 682. This suggests that n_2 is not constant but is probably dependent on the drug concentration. Therefore, it is inappropriate to use Karush's equation for the description of salicylate–BSA binding data. On the other hand, analysis of the data obtained from our experiments performed over a wide range of concentration by the use of Eq. 3 seems to give appropriate values of binding parameters, *i.e.*, n_0, K and $\log K_{1:1}$. Thus, it is considered that Eq. 3 can express the binding well in spite of being only a three-parameter equation.

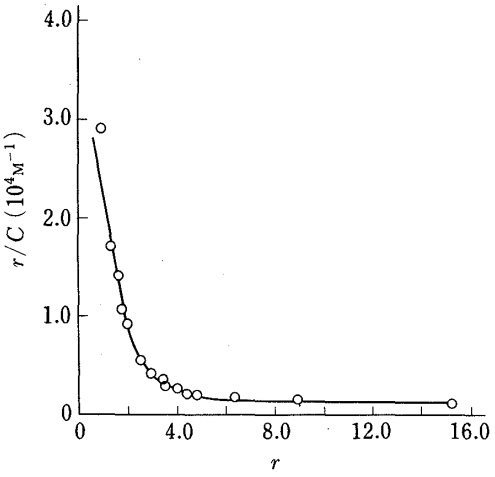

Fig. 1. Scatchard Plot for the Binding of Salicylate to BSA

Each data point represents the average of two experiments. The solid line is the calculated curve based on Eq. 3.

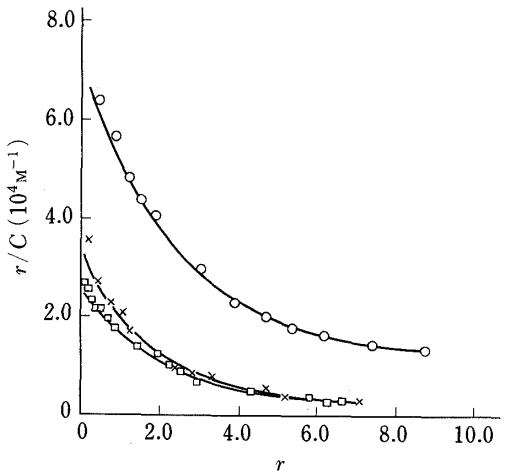

Fig. 2. Scatchard Plot for the Binding of HABCA to BSA at Various pH Values

○, pH 5.0; □, pH 7.0; ×, pH 8.0.
Each data point represents the average of two experiments. The solid lines are the calculated curves based on Eq. 3.

TABLE II. Parameters for Binding of Salicylate with Albumins

Salicylate concn. (10^{-4} M)	Protein concn. (10^{-4} M)	Karush's model (Eq. 2)			
		n_1	n_2	K_1 (10^4 M^{-1})	K_2 (10^2 M^{-1})
Other data					
0.21—26.7[a]	1.0 (BSA)	0.37	3.5	3.0	8.0
0.11—0.488[b]	5.88 (BSA)[d]	1.04	6.27	5.0	2.98
1.81—36.2[c]	5.0[e]	1.99[f]	682.0[f]	1.19[f]	0.012[f]
		2.51[g]	675.0[g]	0.95[g]	0.016[g]

Salicylate concn. (10^{-4} M)	Present model (Eq. 3)			
	n_0	K (10^4 M^{-1})	α (10^3)	$\log K_{1:1}$
This study				
0.5—120.0 (BSA)	2.54	1.28	1.10	4.52

a) Ref. 9. *b*) Ref. 10. *c*) Ref. 11. *d*) 4% BSA (MW: 68000). *e*) Human plasma (albumin concn. 5.0×10^{-4} M). *f*) By microultrafiltration. *g*) By spectrophotofluorometry.

TABLE III. Parameters for Binding of HABCA with BSA

pH	n_0	K (10^4 M^{-1})	α (10^3)	$\log K_{1:1}$
5.0	3.59	1.91	8.98	4.85
6.0	4.96	0.94	1.01	4.67
7.0	4.04	0.51	1.23	4.32
8.0	3.67	0.67	1.64	4.40
9.0	3.64	0.49	1.31	4.26

Next, the binding of 4′-hydroxyazobenzene-2-carboxylate (HABCA) to BSA was measured at various pH values. Examples of the Scatchard plots, along with the calculated curves based on Eq. 3, are shown in Fig. 2. Equation 3 well describes the binding data. In contrast, judging from the shape of the curves, it is apparent that Karush's equation does not fit the data, just as in the case of salicylate–BSA binding. The binding parameters calculated by using Eq. 3 are listed in Table III. The highest binding was observed at pH 5.0 and the binding generally decreased with elevation of pH. An outstandingly high value for α was obtained at pH 5.0. This may reflect the fact that at pH 5.0, approximately the isoelectric point for BSA, the protein takes a reduced shape which is sensitive to conformational changes induced by ligand binding. An expansion of the volume of BSA caused by the binding of dodecyl sulfate to the first high-affinity binding sites at pH 5.1 was reported by Katz and coworkers.[2,12]

Characteristics of Binding Parameters

The binding of 2-naphthoate, benzoate, and 4-substituted derivatives of benzoate to BSA was investigated to elucidate the characteristics of the binding parameters in Eq. 3. The binding parameters obtained are shown in Table IV.

Data for eleven compounds were examined by the use of multiple regression analysis to determine by what factors the binding to BSA is governed. In the analysis, n_0, $\log\alpha$, and $\log K_{1:1}$ as the criterion variables and van der Waals volume[5] (V_W), hydrophilic effect[5] (V_H),

TABLE IV. Parameters for Binding of 2-Naphthoate, Benzoate and 4-Substituted Derivatives of Benzoate with BSA

Compound	n_0	K (10^4 M^{-1})	α (10^3)	$\log K_{1:1}$	V_W[a]	V_H[a]	π[b]	pK_a[c]
2-Naphthoate	2.18	21.10	4.05	5.63	1.205	0.0	1.32	4.16
Benzoate	1.48	1.41	1.03	4.35	0.785	0.0	0.0	4.18
4-Cl-Benzoate	2.56	5.48	1.04	5.15	0.950	0.0	0.71	3.98
4-NO_2-Benzoate	2.01	2.93	1.13	4.78	0.994	0.31	−0.28	3.43
4-OH-Benzoate	2.67	0.24	0.60	3.83	0.853	0.32	−0.67	4.57
4-NH_2-Benzoate	2.16	0.07	0.57	3.25	0.884	0.59	−1.23	4.85
4-CN-Benzoate	2.41	0.68	0.69	4.22	0.962	0.44	−0.57	3.55
4-CH_3-Benzoate	1.80	3.73	0.87	4.83	0.939	0.0	0.56	4.37
4-CH_3O-Benzoate	2.08	4.17	1.00	4.94	1.020	0.29	−0.02	4.47
4-CH_3CO-Benzoate	2.85	1.27	1.40	4.57	1.114	0.56	−0.55	3.70
4-$(CH_3)_2N$-Benzoate	2.52	1.09	1.09	4.45	1.208	0.45	0.18	6.03[d]

a) Ref. 5. *b*) Ref. 6. *c*) Ref. 7. *d*) Ref. 13.

TABLE V. Multiple Regression Equations for Characterization of the Binding Parameters

$$\log\alpha = 1.298(\pm0.667)V_W - 0.527(\pm0.391)V_H + 1.870(\pm0.658) \quad (8)$$
$$n=11,\ r=0.874,\ \text{S.D.}=0.123$$

$$\log\alpha = 0.235(\pm0.157)\pi + 3.028(\pm0.108) \quad (9)$$
$$n=11,\ r=0.749,\ \text{S.D.}=0.159$$

$$\log K_{1:1} = 3.441(\pm1.081)V_W - 1.971(\pm0.626)V_H - 0.284(\pm0.205)pK_a + 2.883(\pm1.217)$$
$$n=11,\ r=0.969,\ \text{S.D.}=0.190 \quad (10)$$

$$\log K_{1:1} = 3.122(\pm1.532)V_W - 2.100(\pm0.898)V_H + 2.013(\pm1.513) \quad (11)$$
$$n=11,\ r=0.920,\ \text{S.D.}=0.283$$

$$\log K_{1:1} = 1.314(\pm1.211)V_W - 0.693(\pm0.222)\pi - 0.315(\pm0.206)pK_a + 4.629(\pm1.308)$$
$$n=11,\ r=0.969,\ \text{S.D.}=0.192 \quad (12)$$

$$\log K_{1:1} = 0.802(\pm0.253)\pi - 0.257(\pm0.253)pK_a + 5.692(\pm1.101) \quad (13)$$
$$n=11,\ r=0.938,\ \text{S.D.}=0.250$$

$$\log K_{1:1} = 0.798(\pm0.303)\pi + 4.585(\pm0.210) \quad (14)$$
$$n=11,\ r=0.893,\ \text{S.D.}=0.307$$

hydrophobic constant[6] (π) and pK_a[7] as the predictor variables (Table IV) were used; pK_a was utilized as a parameter for electrostatic property. The regression equations obtained are listed in Table V. In these equations, the figures in parentheses are the 95% confidence limits, n is the number of data points, r is the correlation coefficient, and S.D. is the standard deviation of errors.

As regards n_0, no equation where all the predictor variables were significant at $p<0.05$ was obtained. It is suggested that n_0 is not dependent on the physicochemical properties of individual drug molecules in a homologous series. In fact, the observed values of n_0 for the 11 derivatives (Table IV) are at roughly the same level; n_0 may be related to the number of initial affinity sites (located on the surface of the protein) for a series of congeners.

As regards $\log\alpha$, Eqs. 8 and 9 of Table V suggest that molecular size and hydrophobic character contribute to $\log\alpha$. This characteristic of $\log\alpha$ is consistent with our assumption that α is related to the structural changes of BSA caused by ligand binding.

Regarding $\log K_{1:1}$, Eqs. 10—14 were obtained as significant equations (Table V). It is clear from Eqs. 10 and 12 that $\log K_{1:1}$ is highly correlated to combinations of V_W, V_H, and pK_a and of V_W, π, and pK_a. Thus, the binding of benzoates to BSA is governed especially by

hydrophobic character in addition to molecular size and pK_a.

In conclusion, we propose a three-parameter equation for the description of ligand binding behavior to protein over a wide range of ligand concentration. The equation well describes the binding data of BSA with salicylate and HABCA, to which Karush's equation is not applicable. Multiple regression analysis of the binding data of BSA with 11 benzoates showed that the novel parameter α in our equation is significantly correlated with the molecular size and hydrophobicity of ligands; this supports our assumption that α is related to the increase in binding capacity of SA caused by binding of ligands.

In this paper, a comparison of our three-parameter equation with the stepwise model described by Klotz *et al.*[14] and Spector *et al.*[15] in the expression of binding data is not included; this remains to be done.

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