A Structure-Activity Relationship in the Binding of Multicharged Anionic Azo and Pyrene Dyes to Serum Albumin

Kiyofumi MURAKAMI,* Miki AKAMATSU,† and Takayuki SANO††
Department of Chemistry, Faculty of Education, Yamaguchi University, Yamaguchi 753
† Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kyoto 606-01
†† Department of Materials Science, Faculty of Science, Hiroshima University, Higashi-Hiroshima 724
(Received March 23, 1994)

The binding of 11 azo and pyrene dyes (which have 1 to 4 sulfonato groups) to bovine serum albumin at pH=7.0 and 25 °C has been studied by spectrophotometry, equilibrium dialysis, and/or ultrafiltration method. Binding parameters were determined assuming one or two classes of independent binding sites. By combining with data reported before, we examined the effects of hydrophobicity and net charge number of ligands on the binding parameters for a total of 19 dyes. The ratio of the binding ability to the primary sites to the total binding ability, a measure of binding specificity, was found to increase with an increase in the anionic charge number of ligands. The values of binding constant for nonspecific binding of the monoanionic dyes and for the primary binding of the multicharged dyes were found to have a strong correlation to both the hydrophobicity and the anionic charge number of ligands. A multiple regression analysis has derived the equation:

$$\log K = 0.86 \log P + 2.00 \sum_{i=1}^{m} \alpha_i - 0.81,$$

where K is a binding constant, P is the partition coefficient in 1-octanol/water system, and m and α_i are respectively the number of anionic groups and the degree of dissociation of the i-th group. From this equation, the contribution of electrostatic interactions due to a unit net charge on ligands to the standard free energy change of binding was estimated to be -11.4 kJ mol^{-1}. The role of electrostatic interactions has been discussed in detail.

To elucidate the origin of the inherent role of serum albumin as the carrier of many kinds of organic molecules, it is essential to study the relationship between the structure of the protein and ligands and their mutual binding properties. The method of quantitative structure–activity relationship,[1—3] which has been successfully applied to presumption of action mechanism and prediction of biological activity of many chemical compounds[4,5] and to design of medical substances,[6,7] has also been applied to albumin-ligand binding.

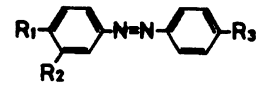
The conventional way to express activity has been the binding ability, which is defined as the reciprocal of free ligand concentration required for the formation of 1 : 1 complex.[8—10] However, this quantity is not a direct measure of binding constants and is liable to underestimate the binding ability for the case in which a strong binding to one site is predominant. Therefore, it is better to analyze overall binding isotherms and to use binding constant or standard free energy change of binding as the activity expression. It is also desirable for the determination of the binding isotherms to use the data of equilibrium dialysis or ultrafiltration, which measure directly the concentration of free ligand in equilibrium with the bound one, rather than the data from indirect methods such as spectrophotometric or fluorometric probe techniques.[11—15]

The structural character of ligands is often given by the partition coefficient of an acid form in 1-octanol/water system.[8—10] A linear relationship in the logarithm plot between reciprocal free-ligand concentration required for the 1 : 1 complex formation or binding constant and the partition coefficient is expected; that is, the higher the hydrophobicity of ligands, the more the binding ability of them to albumin molecules increases. On the other hand, the importance of electrostatic interactions due to ionized groups on ligands has sometimes been pointed out,[16—18] and their contribution to the free energy change of binding has also been estimated.[19—22] Their effect on binding properties has been studied from the viewpoint of quantitative structure–activity relationships.[12,23,24] However, at present, no clear-cut quantitative results concerning the role of the electrostatic interactions have yet been presented. This is because these studies have not necessarily succeeded in separating this effect from the other ones, due to the limitation to monoanionic ligands.

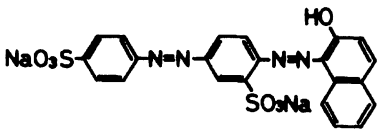
In the present study, the effects of net charge number of ligands upon the number of binding sites and a quantitative relationship between binding constant, ligand's hydrophobicity and charge number have been studied for bovine serum albumin (BSA), through determinations of binding isotherms and binding parameters for the binding of anionic azo and pyrene dyes, which have 1 to 4 sulfonato groups.

Experimental

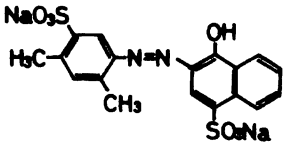
Materials. BSA samples (Fraction V and Fatty acid free <0.02%) were purchased from Armour. The former was defatted by means of charcoal treatment in an acid solution[25] and lyophilized. The latter was used without further purification. The molar concentration of BSA solutions was determined from the absorbance at 280 nm ($E_{1cm}^{1\%}$=



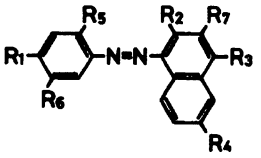
No.	Dye	R_1	R_2	R_3
1	Methyl Orange	SO_3Na	H	$N(CH_3)_2$
5	Metanil Yellow	H	SO_3Na	NHC_6H_5
6	Tropaeolin OO	SO_3Na	H	NHC_6H_5



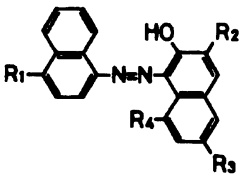
No.13 Biebrich Scarlet



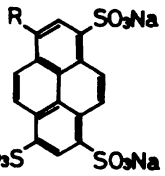
No.17 Ponceau SX



No.	Dye	R_1	R_2	R_3	R_4	R_5	R_6	R_7
2	Orange I	SO_3Na	H	OH	H	H	H	H
3	Orange II	SO_3Na	OH	H	H	H	H	H
4	Ponceau 4G	H	OH	H	SO_3Na	H	H	H
7	Sunset Yellow FCF	SO_3Na	OH	H	SO_3Na	H	H	H
8	Ponceau 2R	CH_3	OH	H	SO_3Na	CH_3	H	SO_3Na
12	Ponceau 3R	CH_3	OH	H	SO_3Na	CH_3	CH_3	SO_3Na



No.	Dye	R_1	R_2	R_3	R_4
9	Acid Red 13	SO_3Na	H	SO_3Na	H
10	Bordeaux Red	H	SO_3Na	SO_3Na	H
11	Crystal Scarlet	H	H	SO_3Na	SO_3Na
15	Amaranth	SO_3Na	SO_3Na	SO_3Na	H
16	Ponceau 4R	SO_3Na	H	SO_3Na	SO_3Na
19	Ponceau 6R	SO_3Na	SO_3Na	SO_3Na	SO_3Na



No.	Dye	R
14	Solvent Green 7	OH
18	Tetra sodium 1,3,6,8-pyrene tetrasulfonate	SO_3Na

Fig. 1. Chemical structure of dyes.

$6.6^{26)}$), assuming a molecular weight of 67 kDa (1 Da= 1.66054×10^{-27} kg). Sodium 3-[4-(phenylamino)phenylazo]-benzenesulfonate (Metanil Yellow), sodium 4-[4-(phenylamino)phenylazo]benzenesulfonate (Tropaeolin OO), disodium 3-hydroxy-4-(2,4-dimethylphenylazo)-2,7-naphthalenedisulfonate (Ponceau 2R), disodium 3-hydroxy-4-(2,4,5-trimethylphenylazo)-2,7-naphthalenedisulfonate (Ponceau 3R) and disodium 3-(2,4-dimethyl-5-sulfonatophenylazo)-4-hydroxy-1-naphthalenesulfonate (Ponceau SX) were purchased from Wako Pure Chemical Industries. Disodium 4-(2-hydroxy-1-naphthylazo)-5-(4-sulfonatophenylazo)benzenesulfonate (Biebrich Scarlet) was purchased from Chroma. Disodium 7-hydroxy-8-(1-naphthylazo)-1,3-naphthalenedisulfonate (Crystal Scarlet) was purchased from Aldrich Chemical Company. Disodium 3-hydroxy-4-(1-naphthylazo)-2,7-naphthalenedisulfonate (Bordeaux Red) was purchased from Tokyo Chemical Industry Co., Ltd. Disodium 6-hydroxy-5-(4-sulfonatonaphthylazo)-2-naphthalenesulfonate (Acid Red 13) was prepared by coupling sodium 4-amino-1-naphthalenesulfonate with sodium 6-hydroxy-2-naphthalenesulfonate. All of these samples were purified by three recrystallizations from an aqueous ethanol solution, and were dried at 110 °C in a vacuum for 20 h. Tetrasodium 1,3,6,8-pyrenetetrasulfonate and trisodium 8-hydroxy-1,3,6-pyrenetrisulfonate (Solvent Green 7) were purchased from Eastman Kodak Company, and used without further purification. pK_a values of the hydroxyl group of these dyes have been determined by a series of spectrophotometric titration. From the concentration dependence of absorption spectrum at pH=7.0, molar extinction coefficients of these dyes were also determined. Figure 1 shows the chemical structures of the dyes which were supplied to the present analysis of a structure–activity relationship on albumin binding. All the other chemicals used were reagent-grade. All the sam-

ple solutions were prepared in a 0.1 mol dm^{-3} Na_2HPO_4–NaH_2PO_4 buffer of pH=7.00±0.02.

Methods. The extent of binding of ligands was determined by the equilibrium dialysis and/or ultrafiltration techniques. The details of the equilibrium dialysis technique have been reported elsewhere.[18] The apparatus and method for the ultrafiltration were similar to that reported by Imamura et al.[27] A 5 ml sample of BSA-dye solution placed inside a dialysis tubing (Visking 18/32) was centrifuged for 2 h at 4500 rpm using Marusan 45-CFS centrifuge. During the centrifugation, the temperature of the sample was maintained at 25 °C. An aliquot of the ultrafiltrate was supplied to spectrophotometric determination of free ligand concentration. The extent of binding was estimated by taking into account the amount of absorbed dye on the tubing, which was determined by a series of blank measurements without the protein. Spectrophotometric measurements were carried out with Shimadzu UV-200S and Hitachi U-2000 spectrophotometers. All the experiments were performed at 25±0.2°C. Nonlinear regression analysis for the evaluation of binding parameters was carried out with a program of Nakagawa and Oyanagi[28] on an Acos-850 computer system (NEC Corporation). log P values of the dyes in 1-octanol/water system were calculated using a program of CLOGP version 3.54.

Results and Discussion

Binding Isotherms and Binding Parameters. Figure 2 illustrates Scatchard plots[29] of the binding of Metanil Yellow, Bordeaux Red, and Ponceau SX to BSA at pH=7.0 and 25 °C, in which $\bar{\nu}$ and L are the average number of bound ligand per one molecule of the protein and the free ligand concentration, respectively. The linear dependence of $\bar{\nu}L^{-1}$ on $\bar{\nu}$ for BSA-Metanil Yellow system shows the existence of one class of equivalent binding sites. This type of binding is called nonspecific binding. On the other hand, the curved plots for the latter two dyes show that BSA has at least two classes of binding sites. Assuming that all of the binding sites are independent of each other, $\bar{\nu}$ can be expressed as[30]

$$\bar{\nu} = \sum_{i=1}^{n_c} \frac{n_i K_i L}{1 + K_i L}, \quad (1)$$

where n_i and K_i are the number of binding sites and the binding constant, respectively. The subscript i refers to the i-th class of binding sites, and n_c is the number of classes. The data were fitted to Eq. 1 by the nonlinear regression analysis for n_c=1 or 2. The determined values of the binding parameters are listed in Table 1. The solid curves in Fig. 2 represent the theoretical ones calculated using these values. The values of log P and the average number of anionic charges on a ligand, which was estimated as the sum of degrees of dissociation of ionizable groups in the present conditions ($\sum_{i=1}^{m} \alpha_i$), are also listed in this table. Ponceau SX is exceptionally classified as a trianionic ligand, whereas it is dianionic in the free state under the present conditions. This is because the hydroxyl group of this dye was found from a spectrophotometric titration to be deprotonated in the bound state at the primary site.

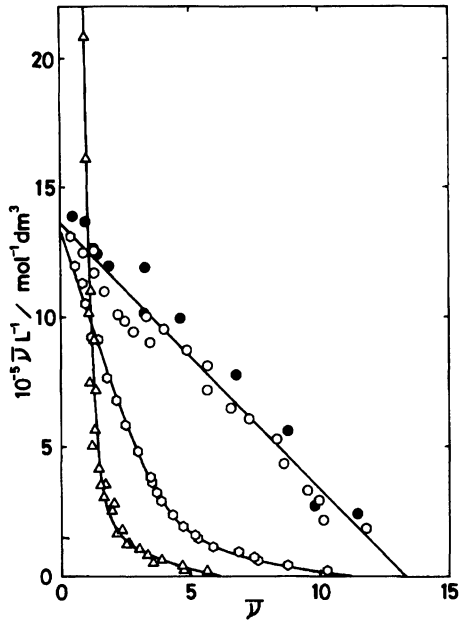


Fig. 2. Scatchard plots of the binding of Metanil Yellow (circle), Bordeaux Red (hexagon), and Ponceau SX (triangle) to BSA at 25 °C and pH=7.0. The open symbols show the data of equilibrium dialysis, and the filled ones show those of ultrafiltration. The solid lines represent the theoretical curves calculated using the values of the binding parameters listed in Table 1.

Binding Specificity. As for the monoanionic ligands, there are two cases of binding: (1) a small number of primary sites and a large number of nonspecific secondary sites coexist, (2) the typical nonspecific case in which there exists only one class of a large number of binding sites. From the similarity in the number of binding sites, the binding parameters of the second case are listed as the secondary sites in Table 1. For the ligands having more than one anionic charges, there commonly exist two classes of binding sites. However, the number of the secondary sites has a tendency to decrease with an increase in the number of anionic charges of ligands.

Figure 3 illustrates the dependence of the number of sites on the anionic charge number of ligands. This shows that the total number of binding sites extensively decreases with an increase in the number of anionic charges; this is due to the reduction of the number of the secondary sites. The ratio of the binding ability to the primary sites to the total binding ability;

$$RS1 = \frac{n_1 K_1}{n_1 K_1 + n_2 K_2}, \quad (2)$$

shows the relative importance of the primary sites in the overall binding of a ligand, and may be used as a measure of the degree of binding specificity. The insert in Fig. 3 shows that the ligand-binding specificity strongly increases with an increase in the number of an-

Table 1. Binding Parameters

No.	Dye	n_1	$10^{-4}\ K_1$ / $mol^{-1}\ dm^3$	n_2	$10^{-4}\ K_2$ / $mol^{-1}\ dm^3$	log P	$\sum_{i=1}^{m} \alpha_i$	Reference
1	Methyl Orange[a)]	—	—	17	0.34	2.633	1	31,32,33
2	Orange I	—	—	15.6	0.97	3.274	1	34
3	Orange II	2.01	18.0	20.8	1.13	3.274	1	35
4	Ponceau 4G	1.98	17.3	21.4	1.06	3.274	1	35
5	Metanil Yellow	—	—	13	10	4.259	1	This work
6	Tropaeolin OO	0.61	270	18	3.3	4.259	1	This work
7	Sunset Yellow FCF	1.6	6.2	3.2	0.60	1.417	2	35
8	Ponceau 2R	3.3	6.9	5.4	1.2	2.415	2	This work
9	Acid Red 13	3.5	19	5.0	1.7	2.591	2	This work
10	Bordeaux Red	3.84	33.1	7.57	0.85	2.591	2	This work
11	Crystal Scarlet	3.7	32	8.9	0.20	2.591	2	This work
12	Ponceau 3R	0.63	51	7.0	3.2	2.864	2	This work
13	Biebrich Scarlet	3.3	130	7.8	4.9	3.125	2	This work
14	Solvent Green 7	1.6	3.2	2.8	0.39	−1.228	3.36	This work
15	Amaranth	0.9	60	2.4	1.2	0.734	3	18
16	Ponceau 4R	1.0	140	1.8	1.8	0.734	3	18
17	Ponceau SX	1.2	690	4.8	2.1	2.415	3	This work
18	Tetrasodium 1,3,6,8-pyrene-tetrasulfonate	1.5	3.6	1.5	0.16	−2.478	4	This work
19	Ponceau 6R	1.0	960	1.4	0.8	−1.123	4	18

a) The values of the binding parameters of this dye are the averages of the cited reference data.

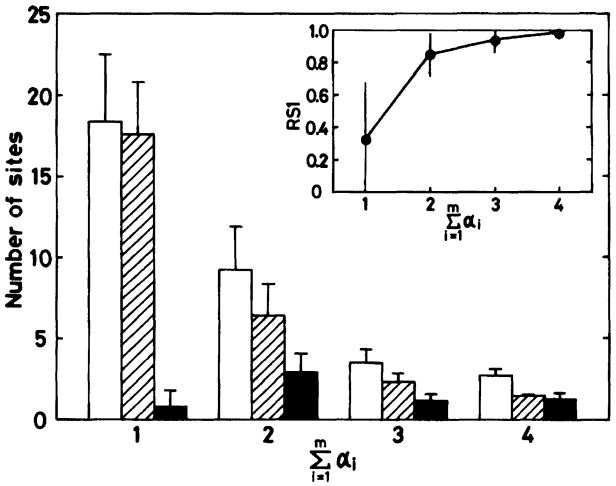


Fig. 3. Dependence of the number of binding sites on $\sum_{i=1}^{m} \alpha_i$: the total number of binding sites (open square), the number of primary sites (filled square), and the number of secondary sites (shaded square). The insert shows the dependence of RS1 defined by Eq. 2 on $\sum_{i=1}^{m} \alpha_i$.

ionic charges on ligands; it reaches almost 100% for the tetranionic ligands.

One of the origins of this tendency is obviously a relative increment of the number of primary sites due to a reduction of the number of secondary sites. A possible mechanism of the reduction for a dianionic ligand has been proposed by Murakami.[35] That is, nonspecific binding sites are composed of a hydrophobic region and at least one positively-charged residue and one of the sulfonato groups of the ligand prohibits an approach of its hydrophobic part to the hydrophobic region of the sites, resulting in a considerable weakening of hydrophobic interaction. This mechanism seems to apply also to the cases of multi-charged ligands in Fig. 3. On the other hand, one can see from Table 1 that hydrophobicity of ligands does not directly relate to the number of binding sites.

Multiple Regression Analysis of Binding Constants. From Fig. 4, the plots of log K vs. log P, one can see that the value of log K in each group of charge numbers and binding classes linearly increases with increasing values of log P, and the slopes are similar, while those of the secondary sites for the multicharged ligands seems somewhat reduced. If one pays attention to the nonspecific secondary sites of monoanionic ligands (which exist for all the monoanionic ligands) and the primary sites of multicharged ligands, it is further found that the value of log K also increases linearly with increasing number of charges of ligands. On the basis of this observation, we analyzed these data by a multiple-regression analysis, using log P and $\sum_{i=1}^{m} \alpha_i$ as independent parameters. It would be relevant here to note the reason of using log P of the neutral forms of ligands, which are neither the most abandant forms (the anionic forms) nor the forms transferring from water to 1-octanol phase (the ion-pairs with the salt cations[36—38]) under the present conditions. In order to consider the contribution of the net charges of ligands to log K as an independent term of $\sum_{i=1}^{m} \alpha_i$, we must use log P of the neutral forms rather than that of the latter two forms,

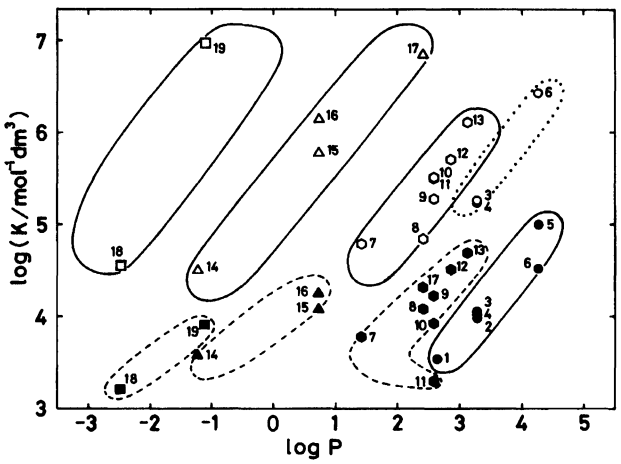


Fig. 4. Plots of $\log K$ vs. $\log P$ for monoanionic (circle), dianionic (hexagon), trianionic (triangle), and tetranionic (square) ligands. The designated numbers mean the dye numbers which are listed in the first column of Table 1. The open and filled symbols show the data for the primary and secondary sites, respectively. The data encircled by solid lines have been supplied to the present analysis of a quantitative structure-activity relationship (see text).

which already contain the effect of charges of the ligands. The result is

$$\begin{aligned} \log K = {} & 0.861 \log P + 1.997 \sum_{i=1}^{m} \alpha_i - 0.805 \\ & (0.206) \qquad (0.378) \qquad (1.193) \\ & nd = 19, \quad s = 0.113, \\ & r = 0.945, \quad F = 66.57, \end{aligned} \tag{3}$$

where nd, s, r, and F are the number of analyzed data, the standard deviation, the multiple correlation coefficient, and the F-value, respectively. The numerical values in the parentheses show the 95% confidence intervals of the parameters. The values of the partial correlation coefficients of $\log P$ and $\sum_{i=1}^{m} \alpha_i$ became 0.912 and 0.942, respectively. Figure 5 shows the plot of observed $\log K$ vs. the value calculated with Eq. 3. These results show that the observed data are well interpreted by the equation. If the data set in which Ponceau SX is counted as dianionic (charge number in free state) was used for the analysis, the resultant values of the statistical parameters (s=0.226, r=0.877, F=29.4) were less satisfactory than Eq. 3. This also confirms that Ponceau SX is in the trianionic form in the bound state at the primary site. This fact is very interesting from the view points of presumption of charged states of bound ligands and effectiveness of charges of ligands for the stabilization of the complexes. That is, one can presume their ionization states from Eq. 3 using the data of their binding constant and hydrophobicity. Further, it can be seen from Fig. 4 that the binding constant of the secondary sites for the multicharged ligands depends also on the number of charges on ligands. From this, one can also presume that Ponceau SX (Data No. 17 in Fig. 4) bound to

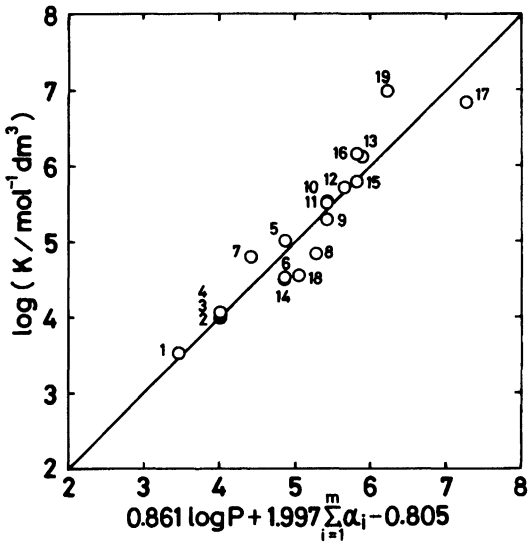


Fig. 5. Plot of observed $\log K$ vs. calculated one from Eq. 3.

the secondary sites exists in the dianionic form, unlike the primary site, and one of the two sulfonato groups of Crystal Scarlet (Data No. 11 in Fig. 4) bound to the secondary sites does not contribute to the stabilization of the complexes.

Role of Electrostatic Interactions on Ligand Binding. In many studies concerning ligand-binding to serum albumin, much attention has been paid to the role of hydrophobic interaction.[8—10,39] However, Eq. 3 shows that the electrostatic interaction due to net charges of ligands in addition to the hydrophobic one plays an important role in this binding. In terms of standard free energy change of binding, $\log K$ may be expressed as

$$\log K = -\frac{\log e}{RT} \Delta G^\circ. \tag{4}$$

According to Hansch and Helmer,[40] this may be factored, as a first approximation, into some contributions:

$$\begin{aligned} \log K = -\frac{\log e}{RT} \Big(& \Delta G^\circ_{(\text{hydrophobic})} \\ & + \Delta G^\circ_{(\text{net-charge electrostatic})} \\ & + \Delta G^\circ_{(\text{other electrostatic})} \\ & + \Delta G^\circ_{(\text{other})} \Big), \end{aligned} \tag{5}$$

where $\Delta G^\circ_{(\text{hydrophobic})}$ means the contribution of a hydrophobic interactions, $\Delta G^\circ_{(\text{net-charge electrostatic})}$ and $\Delta G^\circ_{(\text{other electrostatic})}$ are the contribution of electrostatic interactions arising from the net-charges on ligands and that of other electrostatic interactions, respectively. The last term ($\Delta G^\circ_{(\text{other})}$) means the contribution of other effects such as steric factor. The second term of Eq. 3 corresponds to the second term of Eq. 5. Therefore, one can estimate the contribution of a unit net-charge on ligands to the standard free energy change to be -11.4 kJ mol^{-1}. This value is similar to that reported for other systems;[19—22] it may

be more reliable since the present procedure has separated the electrostatic effect due to net charges from the other ones. Using this value, one can evaluate the percentage of the contribution of the net-charge electrostatic interactions to the standard free energy change as 40—50% for the nonspecific binding of the monoanionic dyes, 70—80% for the primary binding of the dianionic dyes, 90—100% for the primary binding of the trianionic dyes, and almost 100% for the primary binding of the tetranionic dyes. That is, as the number of anionic charges of ligands increases, the electrostatic interactions become more and more important. The fact that charges on a ligand having more than one sulfonato group contribute to the standard free energy change in an additive manner (Eq. 3) shows that the electrostatic interactions due to the charges operate independently of each other. These binding properties of the multianionic ligands suggest that their primary sites are composed of several cationic residues, and their relative arrangement in space is flexible to allow effective electrostatic bonds with the anionic charges of ligands. This expectation agrees well with the result of the recent crystallographic study by He and Carter,[41] that is, two principal ligand-binding regions have been shown to be hydrophobic cavities containing three or four cationic residues at their entrances.

At the present stage, application of Eq. 3 is limited to the primary binding of multianionic ligands and the nonspecific binding of monoanionic ligands. The appearance of the specific binding for monoanionic ligands seems to be related to some steric factor, because it is governed by a slight difference in the position of the functional groups (compare the data of Orange I and Orange II and those of Metanil Yellow and Tropaeolin OO). In order to modify Eq. 3 so as to describe the specific binding for the monoanionic ligands as well, it would therefore be necessary to introduce another new parameter which takes into account the steric factor. This will be achieved by another set of binding studies, and the clarification of the relation between these results and the information about binding regions and their tertiary structures is an important subject for future research.

The authors wish to thank emeritus Professor Toshio Fujita of Kyoto University for his helpful advice about this work. They also wish to thank Mr. Susumu Kubota, Ms. Keiko Suehiro, Ms. Yumiko Uchiyama, and Ms. Yoshiko Hakuno of Yamaguchi University for their assistance with the measurements.

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