Cooperative Ligand Binding to Globular Protein(II). Binding of Dianionic Azo Dyes to Hen Egg White Lysozyme

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The binding of five dianionic azo dyes: disodium 3-hydroxy-4-(2,4,5-trimethylphenylazo or 1-naphthylazo)-2,7-naphthalenedisulfonate, disodium 6-hydroxy-5-(4-sulfonatophenylazo or 4-sulfonato-1-naphthylazo)-2-naphthalenesulfonate, and disodium 2-(2-hydroxy-1-naphthylazo)-5-(4-sulfonatophenylazo)benzenesulfonate, to hen egg white lysozyme has been studied by an equilibrium dialysis method at pH = 7.0 (0.1 mol dm^{-3} phosphate buffer) and 25 °C. The measured binding isotherms showed that all of these dyes cooperatively bind to the protein, but the degree of the cooperativity differs from dye to dye. From the result that the maximum binding number (6) was about half of those for monoanionic dyes, these dye molecules were found to bind bifunctionally with their two sulfonato groups to the cationic residue pairs on the protein. The binding isotherms were analyzed on the basis of the stepwise binding scheme by a nonlinear regression analysis, and all the binding constants have been evaluated. The differences in the cooperative-binding behavior between dyes have been examined from the viewpoints of the structural difference between dyes and of the distribution of cationic residues on the protein. It was found that the hydrophobicity of each ligand plays a central role for the cooperativity, and that the orientation of the hydrophobic part of ligand in bound state as well as the distribution of charged residues on the protein also affect the binding behavior.

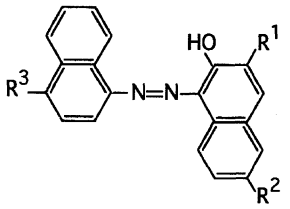
Cooperative binding of amphiphiles such as surfactants and ionic dyes to proteins has attracted the attention of many researchers from the viewpoints of colloidal and denaturation properties of the proteins.[1—3] From recent studies for surfactant systems,[4—11] scientists have recognized that both the electrostatic specific binding between the charged group of ligand and the oppositely charged residues on protein and the hydrophobic interaction between bound ligands play essential roles in the cooperative binding behavior.

In a previous paper, Murakami has reported the cooperative binding of two kinds of monoanionic azo dyes to lysozyme at neutral pH region.[12] The aromatic azo dyes were found to bind in one-stage cooperativity, while the dyes to which alkyl chains are attached bind in two-stage cooperativity with the same binding number. In the latter case, the bilayer-like structure has been proposed from the consideration of the binding number and the values of the binding parameters. Quite recently, Murakami has explored a statistical mechanical theory based on the simple geometrical model for the analysis of cooperative binding of monoionic ligands to globular protein, and has applied it to the binding of several anionic ligands to hen egg white lysozyme.[13] The evaluated model parameters were found to be consistent with the structures of the ligands and the protein and with the nature of ligand–ligand hydrophobic interaction, and the real image of the cooperativity has become pictured by combining the further detailed information such as species fraction, variance, and binding site identification. All of these studies are, however, concerned with singly-charged ligands. On the other hand, it is very interesting to learn how multicharged ligands cooperatively bind to globular proteins.

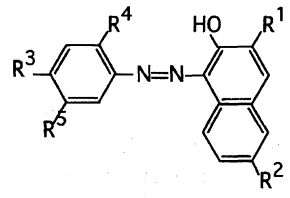
The present paper describes the binding of five dianionic azo dyes, whose backbone structures or position of charged groups are different, to hen egg white lysozyme. We will show the roles of the hydrophobicity of ligand and the charge distributions on both of the ligand and the protein in the binding mechanism.

Experimental

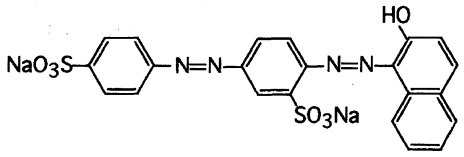
Materials. Hen egg white lysozyme (six times recrystallized) was purchased from Seikagaku Kogyo Co., Ltd. and was used without further purification. The molar concentration of lysozyme samples was measured from the absorbance at 280 nm using the molar extinction coefficient of 3.77×10^4 mol^{-1} dm^3 cm^{-1}.[14] Disodium 6-hydroxy-5-(4-sulfonatophenylazo)-2-naphthalenesulfonate (Sunset Yellow FCF) and disodium 3-hydroxy-4-(1-naphthylazo)-2,7-naphthalenedisulfonate (Bordeaux Red) were purchased from Tokyo Chemical Industry Co., Ltd. Disodium 3-hydroxy-4-(2,4,5-trimethylphenylazo)-2,7-naphthalenedisulfonate (Ponceau 3R) was purchased from Wako Pure Chemical Industries. Disodium 2-(2-hydroxy-1-naphthylazo)-5-(4-sulfonatophenylazo)benzenesulfonate (Biebrich Scarlet) was purchased from Chroma. Disodium 6-hydroxy-5-(4-sulfonato-1-naphthylazo)-2-naphthalenesulfonate (Acid Red 13) was prepared by coupling sodium 4-amino-1-naphthalenesulfonate with sodium 6-hydroxy-2-naphthalenesulfonate. These dyes were purified by three recrystallizations from an aqueous sodium acetate solution and by additional three recrystallizations from an aqueous ethanol solution, they were dried at 110 °C in a vacuum for 20 h. Figure 1 shows the chemical structure of these dyes. Molar extinction coefficients of these dyes were measured from the concentration dependence of absorption spectrum at pH = 7.0, and the values were used for the measurements of free-



Dye	R^1	R^2	R^3
Bordeaux Red	SO_3Na	SO_3Na	H
Acid Red 13	H	SO_3Na	SO_3Na



Dye	R^1	R^2	R^3	R^4	R^5
Ponceau 3R	SO_3Na	SO_3Na	CH_3	CH_3	CH_3
Sunset Yellow FCF	H	SO_3Na	SO_3Na	H	H



Biebrich Scarlet

Fig. 1. Chemical structure of dyes.

ligand concentrations. All the sample solutions were prepared in a 0.1 mol dm^{-3} phosphate buffer of pH = 7.00±0.02.

Methods. The extent of binding was measured using the equilibrium dialysis technique, as in the previous report.[12] Spectrophotometric measurements were done with Hitachi U-2000 spectrophotometer. All the experiments were done at 25±0.2°C. The log *P* values of the dyes in 1-octanol/water system were calculated using a program of Maclog *P* 2.0.3 (BioByte Corp.).

Results

Figure 2 shows the $\overline{\nu}$ vs. log [L] plot (Fig. 2(A)) and Scatchard plot[15] (Fig. 2(B)) for the binding of the five dianionic azo dyes to hen egg white lysozyme at pH = 7.0 and 25 °C. It can be seen from these figures that all the five dyes cooperatively bind to lysozyme, but the degree of cooperativity differs from dye to dye. Especially, the cooperative effect in the binding of Sunset Yellow FCF is small compared with those for the other systems. The maximum binding number was estimated from the asymptotic values of $\overline{\nu}$ as 6 for these dyes.

Based on the maximum binding number, the following stepwise binding scheme has been assumed

$$\begin{aligned} &P + L \rightleftarrows PL, \\ &PL + L \rightleftarrows PL_2, \\ &\quad\cdots\cdots \\ &PL_5 + L \rightleftarrows PL_6, \end{aligned} \tag{1}$$

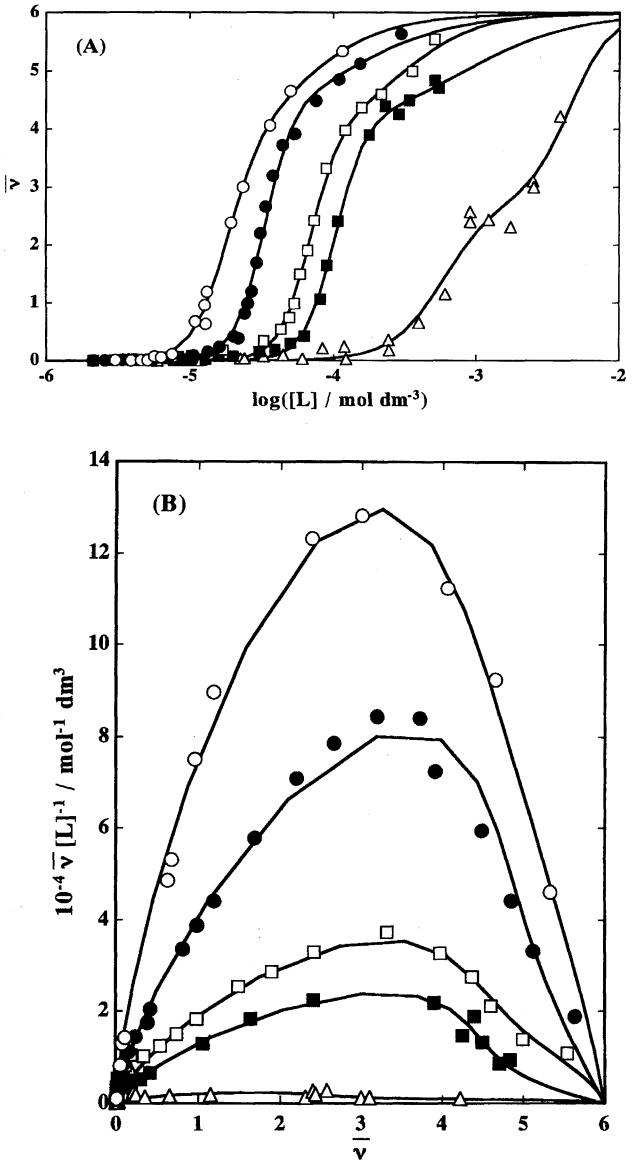


Fig. 2. Binding isotherms for the binding of dianionic azo dyes to hen egg white lysozyme at pH = 7.0 and 25 °C. (A): $\overline{\nu}$ vs. log [L] plots and (B): Scatchard plots. The symbols denote Biebrich Scarlet (open circle), Bordeaux Red (filled circle), Acid Red 13 (open square), Ponceau 3R (filled square), and Sunset Yellow FCF (triangle). The solid curves represent the theoretical ones calcultated using Eq. 3 and the values of the parameters listed in Table 1.

where P, L, and PL_i show free protein, free ligand, and complex species, respectively. The binding constants for these steps are expressed by

$$\begin{aligned} K_1 &= [PL]/[P][L], \\ K_2 &= [PL_2]/[PL][L], \\ &\cdots\cdots \\ K_6 &= [PL_6]/[PL_5][L]. \end{aligned} \tag{2}$$

Using these equations, the average number of bound ligands per molecule of protein can be expressed by[16]

$$\bar{\nu} = \frac{K_1[\mathrm{L}] + 2K_1K_2[\mathrm{L}]^2 + \cdots + 6K_1K_2\cdots K_6[\mathrm{L}]^6}{1 + K_1[\mathrm{L}] + K_1K_2[\mathrm{L}]^2 + \cdots + K_1K_2\cdots K_6[\mathrm{L}]^6}. \quad (3)$$

The data were fitted to Eq. 3 by using a nonlinear regression analysis, considering the binding constants K_1, K_2, $\cdots$, and K_6 as independent parameters. The values of the binding constants evaluated and the corresponding standard free energy changes of binding are listed in Table 1. As can be seen in Fig. 2, the observed data fall on the theoretical curves calculated using these values. The dependence of $\log K_i$ on the binding step is shown in Fig. 3. From each term in Eq. 3, the species fraction may be calculated as

$$SF_i = \frac{K_1K_2\cdots K_i[\mathrm{L}]^i}{1 + K_1[\mathrm{L}] + K_1K_2[\mathrm{L}]^2 + \cdots + K_1K_2\cdots K_6[\mathrm{L}]^6}. \quad (4)$$

Further, the variance in $\bar{\nu}$ can be calculated by[17)]

$$\sigma^2 = \overline{(\nu - \bar{\nu})^2} = [\mathrm{L}](\partial\bar{\nu}/\partial[\mathrm{L}]). \quad (5)$$

The concentration dependence of the species fractions and the square root of variance in $\bar{\nu}$ for each dye is shown in Fig. 4.

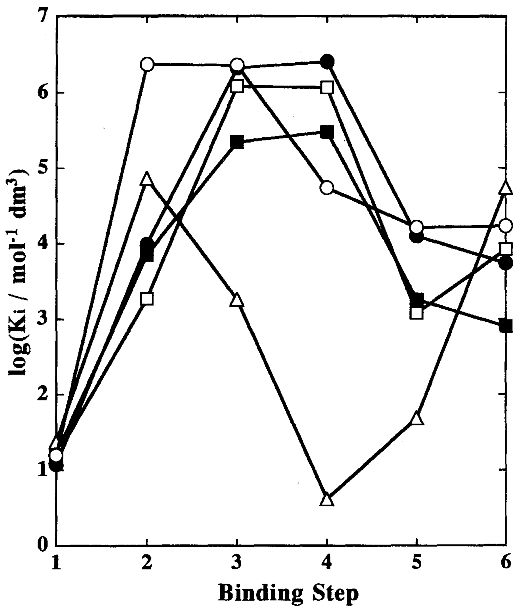


Fig. 3. Dependence of $\log K_i$ on binding step, for Biebrich Scarlet (open circle), Bordeaux Red (filled circle), Acid Red 13 (open square), Ponceau 3R (filled square), and Sunset Yellow FCF (triangle).

Table 1 shows that K_1 takes similar values of the order of 10 $\mathrm{mol^{-1}\,dm^3}$ for these dyes, indicating that all the dye species are interacting with the binding sites with similar magnitudes of binding force in the first step. Figure 3 shows that there is the common tendency for the later steps that K_i increases at the early steps, reaches a maximum, and then decreases toward the last step, but the maximum point of K_i lies at the different steps: the second step for Sunset Yellow FCF, the second or third step for Biebrich Scarlet, and the third or fourth step for the other dyes. In the case of Sunset Yellow FCF, K_i exceptionally reincreases after the fourth step. Figure 4 shows that a species which binds only one ligand molecule is absent at any concentration for all dyes, and that the later the step at which K_i takes the maximum value becomes, the larger the numbers of bound ligands of the existing intermediate species become. In the case of Sunset Yellow FCF, only the two-ligand and three-ligand species are present as the intermediate species. The variance has one peak at the concentration at which the intermediate species appear at the expense of the bare protein and the other peak (Sunset Yellow FCF) or the shoulder (Ponceau 3R, Acid Red 13, Bordeaux Red, and Biebrich Scarlet) at the higher concentration region at which the full-loaded protein develops with the disappearance of the intermediates.

Discussion

The order of magnitude of K_1 (Table 1) is appropriate to the electrostatic bond between the sulfonato groups on the dye molecules and the positively charged groups on the protein under a somewhat reduced rocal dielectric constant.[18,19)] The maximum binding number 6 is about half of the binding number (10—14) for the binding of the monoanionic ligands such as anionic surfactants and anionic dyes at the neutral pH region.[9,10,12)] This fact strongly suggests that these dianionic dye molecules bifunctionally bind to cationic-residue pairs on the protein with their two sulfonato groups by the electrostatic interactions. This state may electrostatically be more stable than the state in which only one sulfonato group is bound, if the distances between positively charged residues on the protein are in agreement with the distances between sulfonato groups on the dye molecules. The distances between sulfonato groups of the dyes are about 7.5 Å

Table 1. Binding Parameters for the Binding of the Dianionic Azo Dyes to Hen Egg White Lysozyme at pH = 7.0 and 25 °C[a)]

Step	Sunset Yellow FCF		Ponceau 3R		Acid Red 13		Bordeaux Red		Biebrich Scarlet	
	$10^{-4}K_i$	$-\Delta G_i°$	$10^{-4}K_i$	$-\Delta G_i°$	$10^{-4}K_i$	$-\Delta G_i°$	$10^{-4}K_i$	$-\Delta G_i°$	$10^{-4}K_i$	$-\Delta G_i°$
	$\mathrm{mol^{-1}\,dm^3}$	$\mathrm{kJ\,mol^{-1}}$	$\mathrm{mol^{-1}\,dm^3}$	$\mathrm{kJ\,mol^{-1}}$	$\mathrm{mol^{-1}\,dm^3}$	$\mathrm{kJ\,mol^{-1}}$	$\mathrm{mol^{-1}\,dm^3}$	$\mathrm{kJ\,mol^{-1}}$	$\mathrm{mol^{-1}\,dm^3}$	$\mathrm{kJ\,mol^{-1}}$
1	0.0024	7.8	0.0016	6.9	0.0015	6.7	0.0012	6.2	0.0016	6.8
2	7.31	27.8	0.71	22.0	0.187	18.7	0.970	22.8	237.	36.4
3	0.183	18.6	22.2	30.5	122.	34.7	213.	36.1	231.	36.3
4	0.0004	3.5	30.5	31.3	118.	34.7	256.	36.6	5.51	27.1
5	0.0049	9.6	0.182	18.6	0.121	17.6	1.25	23.4	1.63	24.0
6	5.49	27.1	0.080	16.6	0.835	22.4	0.55	21.3	1.69	24.1

a) The errors of the binding constants were estimated as about 20% of those values.

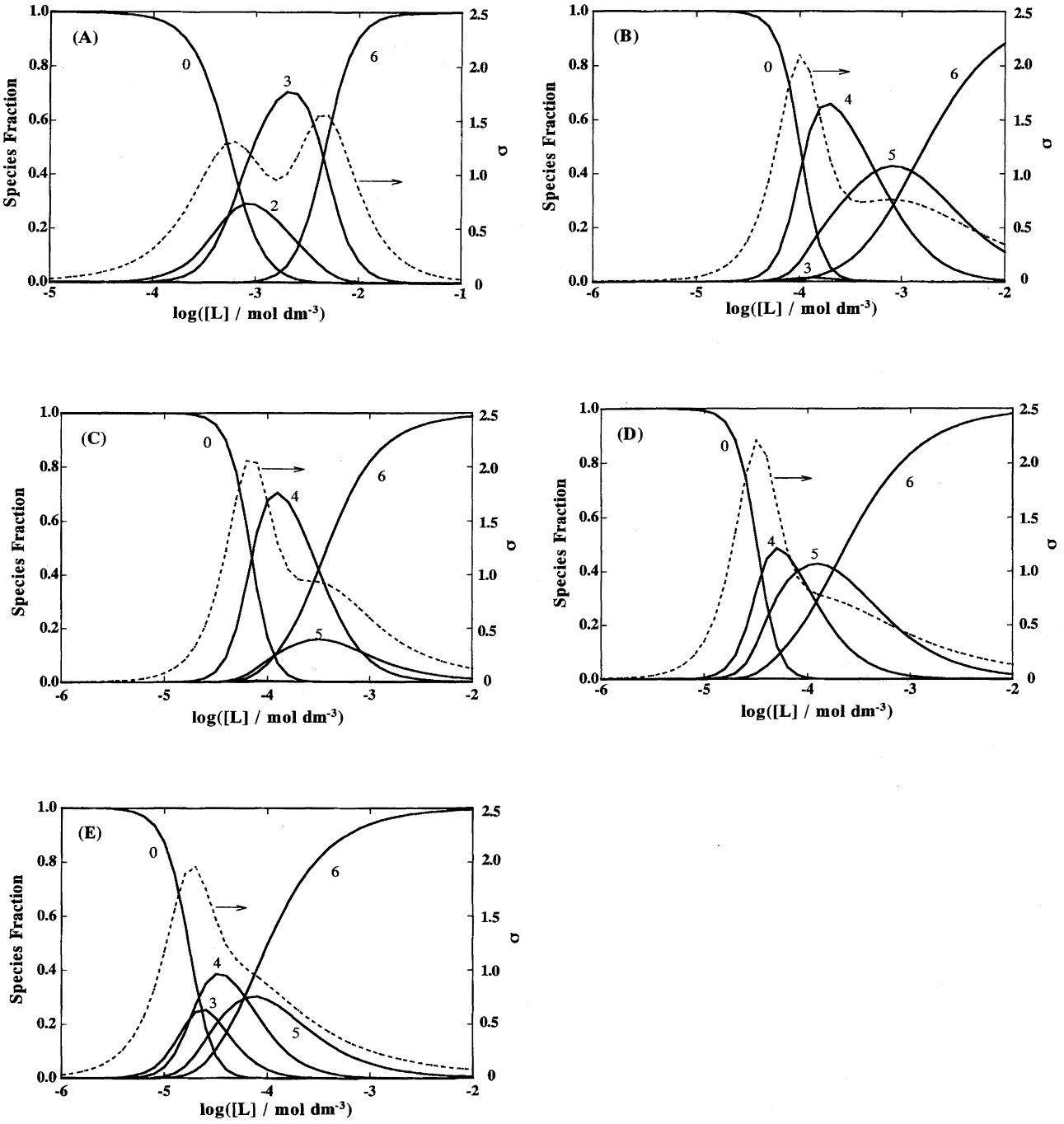


Fig. 4. Free-ligand concentration dependence of the species fractions and the square root of variance in $\overline{\nu}$, for Sunset Yellow FCF (A), Ponceau 3R (B), Acid Red 13 (C), Bordeaux Red (D), and Biebrich Scarlet (E). The solid curves show the species fractions; the number affixed to each curve means the number of bound ligands in the species. The broken curve shows the square root of variance in $\overline{\nu}$.

(Bordeaux Red, Ponceau 3R), 11 Å (Biebrich Scarlet), and 12.5 Å (Acid Red 13, Sunset Yellow FCF). On the other hand, lysozyme has 7 lysyl residues, 11 arginyl residues, and one histidyl residue as the basic residues. Among these, the histidyl residue does not serve as the binding site at pH = 7, since the pK_a value of the histidyl residue is about 6.[20] The alkyl side chains of the lysyl and arginyl residues are mobile around their β-carbons, if the cationic groups are not fixed by salt or hydrogen bond with neighboring anionic groups. Considering the mobility of the alkyl side chains and the above mentioned distances between sulfonato groups on the dye molecules, we may estimate the number of the cationic-residue pairs which are able to form bifunctional binding to be as much as 60 from the three dimensional coordinates[21] (6LYZ) registered in Brookhaven Protein Data Bank. All of these pairs could not be seen as equal from the view point of free energy; rather the pairs which match the distances between sulfonato groups of the dyes may be more suitable

as the binding sites.[22]

Considering the large negative values of the free energy changes of binding after the first step (Table 1), the observed cooperative effect could not be interpreted only by the above-mentioned electrostatic interactions. One well-known mechanism of appearance of cooperative effect in ligand binding is the so-called allosteric mechanism. It has been established for the proteins having subunit structures such as hemoglobin and regulatory enzymes. A large amount of hydrophobic groups must be exposed when one ligand molecule is bound, so that the cooperative effect for the present ligands appears by this mechanism. Lysozyme does not have a subunit structure, but rather has been thought to be very rigid and stable over the wide range of pH (from 2 to 11).[23] Therefore, it is difficult to see how the observed cooperative effect arises from this mechanism, even if some minor structural changes may occur when the ligands bind. It seems rather reasonable to consider that the cooperative effect arises from the hydrophobic interactions between bound ligands. Murakami et al.[24] have recently reported the value of the standard free energy change of dimerization of Biebrich Scarlet in solution in the presence of 0.05 mol dm^{-3} NaCl as -26.4 kJ mol^{-1}. The average value of the standard free energy changes of binding from the second to the sixth step for the Biebrich Scarlet-lysozyme system (Table 1) becomes -29.6 kJ mol^{-1}, somewhat larger than the above value for the dimerization in solution. Considering that the ionic strength of the present study is larger than that of the dimerization study and that the energetically disadvantageous electrostatic repulsions between dye molecules in the dimerization reaction may disappear when bound ligands interact with each other, the value is reasonable for the hydrophobic interactions between bound ligands.

To examine the correlation between the hydrophobicity of the dyes and the cooperative effect would make this point more clear. The hydrophobicity parameter log P of the dyes (P: the partition coefficient in 1-octanol–water system) may be a good measure of the hydrophobic interaction, since this parameter is concerning the acid forms (neutral forms) which may represent the state of bound ligands being neutralized by the electrostatic bonds. log P values of these dyes were calculated as -0.443 (Sunset Yellow FCF), 0.731 (Bordeaux Red and Acid Red 13), 1.00 (Ponceau 3R), and 1.27 (Biebrich Scarlet). The average cooperative effect may be defined in terms of the free energy changes of binding as

$$\textit{Average Cooperative Effect} = -\left(\frac{1}{5}\sum_{i=2}^{6}\Delta G_i^\circ - \Delta G_1^\circ\right), \quad (6)$$

i.e., the average value of the free energy changes of binding for the steps after the first one referred to that for the first step. Figure 5 shows the plot of the average cooperative effect vs. log P, indicating that the average cooperative effect increases linearly with an increase in log P. This shows that the stability of the complex due to the interaction between bound ligands increases with an increase in the hydrophobicity of the ligand, i.e., the observed cooperative effect is basically governed by the ligand's hydrophobicity. The six bound ligands must

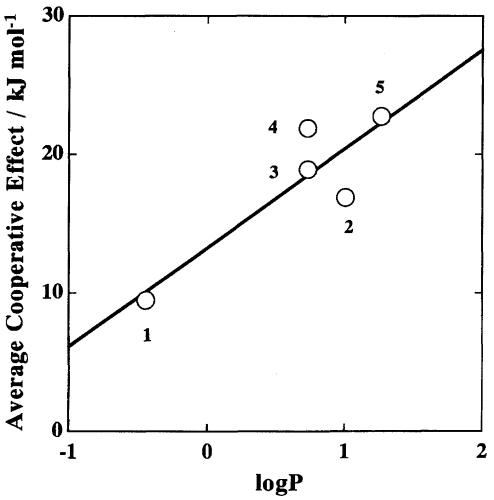


Fig. 5. Plot of the average cooperative effect vs. log P. The number shows the dye species; 1: Sunset Yellow FCF, 2: Ponceau 3R, 3: Acid Red 13, 4: Bordeaux Red, and 5: Biebrich Scarlet. The solid line represents the least-square fit to the data, the slope of which is 7.15 kJ mol^{-1}.

be placed in the restricted region in which they are able to interact with each other, so that the cooperativity appears by this mechanism. In the previously cited study,[13] Murakami has identified two cooperative-binding regions on hen egg white lysozyme for dodecyl sulfate at pH = 3.2: i.e., one is the highly-cooperative large region involving the 15 cationic residues (Lys1α, Lys1ε, Arg5, Lys13, Arg14, His15, Arg21, Lys33, Lys96, Lys97, Arg112, Arg114, Lys116, Arg125, and Arg128) and the other is the poorly-cooperative small region composed of the four cationic residues (Arg45, Arg61, Arg68, and Arg73), which are placed on both sides of the active-site groove of the protein. Considering the number of cationic residues involved in the cooperative binding for the present systems, one can conclude that the cooperative-binding region for these systems is in the large cooperative-binding region.

Figures 3 and 4 shows that the binding manner of Sunset Yellow FCF is different from those of the other four dyes. From Fig. 3 we can see that all the values of $K_2,\cdots,K_6$ for the other dyes are larger than the K_1 value; this shows that all the six dyes cooperatively bind by interacting with each other. In the case of Sunset Yellow FCF, on the other hand, although the values of K_2 and K_3 are larger than that of K_1, the value of K_4 is smaller than K_1; further, the values of K_5 and K_6 becomes larger again. This shows that the earlier three dye molecules bind cooperatively with each other, but the fourth molecule binds independently to them, and further the fifth and sixth dye molecules cooperatively bind with the fourth one again, i.e., there are two stages of cooperativity for Sunset Yellow FCF. From the similarity between these dye structures, it is difficult to consider that Sunset Yellow FCF binds to a quite different binding region. The behavior may therefore be interpreted by considering that the large cooperative-binding region for the other four dyes is composed of two small regions which are in some degree apart

from each other. In such a situation, if the distance between the small regions is larger than the distance in which bound ligands are able to interact with each other (in other words, the "interaction distance"), the two ligands bound in the different small regions could not interact with each other. The interaction distance may depend on both the orientation of the hydrophobic part in bound state (Fig. 6) and its hydrophobicity; the latter factor contributes to bring the interacting bound ligands close together. Because Sunset Yellow FCF is inferior to the other dyes in both of the above two factors, its interaction distance may be smaller than the other dyes. This may be the cause of the appearance of the two-stage cooperativity for Sunset Yellow FCF. This interpretation agrees with the appearance of the two peaks in the variance and of the two-ligands and three-ligands intermediate species for Sunset Yellow FCF (Fig. 4). Although the second peak in variance does not explicitly appear for the other four dyes, they have shoulders instead at the higher concentration region. This behavior also agrees with the existence of the two small regions.

Although Biebrich Scarlet has the largest hydrophobicity parameter (1.27) among the dyes, its orientation in bound state would be a disadvantage for the interaction distance. This may explain the appearance of the three-ligands intermediate species which is absent for Ponceau 3R, Acid Red 13, and Bordeaux Red (Fig. 4). Comparing the values of the binding constants for Bordeaux Red and Acid Red 13, which have the same backbone structure and the same log P value (0.731), all the values for Bordeaux Red are larger than those for Acid Red 13, except for the values of K_6, which are comparable to each other (Table 1 and Fig. 3). This difference is solely attributable to the differences in the position of the sulfonato groups, and may be interpreted by the differences in the interaction distance due to the above orientation factor. On the other hand, although the positions of the sulfonato groups of Bordeaux Red and Ponceau 3R are the same and the value of the hydrophobicity parameter of Bordeaux Red has the smaller value (0.731) than Ponceau 3R (1.00), the average cooperative effect of Bordeaux Red is larger than that of Ponceau 3R (Fig. 5). This discrepancy is due to the difference between the naphthyl group and the trimethylphenyl group, suggesting that the face-to-face contact between the plane aromatic groups is more of an advantage than the contact between the bulky trimethylphenyl groups for the interaction.

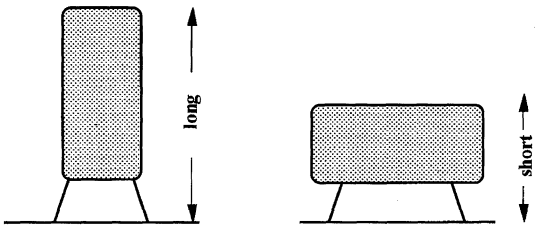


Fig. 6. Schematic representation of the typical orientations of the hydrophobic part of ligand in bound state. (A): longitudinal orientation, the case for Ponceau 3R and Bordeaux Red, (B): lateral orientation, the case for Sunset Yellow FCF, Acid Red 13, and Biebrich Scarlet. The interaction distance for the longitudinal orientation is larger than that for the lateral one.

Holler et al.[25] measured the binding of Biebrich Scarlet to lysozyme using the spectrophotometric titration technique at the similar experimental conditions: pH = 7.6, 24 °C, and ionic strength = 0.1. They analyzed the data by applying the simple one-step bimolecular binding scheme and evaluated the binding constant to be 6.7×10^3 mol^{-1} dm^3, which is larger by about two orders of magnitude than the K_1 values in this study (Table 1). Figure 4(E) shows that the amounts of the species which bind one or two molecule(s) of the dye are negligibly small compared to those of the other species in any concentration region and the species which bind more than two ligand molecules are in equilibrium with the bare protein species. This fact suggests that the large value of the binding constant in their study is an apparent one due to the over-simplified binding scheme which does not take account of the cooperative effect.

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22) In this argument, the possibility of unifuntional binding (binding of dye molecules only by one sulfonato groups) is not taken into account. While the maximum binding number 6 for the present systems is about half of those of the monoanionic ligands 10—14 (see text), the number 6 is strictly speaking somewhat larger than 5 which may be expected from the binding number of Orange I and Orange II, which have similar backbone structures as those of the present ligands. Although this difference suggests that we clould not completely deny the possibility of partial unifunctional bindings, the major component may be the bifunctionally bound state.

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