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

Kiyofumi Murakami

Department of Science Education, Faculty of Education, Yamaguchi University, Yamaguchi 753

(Received March 16, 1998)

Binding of sodium 4-(2- or 4-hydroxy-1-naphthylazo)benzenesulfonate and sodium 4-[4-(amino or dialkylamino)-phenylazo]benzenesulfonate (alkyl: methyl, ethyl, or butyl) 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. In the case of the (2- or 4-hydroxy-1-naphthyl-azo)benzenesulfonate system, a single cooperative binding stage has been observed with a maximum binding number of 10. In the case of the 4-[4-(dialkylamino)phenylazo]benzenesulfonate systems, two cooperative binding stages have been observed for the dyes having alkyl groups longer than a methyl group. The maximum binding numbers of the first stage are 14 and 13 for 4-[4-(diethylamino)phenylazo]benzenesulfonate and 4-[4-(dibutylamino)phenylazo]benzenesulfonate, respectively, and those of the second stage were the same as those for the first one. The binding isotherms have been well interpreted by the stepwise binding model, which takes account of the free energy decrease due to interactions between bound ligands and of its reduction as the binding step proceeds, and binding constants have been evaluated. On the basis of the binding constants and the free energy changes of binding, the detailed binding mechanism is discussed.

Cooperative ligand binding to proteins is an important phenomenon in the study of physicochemical and colloidal properties of the proteins. Especially, binding of surface active agents to globular proteins such as serum albumin and lysozyme, which are the typicals of acidic and basic proteins, has extensively been studied by many authors,[1—9] also from the growing interest in protein denaturation. It has been known, in many cases, that the initial specific bindings to the oppositely charged residues on protein molecules, which are cooperative or noncooperative depending on the systems, are followed by subsequent cooperative bindings with a large numbers of ligands to form micelle-like structures. It is also known that some anionic dyes and aromatic substances bind to those proteins in cooperative or noncooperative manners.[10—13]

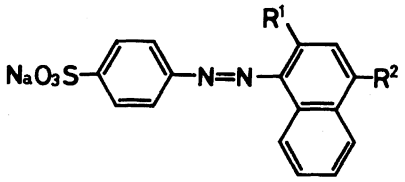
In the analysis of the binding isotherms of the cooperative binding systems, the concept of Wyman's binding potential[14] has often been used.[7,9,12,15—17] In this treatment, existence of only two protein species, i.e., one is the bare protein and the other is the full-loaded protein with ligands, is assumed in the limit of strong cooperativity. And the apparent binding constants have been derived from the binding isotherms, in further assumption that there exists a predominant species that binds average-bound-number ligands. From the apparent binding constants, however, we could not obtain a real image of the cooperativity except in the highly bound region, because these two assumptions contradict each other. It is rather desirable to discuss this on the basis of the stepwise binding model.

In this study, cooperative bindings of two kinds of monoanionic azo dyes (hydroxynaphthylazobenzenesulfonates and 4-[4-(dialkylamino)phenylazo]benzenesulfonates) to hen egg white lysozyme have been examined by the equilibrium dialysis method. In addition to the new experimental feature on the effects of alkyl chains attached to the aromatic substance, the detailed binding mechanism has been discussed on the basis of the binding constants calculated using the stepwise binding model.

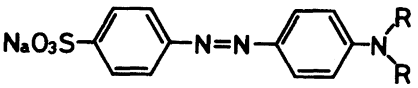
Experimental

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 calculated from the absorbance at 280 nm using the molar extinction coefficient of 3.77×10^4 mol^{-1} dm^3 cm^{-1}.[18] Sodium 4-(4-hydroxy-1-naphthylazo)benzenesulfonate (Orange I), sodium 4-(2-hydroxy-1-naphthylazo)benzenesulfonate (Orange II) and sodium 4-[4-(dimethylamino)phenylazo]-benzenesulfonate (Methyl Orange) were purchased from Wako Pure Chemical Industries, Ltd. Sodium 4-(4-aminophenylazo)benzenesulfonate and sodium 4-[4-(diethylamino)phenylazo]benzenesulfonate (Ethyl Orange) were purchased from Tokyo Chemical Industry Co., Ltd. Sodium 4-[4-(dibutylamino)phenylazo]benzenesulfonate (Butyl Orange) was synthesized from sulfanilic acid and *N*,*N*-dibutylaniline, washed with toluene, and purified by three times recrystallizations from a mixture of 0.05 mol dm^{-3} HCl and ethanol. These dyes were purified by three recrystallizations from an aqueous sodium acetate solution and by three further recrystallizations from an aqueous ethanol solution, and were dried at 110 °C in a vacuum for 20 h. Figure 1 shows the chemical structure of the dyes. Molar extinction coefficients of these dyes were measured from the concentration dependence of the absorption spectrum at pH = 7.0. 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 ligand binding was measured using the equilibrium dialysis technique.[19] Visking dialysis tubing (24/32)



Dye	R^1	R^2
Orange I	H	OH
Orange II	OH	H



Dye	R
Sodium 4-(4-aminophenylazo)-benzenesulfonate	H
Methyl Orange	CH_3
Ethyl Orange	C_2H_5
Butyl Orange	C_4H_9

Fig. 1. Chemical structure of dyes.

cut to a length of 9 cm was treated twice with a 1% $NaHCO_3$ aqueous solution at 100 °C for 40 min and subsequently immersed in a 95% aqueous ethanol solution overnight, soaked in a warm 1% EDTA solution for 30 min, and then exhaustively rinsed with distilled water. A 5-ml sample of lysozyme solution ([lysozyme] = 5.0×10^{-5} mol dm^{-3}) placed inside the dialysis tubing was dialyzed against 10 ml of the dye solution at 25 °C for 48 h. After equilibration, the concentration of the dye solution outside the dialysis tubing was measured spectrophotometrically using the molar extinction coefficients measured before. The extent of binding was calculated taking into account the amounts of adsorbed dye on the dialysis tubing, which were measured by a series of blank measurements without lysozyme. Leakage of the protein out of the tubing has also been examined, and it was found that the extent was negligibly small.

Spectrophotometric measurements were done with Shimadzu UV-200S and Hitachi U-2000 spectrophotometers. All the experiments were done at 25±0.2°C.

Nonlinear regression analysis for the evaluation of binding parameters was done with a program of Nagasawa and Oyanagi[20] on an Acos-850 computer system (NEC Corporation).

Results

Binding Isotherms. The binding isotherms for the binding of the two hydroxynaphthylazobenzenesulfonates and four 4-[4-(dialkylamino)phenylazo]benzenesulfonates to hen egg white lysozyme at pH = 7.0 and 25 °C are shown in Figs. 2 and 3, respectively. From these figures, it can be seen that these two types of ligand cooperatively bind to lysozyme in different manners. The hydroxynaphthylazobenzenesulfonates cooperatively bind in one stage. From the asymptotic value of $\bar{\nu}$, the maximum binding number was evaluated as 10. On the other hand, the 4-[4-(dialkylamino)phenylazo]-benzenesulfonates having longer alkyl chains (ethyl and butyl groups) cooperatively bind in two stages. The maximum binding numbers of the first and second stages agree with each other, and those for Ethyl Orange and Butyl Orange are 14 and 13, respectively. As has been observed in the binding of anionic surfactants to lysozyme,[8] precipitation was observed at the end region of the first stage for both of the two types of ligands, while the precipitate has disappeared in the end region of the second stage for the Ethyl Orange

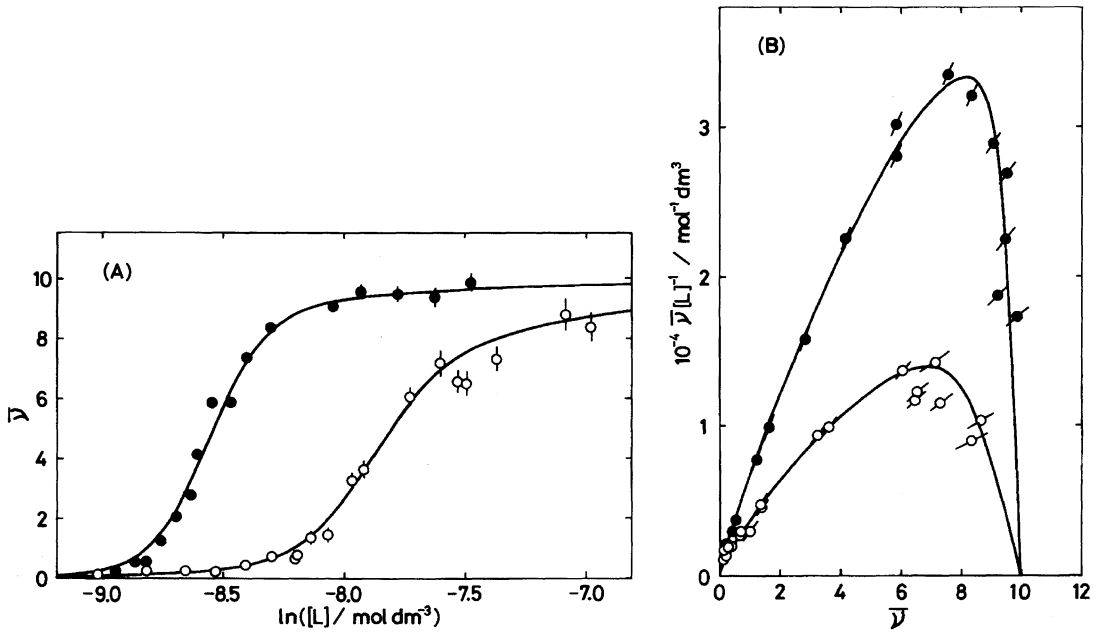


Fig. 2. Binding isotherms for the binding of hydroxynaphthylazobenzenesulfonates to hen egg white lysozyme at pH = 7.0 and 25 °C. (A): $\bar{\nu}$ vs. ln [L] plots and (B): Scatchard plots,[21] for Orange I (circle) and Orange II (filled circle). The solid lines represent the theoretical curves calculated using Eq. 7 and the values of the parameters listed in Table 1.

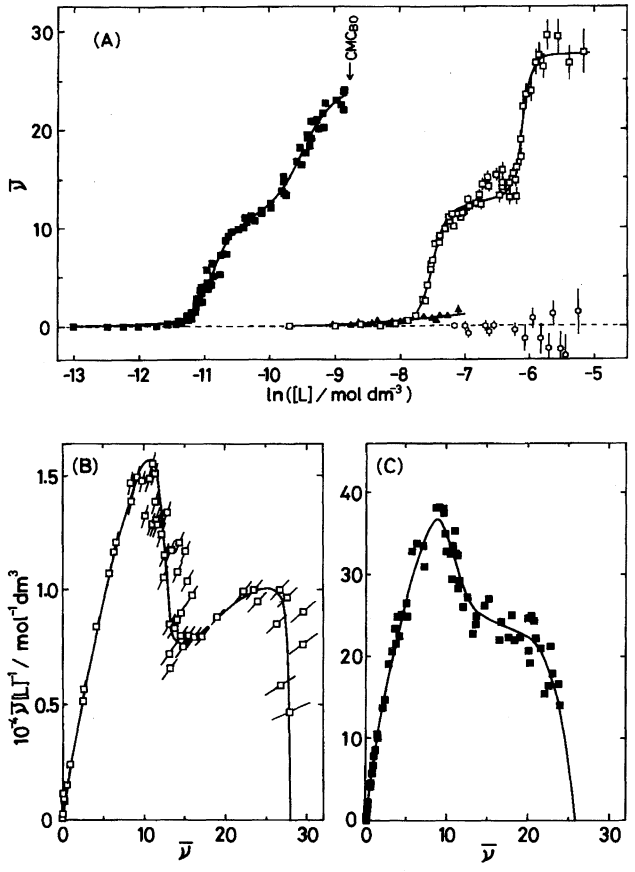


Fig. 3. Binding isotherms for the binding of 4-[4-(dialkylamino)phenylazo]benzenesulfonates to hen egg white lysozyme at pH = 7.0 and 25 °C. (A): $\bar{\nu}$ vs. ln [L] plots, (B): Scatchard plots for Ethyl Orange, and (C): Scatchard plots for Butyl Orange. The symbols denote 4-(4-aminophenylazo)benzenesulfonate (hexagone), Methyl Orange (filled triangle), Ethyl Orange (square), and Butyl Orange (filled square). The solid lines represent the theoretical curves calculated using Eq. 9 and the values of the parameters listed in Table 1, except for the line of Methyl Orange which was calculated under the assumption of independent and equivalent binding sites (see text). The arrow in (A) shows the critical micelle concentration of Butyl Orange.

and Butyl Orange systems. The 4-[4-(dialkylamino)phenylazo]benzenesulfonates having shorter alkyl chains than ethyl group did not cooperatively bind to lysozyme. In the case of Methyl Orange, the solubility was smaller than those of the other dyes and the observed binding number was less than 2 below it. In the case of 4-(4-aminophenylazo)benzenesulfonate, no binding was observed for the measured concentration range. As a conclusion, the longer the length of the alkyl chain becomes, the stronger the cooperativity.

Analysis of Binding Parameters. Binding of ligands to protein is generally expressed by the stepwise binding scheme:[22]

$$\begin{aligned} &\mathrm{P} + \mathrm{L} \rightleftarrows \mathrm{PL}, \\ &\mathrm{PL} + \mathrm{L} \rightleftarrows \mathrm{PL}_2, \\ &\cdots\cdots\cdots\cdots \\ &\mathrm{PL}_{n-1} + \mathrm{L} \rightleftarrows \mathrm{PL}_n, \end{aligned} \tag{1}$$

where P, L, and PL_i show free protein, free ligand, and complex species, respectively, and n means the number of binding sites. The macroscopic binding constants for the steps are expressed by[22]

$$\begin{aligned} K_1 &= [\mathrm{PL}]/[\mathrm{P}][\mathrm{L}], \\ K_2 &= [\mathrm{PL}_2]/[\mathrm{PL}][\mathrm{L}], \\ &\cdots\cdots\cdots\cdots \\ K_n &= [\mathrm{PL}_n]/[\mathrm{PL}_{n-1}][\mathrm{L}]. \end{aligned} \tag{2}$$

Using these equations, the average number of bound ligands per molecule of protein can be expressed by[22]

$$\bar{\nu} = \frac{K_1[\mathrm{L}] + 2K_1K_2[\mathrm{L}]^2 + \cdots + nK_1K_2\cdots K_n[\mathrm{L}]^n}{1 + K_1[\mathrm{L}] + K_1K_2[\mathrm{L}]^2 + \cdots + K_1K_2\cdots K_n[\mathrm{L}]^n}. \tag{3}$$

If the number of binding sites is large, it is in general difficult to calculate all the values of the binding constants from observed binding isotherms. When the sites on protein molecules are equivalent and independent of each other, however, the macroscopic binding constants can simply be expressed by the microscopic binding constant k_1 as[22]

$$K_i = (n + 1 - i)k_1/i. \tag{4}$$

The observed positive cooperativity shows that the free energies of the states in which more than one ligand are bound are less than those for the above independent case, due to the interaction between bound ligands. Although analytical expressions of binding isotherm, in which interactions between bound ligands are explicitly taken into account in molecular level, have been obtained for systems having high symmetry such as ligand-binding to linear polymers,[23–26] we have now no useful expression for protein systems because of the lack of such symmetry.

The cooperative bindings on protein surface may be liable to occur at the region where binding sites are closely placed. Among them, the first cooperativity (cooperativity between the ligands bound in the first and second steps) would occur at the nearest-neighbor pair (the closest binding-site pair) in the highest probability. This may result in a decrease in the free energy change of binding in the second step. The third ligand would bind to the next nearest-neighbor site, interacting with the already bound two ligands. In this third step, however, the decrease in the free energy change of binding would be somewhat reduced since the site is more remote from the nearest-neighbor pair. Like this, the cooperativity may extend to a wide area. On the basis of this consideration, we now introduce the following phenomenological relations between microscopic binding constants, as the simplest one to express the situation.

$$k_{i+1} = k_i \exp(-\alpha\beta^{i-1}/RT) \qquad 1 \leqq i \leqq n-1, \tag{5}$$

where k_i is the microscopic binding constant for the binding of i-th ligand. α and β are the parameters which take account of the free energy decrease due to interaction between bound ligands and of the reduction of the free energy-decreasing effect as the binding step proceeds ($0 \leqq \beta \leqq 1$), respectively. Here the rate of reduction is assumed to be

constant. Equation 4 was extended to

$$K_i = (n+1-i)k_i/i. \tag{6}$$

By using Eqs. 3, 5, and 6, $\bar{\nu}$ was written as a function of n, k_1, α, β, and [L] as

$$\bar{\nu} = \frac{\sum_{i=1}^{n} i\left\{\prod_{j=1}^{i}(n-j+1)k_j/j\right\}[\mathrm{L}]^i}{1+\sum_{i=1}^{n}\left\{\prod_{j=1}^{i}(n-j+1)k_j/j\right\}[\mathrm{L}]^i}, \tag{7}$$

where

$$k_j = k_1 \exp\left\{-\alpha\left(\sum_{k=1}^{j-1}\beta^{k-1}\right)/RT\right\}. \tag{8}$$

The data for the hydroxynaphthylazobenzenesulfonates were fitted to this equation, considering k_1, α, and β as independent parameters. The values of the parameters evaluated are listed in Table 1. The value of k_1 for the Orange II system was assumed to be the same as that for the Orange I system, due to the lack of data in the low concentration region before the cooperative effect appears. As can be seen in Fig. 2, the observed data fall on the theoretical curves calculated using these values (solid lines).

For the Ethyl Orange and Butyl Orange systems in which the two cooperative binding stages observed in distinct concentration regions, it is convenient to extend Eqs. 3 and 5 to the next ones (Eqs. 9 and 10), by assuming that the two stages occur independently of each other.

$$\bar{\nu} = \frac{K_{1,1}[\mathrm{L}]+\cdots+n_1K_{1,1}\cdots K_{1,n_1}[\mathrm{L}]^{n_1}}{1+K_{1,1}[\mathrm{L}]+\cdots+K_{1,1}\cdots K_{1,n_1}[\mathrm{L}]^{n_1}} + \frac{K_{2,1}[\mathrm{L}]+\cdots+n_2K_{2,1}\cdots K_{2,n_2}[\mathrm{L}]^{n_2}}{1+K_{2,1}[\mathrm{L}]+\cdots+K_{2,1}\cdots K_{2,n_2}[\mathrm{L}]^{n_2}}, \tag{9}$$

and

$$\begin{aligned} k_{1,i+1} &= k_{1,i}\exp(-\alpha_1\beta^{i-1}/RT) \quad 1 \leqq i \leqq n_1-1, \\ k_{2,i+1} &= k_{2,i}\exp(-\alpha_2/RT) \quad 1 \leqq i \leqq n_2-1, \end{aligned} \tag{10}$$

where the first subscript of the binding constants means the binding stage and the second one the binding step. The independent parameters in this case are $k_{1,1}$, α_1, and β for the first stage and $k_{2,1}$ and α_2 for the second stage. The first stages of these systems correspond to those of the hydroxynaphthylazobenzenesulfonates systems. The β factor is omitted for the second stage to minimize the number of parameters. The values of the parameters, obtained by the fitting procedure, are also listed in Table 1. The theoretical curves calculated using these values reproduce the observed data well (the solid curves in Fig. 3). The dependences of the microscopic binding constants, calculated from the parameters, on the binding step are shown in Fig. 4.

In the Methyl Orange system, we could not observe any marked cooperative effect. Therefore, assuming that all the biding sites are equivalent and independent to each other and that the number of binding sites is equal to that of the first stage for the Ethyl Orange system, the microscopic binding constant k_1 were evaluated as 132 $\mathrm{mol^{-1}\,dm^3}$.

Discussion

It is known that some anionic surfactants bind to lysozyme in a cooperative manner, in which two cooperative binding

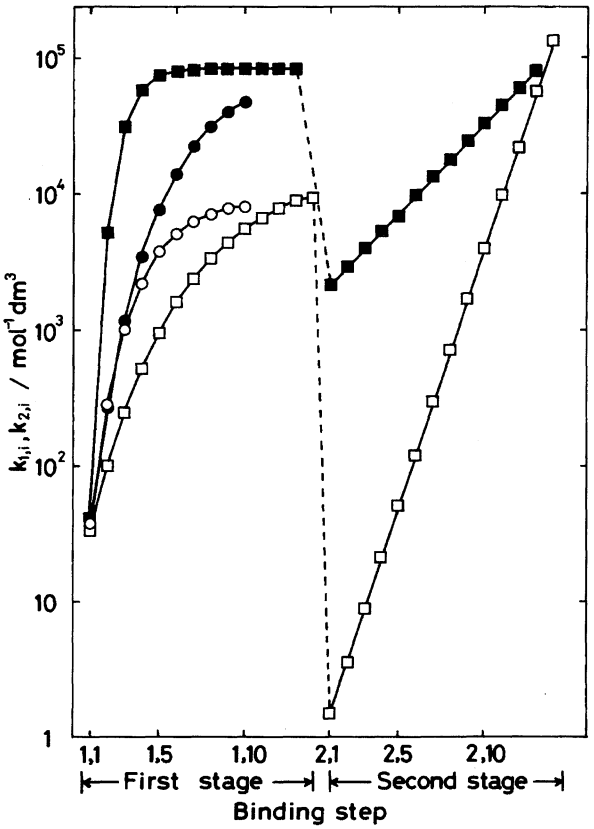


Fig. 4. Dependence of microscopic binding constants on binding step, for Orange I (circle), Orange II (filled circle), Ethyl Orange (square), and Butyl Orange (filled square). The first number of the paired two numbers in the abscissa means the binding stage and the second one the binding step.

Table 1. Binding Parameters for the Binding of Hydroxynaphthylazobenzenesulfonates and 4-[4-(Dialkylamino)phenylazo]benzenesulfonates to Lysozyme at pH = 7.0 and 25 °C

Dye	$k_1, k_{1,1}$ / $\mathrm{mol^{-1}\,dm^3}$	α_1 / $\mathrm{kJ\,mol^{-1}}$	β	$k_{2,1}$ / $\mathrm{mol^{-1}\,dm^3}$	α_2 / $\mathrm{kJ\,mol^{-1}}$
Orange I	38	−4.97	0.631		
Orange II	38	−4.84	0.745		
Methyl Orange	132				
Ethyl Orange	32.9	−2.74	0.820	1.49	−2.18
Butyl Orange	40.2	−12.10	0.365	2150	−0.752

stages have been observed.[7,8,15] The first stage of these systems, in which the maximum binding numbers are comparable to the number of net charge on the protein, is regarded as the specific bindings to cationic residues. On the other hand, the maximum binding numbers of their second stage have been found to be extremely large (more than fifty). Considering the binding number, the first stages observed for these systems correspond to the specific bindings in which the sulfonato groups of the dyes are bound to cationic residues on lysozyme and the hydrophobic parts of them interact with each other. A total of 19 cationic groups including the α-amino group are present on lysozyme molecules, and they all seem to be in the cationic forms at pH = 7.0, judging from their pK_a values.[27] The fact that the observed binding numbers (10 to 14) for the first stage are less than the possible binding number (19) means that not all of the residues effectively act as binding sites. This discrepancy may reasonably be interpreted by the assumption that some of the cationic residues form ionic or hydrogen bonds with neighboring anionic residues and do not serve as effective binding sites. In fact, such ionic or hydrogen bonds have been found in crystals of lysozyme.[28]

Figure 4 shows that the microscopic binding constants for Orange I and Orange II systems increase as the binding steps proceed, and the value for Orange II increasingly exceeds that for Orange I in the steps after the third one. The corresponding free energy of binding ($\Delta G_i^\circ = -RT\ln k_i$) changes from $\Delta G_1^\circ = -9$ kJ mol^{-1} to $\Delta G_{10}^\circ = -22.3$ kJ mol^{-1} (Orange I) and $\Delta G_{10}^\circ = -26.7$ kJ mol^{-1} (Orange II). These results show that the states in which more than three Orange II molecules are bound are more stable than those for Orange I. This large cooperative effect for the Orange II system could be attributed to its stronger stacking tendency.

In the first stage of the 4-[4-(dialkylamino)phenylazo]benzenesulfonate systems, the microscopic binding constant of Butyl Orange steeply increases and reaches a plateau value in the early steps, while that of Ethyl Orange increases more gradually. The free energy of binding changes from $\Delta G_{1,1}^\circ = -9$ kJ mol^{-1} to $\Delta G_{1,14}^\circ = -22.8$ kJ mol^{-1} (Ethyl Orange) and $\Delta G_{1,13}^\circ = -28.1$ kJ mol^{-1} (Butyl Orange). The difference in the free energy of binding between Butyl Orange and Ethyl Orange at the early steps (from the second to sixth step) takes the values in the region of -9.8——-11.9 kJ mol^{-1}. These values means the contribution of the four alkyl groups, which Butyl Orange has in excess of Ethyl Orange, to the free energy change due to the interaction between bound ligands. Converting these values to those per unit alkyl group, we obtain -2.5——-3.0 kJ mol^{-1}. These values agree with the contribution of the unit alkyl group to the free energy change of micelle formation of surfactants, i.e., -2.9 kJ mol^{-1},[29] showing that the alkyl chains of Butyl Orange are in a similar hydrophobic environment to that in the micelles. In addition, it is reasonable to consider that both the aromatic and the alkyl parts of the dyes contribute to the interaction between bound ligands, through the hydrophobic and/or stacking interactions.

Another characteristic of the 4-[4-(dialkylamino)phenylazo]benzenesulfonate systems is in the second stage. The maximum binding numbers of the second stage are the same as those of the first one, while the second stage of surfactant–lysozyme systems has very large binding numbers and is thought of as the formation of micelle-like structures. The free energies of binding for the first steps of the second stage are very different between dyes as $\Delta G_{2,1}^\circ = -1.0$ kJ mol^{-1} (Ethyl Orange) and $\Delta G_{2,1}^\circ = -19.0$ kJ mol^{-1} (Butyl Orange). As the binding step proceeds, both of them decrease linearly and reach the final values of $\Delta G_{2,14}^\circ = -29.3$ kJ mol^{-1} (Ethyl Orange) and $\Delta G_{2,13}^\circ = -28.1$ kJ mol^{-1} (Butyl Orange), being comparable to each other. The charge of the free energy of binding per one-step increase (α_2) was evaluated as -2.18 kJ mol^{-1} (Ethyl Orange) and -0.75 kJ mol^{-1} (Butyl Orange) (Table 1), that is, the magnitude of the free energy of binding for Butyl Orange is larger in negative value than that for Ethyl Orange but the rate of its decrease is smaller than that for Ethyl Orange (about one third). These observations suggest that two factors participate in the binding of the second stage. The first factor is the interaction between the ligands bound in the first stage and the ligands coming in the second stage. In this interaction, the secondary ligands must not migrate into the deep position in the layer formed by the ligands in the first stage, because the magnitude of the free energies of binding for the earlier steps is too small to consider such a situation. The previously cited value (-19.0 kJ mol^{-1}) of the free energies of binding for the first steps of the second stage of Butyl Orange is 80—97% of the value which could be expected if alkyl chains of Butyl Orange are in a micelle-like hydrophobic environment. On the other hand, that value for Ethyl Orange (-1.0 kJ mol^{-1}) is only 8—10% of the value, suggesting that the alkyl groups of the first layer of Ethyl Orange do not provide an effective hydrophobic environment to the secondary ligands. That is, the hydrophobic interaction between alkyl chains may play a central role in this interaction. This idea seems to interpret the facts that the large difference between the free energies of binding for Ethyl Orange and Butyl Orange in the first step and that the second stage was not observed for the hydroxynaphthylazobenzenesulfonates which have no alkyl chain. The second factor is the interaction between bound ligands.

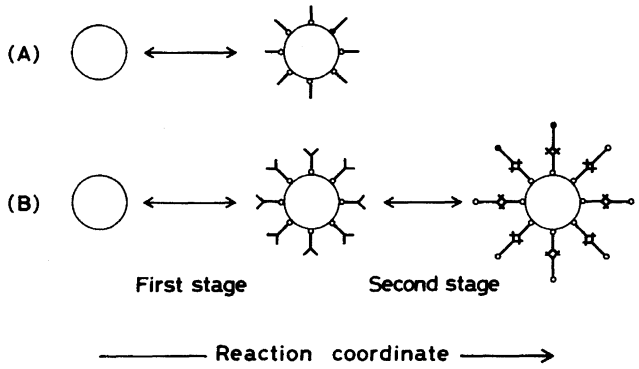


Fig. 5. Schematic representation of the cooperative binding model for the systems of (A) Orange I and Orange II and (B) Ethyl Orange and Butyl Orange.

In the case of Butyl Orange, the orientation of the bound ligands would be highly restricted since the butyl groups are strongly interacting with those in the first layer. This results in a reduction of the lateral interaction between aromatic parts through hydrophobic and/or stacking forces. In the case of Ethyl Orange, this effect may be smaller than the Butyl Orange case. The difference in α_2 between Ethyl Orange and Butyl Orange systems may be interpreted in this way. As a result, the second stages of these systems may be characterized by the completion of the formation of a bilayer-like structure of bound ligands. Figure 5 shows the schematic illustration of this complex formation model. According to this model, the complexes formed in the first stage would be liable to aggreate since the protein charges are neutralized and the hydrophobic parts of bound ligands are exposed to the solvent, on the other hand, the complexes formed in the second stage would be more soluble than the first ones because these are charged up again by the second-layer ligands the charged groups of which are exposed to the solvent. This agrees with the observed precipitation behavior.

The author wishes to thank Mr. Satoshi Tanigawa and Mr. Kohji Uesaka of Yamaguchi University for their assistance with the measurements.

References

1) J. Steinhardt and J. A. Reynolds, "Multiple Equilibria in Proteins," Academic Press, New York (1969), Chap. VII.

2) S. Lapanje, "Physicochemical Aspects of Protein Denaturation," John Wiley & Sons, New York (1978).

3) R. V. Decker and J. F. Foster, *Biochemistry*, **5**, 1242 (1966).

4) T. Takagi, K. Tsuji, and K. Shirahama, *J. Biochem.*, **77**, 939 (1975).

5) K. Hiramatsu, C. Ueda, K. Iwata, K. Arikawa, and K. Aoki, *Bull. Chem. Soc. Jpn.*, **50**, 368 (1977).

6) K. Takeda, M. Shigeta, and K. Aoki, *J. Colloid Interface Sci.*, **117**, 120 (1987).

7) M. N. Jones and P. Manley, *J. Chem. Soc., Faraday Trans. 1*, **75**, 1736 (1979).

8) K. Fukushima, Y. Murata, N. Nishikido, G. Sugihara, and M. Tanaka, *Bull. Chem. Soc. Jpn.*, **54**, 3122 (1981).

9) M. Subramanian, B. S. Sheshadri, and M. P. Venkatappa, *J. Biochem.*, **95**, 413 (1984).

10) K. Murakami, M. Akamatsu, and T. Sano, *Bull. Chem. Soc. Jpn.*, **67**, 2647 (1994).

11) J. R. Colvin, *Can. J. Chem.*, **30**, 320 (1952).

12) M. Subramanian, B. S. Sheshadri, and M. P. Venkatappa, *J. Biosci.*, **5**, 331 (1983).

13) Y. Nakano, T. Seki, H. Iwata, J. Komiyama, and T. Iijima, *Colloid Polym. Sci.*, **260**, 218 (1982).

14) J. Wyman, *J. Mol. Biol.*, **11**, 631 (1965).

15) M. N. Jones, P. Manley, and A. Holt, *Int. J. Biol. Macromol.*, **6**, 65 (1984).

16) M. Subramanian, B. S. Sheshadri, and M. P. Venkatappa, *J. Biochem.*, **96**, 245 (1984).

17) M. Subramanian, B. S. Sheshadri, and M. P. Venkatappa, *J. Biosci.*, **10**, 359 (1986).

18) A. J. Sophianopoulos, C. K. Rhodes, D. N. Holcomb, and K. E. Van Holde, *J. Biol. Chem.*, **237**, 1107 (1962).

19) K. Murakami, *Biophys. Chem.*, **41**, 253 (1991).

20) T. Nagasawa and Y. Oyanagi, "Recent Developments in Statistical Inference and Data Analysis," ed by K. Matushita, North Holland, New York (1980), p. 15.

21) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

22) J. Steinhardt and J. A. Reynolds, "Multiple Equilibria in Proteins," Academic Press, New York (1969), Chap. II.

23) G. Schwartz, *Eur. J. Biochem.*, **12**, 442 (1970).

24) J. D. McGhee and P. H. von Hippel, *J. Mol. Biol.*, **86**, 469 (1974).

25) I. Satake and J. T. Yang, *Biopolymers*, **15**, 2263 (1976).

26) T. L. Hill, "Cooperativity Theory in Biochemistry," Springer-Verlag, New York (1985), Chap. 8.

27) S. Kuramitsu and K. Hamaguchi, *J. Biochem.*, **87**, 1215 (1980).

28) T. Imoto, L. N. Johnson, A. C. T. North, D. C. Phillips, and J. A. Rupley, "The Enzymes," 3rd ed, ed by P. D. Boyer, Academic Press, New York (1972), Vol. 7, p. 665.

29) C. Tanford, "The Hydrophobic Effect," John Wiley & Sons, New York (1973), Chap. 7.