Static and Kinetic Studies of the Interaction of Toluidine Blue with Chondroitin-4-sulfate

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The binding of Toluidine Blue to chondroitin-4-sulfate was investigated by absorption spectra, titration curves, and temperature-jump relaxation spectra. The results were well interpreted by a basic model of binding to a linear lattice, where the cooperative interaction is restricted to the nearest-neighbor binding sites. The parameters g (number of binding sites per disaccharide repeating unit), K (cooperative binding constant), and q (strength factor of cooperativity) in 0.015 M phosphate buffer (pH 7.0) were determined to be 2, 1.02×10^5 M^{-1}, and 270 $\pm$60, respectively. The fastest process of the three relaxations observed in the system of Toluidine Blue-chondroitin-4-sulfate, could be interpreted as the aggregation process for cooperative binding. The rate constant $\tilde{k}_R$ was determined to be 1.6×10^8 M^{-1} s^{-1} at 28 °C.

The cooperative binding of dye to linear biopolymer has been well known as the aggregation of dye molecules on the charged polymer.[1] Schwarz's theory has well interpreted the static and dynamic phenomena of the cooperative binding of dye molecules to such polymers as polyglutamic acid, polyacrylic acid, and polyphosphate.[2-6] Recently, an extensive model for competitive cooperative bindings has been derived theoretically and has been applied to the investigations on the mechanisms of proflavin binding to poly(A) and DNA.[7,8]

The interactions of dyes with glucosaminoglycans, which have been known to adopt helical conformations in the solid state,[9-13] have been studied by the measurements of absorption spectra, optical rotatory dispersion, and circular dichroism.[14-18] One approach employing such measurements and using cationic dye has been performed as a probe of the glucosaminoglycan conformation in dilute aqueous solution. However, the kinetics of the conformational changes of glucosaminoglycans and the interactions of dyes with polymers have not been examined.

In the present paper, the first kinetical attempt to explain the interaction of dye with polysaccharide will be made systematically in the system of Toluidine Blue–chondroitin-4-sulfate by referring to a basic model of cooperative binding of a small ligand to a linear polymer.

Experimental

Chondroitin-4-sulfate (Ch4-S) from whale cartilage was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo). The molecular weight was determined to be 24000 by means of an intrinsic viscosity measurement in 0.015 M phosphate buffer (pH 7.0) containing 0.2 M NaCl at 25 °C, using the following relation:[19]

$$(\eta) = 3.1\times10^{-4} M_w^{0.74},$$

where η is the intrinsic viscosity and M_w is the weight-average molecular weight. Toluidine Blue (TB) was purchased from Chroma Gesellschaft and was purified by washing with chloroform until the chloroform was colorless. Solutions of TB were made fresh for each experiment. The polymer-dye complex was prepared by adding TB to Ch4-S solutions with stirring. The pH was adjusted by the addition of NaOH or HCl. The measurements of the pH dependencies of the absorption spectra and the titration curves were performed without controlling the ionic strength. All sample solutions except those used for these two measurements were prepared with 0.015 M phosphate buffer solution (pH 7.0 and constant ionic strength (μ=0.06)). The concentration of Ch4-S (C_p) was calculated as the mole concentration of disaccharide repeating units. The weighing-in concentration of TB (C_A°) was checked by the measurement of absorbance at 630 nm. The pH values of solutions were measured with a Hitachi-Horiba Type F-5 pH meter. The absorption spectra were observed with a Union Giken SM 401 spectrophtometer. The extinction coefficient (ε) in the solution was obtained from E(absorbance)/C_A°. The extinction coefficients of pure monomer (ε_A) and pure dimer (ε_D) in TB were 4.2×10^4 and 0.13×10^4 M^{-1} cm^{-1} at 630 nm, respectively.

Titrations were performed in a closed 500-ml flask. After the addition of a very small amount of the standardized HCl titrant, a solution of 10 ml was removed for pH measurement. The percent protonation of Ch4-S as a function of pH was computed from the amount of added titrant minus the solvent blank volume.

Details of the Joule-heating temperature-jump apparatus have already been described elsewhere.[20] A temperature-jump of 8 °C was produced by a 20 kV capacitor discharge, giving a heating time of about 50 μs. All static experiments were performed at 25$\pm$0.5 °C.

Results

Static Studies. The absorption spectra of Ch4-S–TB complexes under the various polymer-to-dye ratios ($P/D=C_p/C_A^\circ$) at pH 7.0 are shown in Fig. 1. The solid line at $P/D=0$ exhibits two peaks, around 630 nm (due to the monomer of TB molecular) and 600 nm (due to the dimer). By adding Ch4-S to TB solution, a new band appears around 550 nm. The metachromatic effect becomes prominent with increasing P/D. The metachromatic band shift, however, is not observed within the range of P/D=5—50. With a P/D above 50, the band shifts to the longer wavelength side. The negative metachromatic effect may be attributed to the occurrence of isolated dye molecules from the polymer–dye complexes.

Figure 2 shows that the pH values of TB are approxi-

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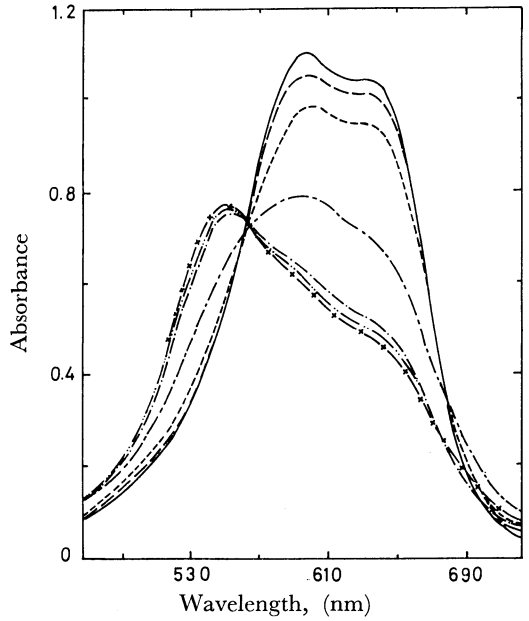

Fig. 1. P/D dependence of absorption spectra of the TB-Ch4-S complex in 0.015 M phosphate buffer (pH 7.0) at 25 °C and $C_A^\circ = 8\times10^{-5}$ M: (——) $P/D=0$; (— —) $P/D=0.1$; (---) $P/D=0.2$; (·——) $P/D=0.5$; (—·—) $P/D=1$; (—··—) $P/D=3$; (—×—) $P/D=6$.

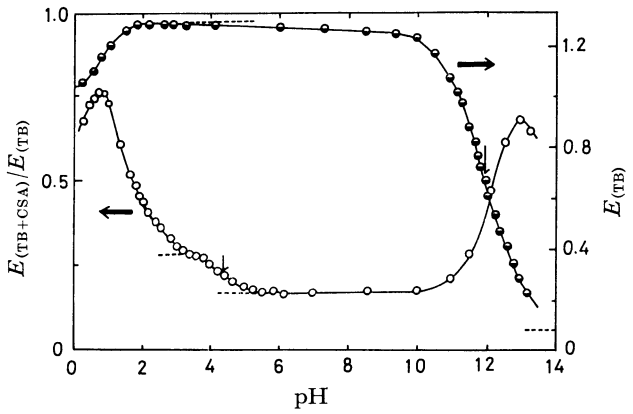

Fig. 2. pH dependencies of absorbances of TB and TB-Ch4-S at 630 nm, where E means the total absorbance: (◒) $C_A^\circ = 4\times10^{-5}$ M; (○) $C_A^\circ = 4\times10^{-5}$ M, $C_P = 2\times10^{-4}$ M.

mately 12 and 1, and that a small shoulder appears over the pH range of 3.5—5.5 in the Ch4-S–TB system. The titration curves of the Ch4-S and Ch4-S–TB complexes are demonstrated in Fig. 3 by plots of (pH—log $\alpha/(1-\alpha)$) against α, where α denotes the degree of ionization of the acid. The titration curve (a) suggests that there exist three conformations of Ch4-S in solution at least.** The titration curve (b), however, presents more simple features than Ch4-S alone. The extrapolated lines from the higher α values of the two curves give the pK values of 3.38 for (a) and 4.25 for (b). Their

** The analysis of the corresponding titration curve has been reported in the paper of the potentiometric titration study for the helix-coil transition of polyglutamic acid by M. Nagasawa *et al.*[22]

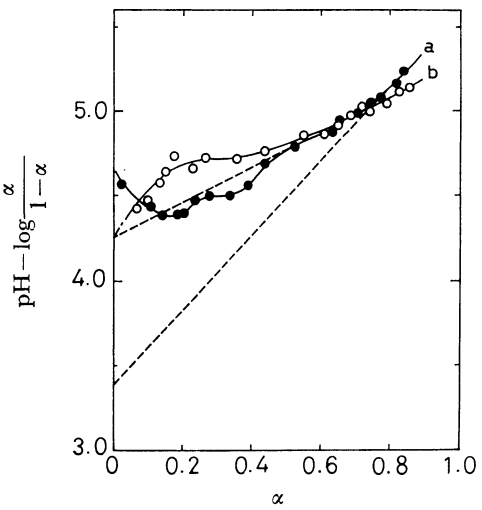

Fig. 3. Titration curves of Ch4-S and TB-Ch4-S complex at 25 °C: (●) $C_P = 2\times10^{-4}$ M; (○) $C_A^\circ = 4\times10^{-5}$ M, $C_P = 2\times10^{-4}$ M. The solid lines are the smooth curves between the experimental points. The dashed lines are the extrapolated lines from the higher α values.

values are in good agreement with the one obtained in 0.1 M NaCl solution by M.B. Mathews[21] for the former and with the one estimated from the pH dependency of the absorbance in Fig. 2 for the latter.

The representations of both measured and interpolated extinction coefficients at 630 nm in the solutions of TB and TB–Ch4-S can be found in Fig. 4. The curve at $P/D=0$ means that the aggregation of dye molecules causes the monomer concentration to decrease: the extinction coefficients (ε) in the variable solutions approach to the (ε_D) of the pure dimer from the (ε_A) of the pure monomer. Such smooth curves, however, do not appear upon adding Ch4-S in TB solution. The reversely sigmoid curves may occur by the cooperative binding of TB to Ch4-S. In the binding process where the cooperative interaction of nearest neighbor binding sites can be considered, there exist at least two different types of intrinsic binding processes:[2]

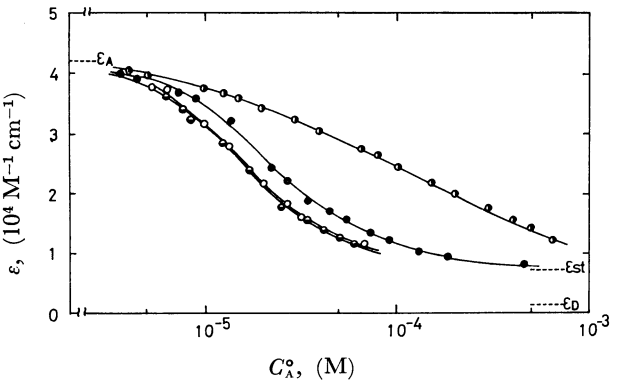

Fig. 4. Plots of molar extinction of TB solutions against C_A° in 0.015 M phosphate buffer (pH 7.0) at 630 nm: (◑) $P/D=0$; (●) $P/D=0.55$; (◒) $P/D=5.85$; (◎) $P/D=55.2$.

I: an initial nucleation of binding $\mathrm{uuu} + \mathrm{A} \overset{K^*}{\rightleftharpoons} \mathrm{uau}$,

II: the aggregation (or growth) process

$$\mathrm{uau} + \mathrm{A} \overset{K}{\rightleftharpoons} \mathrm{uaa},$$

where A, u, and a are the free monomeric ligand, the unoccupide binding site, and the binding site occupied by ligand, respectively. The binding constant for the initial nucleation process is defined to be $K^*=\overline{C}_{\mathrm{uau}}/(\overline{C}_{\mathrm{A}}\cdot\overline{C}_{\mathrm{uuu}})$, while the cooperative binding constant is $K=\overline{C}_{\mathrm{uaa}}/(\overline{C}_{\mathrm{A}}\cdot\overline{C}_{\mathrm{uau}})$. In order to analyse quantitatively the cooperative binding process, some constants should be estimated from the curves of Fig. 4. First, the dimerization constant K_{d} of TB in the absence of Ch4-S was calculated by using the following equation:

$$[(\varepsilon_{\mathrm{A}}-\varepsilon)/C_{\mathrm{A}}^{\circ}]^{1/2} = (2K_{\mathrm{d}}/\Delta\varepsilon)^{1/2}[\Delta\varepsilon-(\varepsilon_{\mathrm{A}}-\varepsilon)], \quad (1)$$

where $\Delta\varepsilon=(\varepsilon_{\mathrm{A}}-\varepsilon_{\mathrm{D}})$. The value of $K_{\mathrm{d}}=6.1\times10^3\ \mathrm{M}^{-1}$ at pH 7.0 and 25 °C is in agreement with that estimated by H. Ushio *et al.*[6] It is also necessary to obtain the extinction coefficient $\varepsilon_{\mathrm{st}}$ of the dye molecules stacked on the Ch4-S molecules. The extinction coefficient is expressed by the following relation:

$$\varepsilon = \overline{\gamma}_{\mathrm{A}}\varepsilon_{\mathrm{A}} + 2K_{\mathrm{d}}C_{\mathrm{A}}^{\circ}\overline{\gamma}_{\mathrm{A}}^{2}\varepsilon_{\mathrm{D}} + \overline{\theta}\,gp\varepsilon_{\mathrm{st}}, \quad (2)$$

where $\overline{\gamma}_{\mathrm{A}}$ is the fraction of free monomeric dye, $\overline{\theta}$ is the fraction of occupied sites of polymer, g is the number of binding sites per disaccharide repeating unit of Ch4-S, and $p=P/D$. With the reduced version of mass conservation

$$\overline{\gamma}_{\mathrm{A}} + 2K_{\mathrm{d}}C_{\mathrm{A}}^{\circ}\overline{\gamma}_{\mathrm{A}}^{2} + \overline{\theta}gp_{\mathrm{st}} = 1, \quad (3)$$

the transformation of Eq. 2 gives the following equation:

$$\varepsilon = \varepsilon_{\mathrm{st}} + (\varepsilon_{\mathrm{A}}-\varepsilon_{\mathrm{st}})\overline{\gamma}_{\mathrm{A}} + (\varepsilon_{\mathrm{D}}-\varepsilon_{\mathrm{st}})2K_{\mathrm{d}}C_{\mathrm{A}}^{\circ}\overline{\gamma}_{\mathrm{A}}^{2}. \quad (4)$$

Quantitative estimations show that the last term of Eq. 4 can be practically neglected. By further introducing the cooperative binding constant K and the variable S $(=\overline{C}_{\mathrm{uaa}}/\overline{C}_{\mathrm{uau}}=K\overline{C}_{\mathrm{A}}=KC_{\mathrm{A}}^{\circ}\overline{\gamma}_{\mathrm{A}})$ for the calculation of equilibrium concentrations of any desired binding state, the following equation is obtained:

$$\varepsilon = \varepsilon_{\mathrm{st}} + (\varepsilon_{\mathrm{A}}-\varepsilon_{\mathrm{st}})(S/K)C_{\mathrm{A}}^{\circ-1}. \quad (5)$$

The plots of ε *versus* $C_{\mathrm{A}}^{\circ-1}$ are shown in Fig. 5. If the parameter S becomes almost constant with respect to changes of C_{A}° at strong cooperativity and medium p, the plots should eventually result in the straight lines. Linear relationships do occur obviously for the smaller values of $C_{\mathrm{A}}^{\circ-1}$. Extrapolation of $C_{\mathrm{A}}^{\circ-1}\to0$ yields $\varepsilon_{\mathrm{st}}=0.7\times10^4\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$.

For obtaining the value of g, Eq. 3 can be rewritten as the following relation:[3]

$$1-\overline{\theta}gp = \overline{\gamma}_{\mathrm{A}}(1+2K_{\mathrm{d}}C_{\mathrm{A}}^{\circ}\overline{\gamma}_{\mathrm{A}}) \equiv \gamma_{\mathrm{A}}^{*}, \quad (6)$$

where γ_{A}^{*} is the total fraction of free dye. The values of γ_{A}^{*} calculated by using Eq. 5 and $\overline{\gamma}_{\mathrm{A}}=(S/K)C_{\mathrm{A}}^{\circ-1}$ are plotted against the P/D at constant C_{A}° in Fig. 6. The intercept on the P/D axis of the common limiting straight line for small P/D (<0.5) yields $g=2.0$. From the value of γ_{A}^{*} of the intersection point of the experimental curve and an auxiliary straight line with half

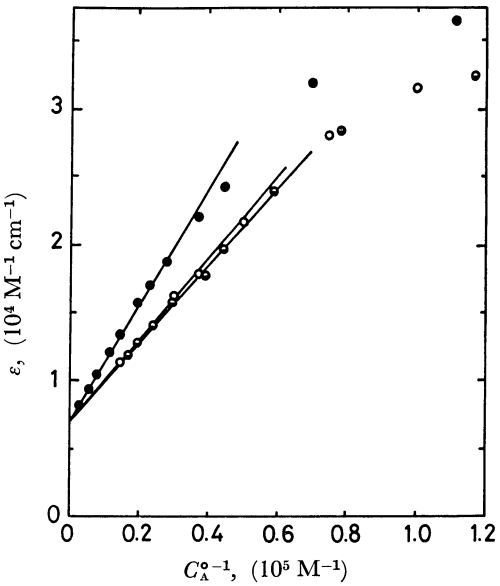

Fig. 5. Plots of molar extinction of TB solutions against $C_{\mathrm{A}}^{\circ-1}$: (●) $P/D=0.55$; (○) $P/D=5.85$; (◒) $P/D=55.2$.

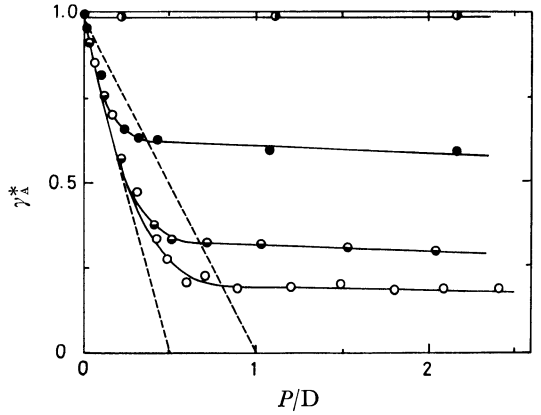

Fig. 6. Plots of total fraction of free dye against various P/D in 0.015 M phosphate buffer (pH 7.0) at 25 °C: (◑) $C_{\mathrm{A}}^{\circ}=9.2\times10^{-6}$ M; (●) $C_{\mathrm{A}}^{\circ}=1.9\times10^{-5}$ M; (◒) $C_{\mathrm{A}}^{\circ}=3.9\times10^{-5}$ M; (○) $C_{\mathrm{A}}^{\circ}=6.7\times10^{-5}$ M.

the slope of the first one in the figure, the value of K was determined to be $1.02\times10^5\ \mathrm{M}^{-1}$ by

$$K = (\overset{\circ}{\gamma}_{\mathrm{A}}^{*}\cdot C_{\mathrm{A}}^{\circ})^{-1} + 2K_{\mathrm{d}}, \quad (7)$$

where $\overset{\circ}{\gamma}_{\mathrm{A}}^{*}$ denotes the γ_{A}^{*} at the intersection point.[3] Since the values of $\overline{\theta}$ at the various initial concentrations of dye are easily accessible from Eq. 6 at constant P/D, the cooperativity parameter $(q=K/K^*)$ was calculated by the following relation:[2]

$$[1-2\overline{\theta}/\{\overline{\theta}(1-\overline{\theta})\}^{1/2}]\cdot\overline{C}_{\mathrm{A}}^{1/2} = (g/K)^{1/2} - (gK)^{1/2}\overline{C}_{\mathrm{A}}. \quad (8)$$

The average value of q was estimated to be 270±60 from the two straight lines shown in Fig. 7.

Using the values of ε_{A}, ε_{D}, $\varepsilon_{\mathrm{st}}$, and K_{d} obtained above, the concentrations of stacked dye molecules $\overline{C}_{\mathrm{st}}$ were estimated by using the following relation:

$$E = \varepsilon_{\mathrm{A}}\overline{C}_{\mathrm{A}} + 2\varepsilon_{\mathrm{D}}\overline{C}_{\mathrm{D}} + \varepsilon_{\mathrm{st}}\overline{C}_{\mathrm{st}}, \quad (9)$$

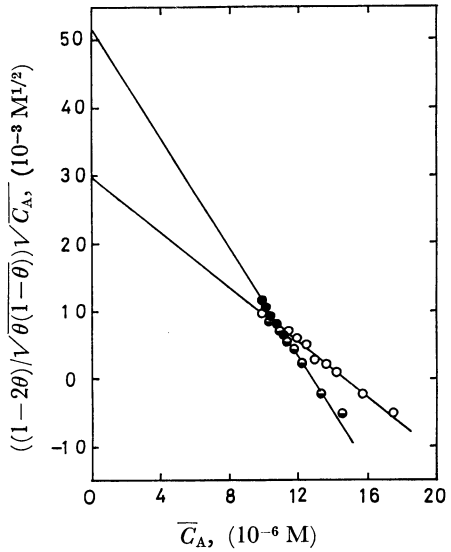

Fig. 7. Plots to determine cooperative parameter q for the TB-Ch4-S system: (●) $C_A^\circ = 1.9\times10^{-5}$ M; (◒) $C_A^\circ = 3.9\times10^{-5}$ M; (○) $C_A^\circ = 6.7\times10^{-5}$ M.

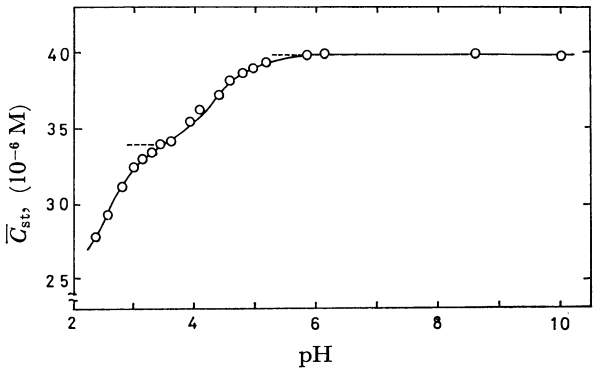

Fig. 8. pH dependence of the concentration of stacked dye molecule $\overline{C}_{st}$ in the TB-Ch4-S system at $C_A^\circ = 4\times10^{-5}$ M, $C_p = 2\times10^{-4}$ M and 25 °C.

where E denotes the total absorbance of the solution. The pH dependency of $\overline{C}_{st}$ is demonstrated in Fig. 8. The two-step saturation curve implies the existence of two interactions involving ionizing groups, that is, TB interacts with a sulfuryl group and a carboxyl group of Ch4-S.

Kinetic Studies. Some typical relaxation curves are shown in Fig. 9. At the metachromatic band (550 nm), a fast single relaxation spectrum was observed with the relaxation time of 10^{-4} s order, as shown in Fig. 9(a), while at 630 nm due to monomeric dye a faster change of absorbance was followed by two slower changes with the relaxation times of the order of 10^{-2} and 10^{-1} s, as shown in Fig. 9(b). This faster change appears to be relaxation with the same order of rate as the one observed at 550 nm. The relaxation amplitude, however, was very small and could not be analyzed. The dichroic effect due to optical anisotropy, which is caused by the induced orientation of the macromolecules in the direction of the electric field, was checked by two observations. At 550 and 630 nm, no change of relaxation amplitudes was observed by using

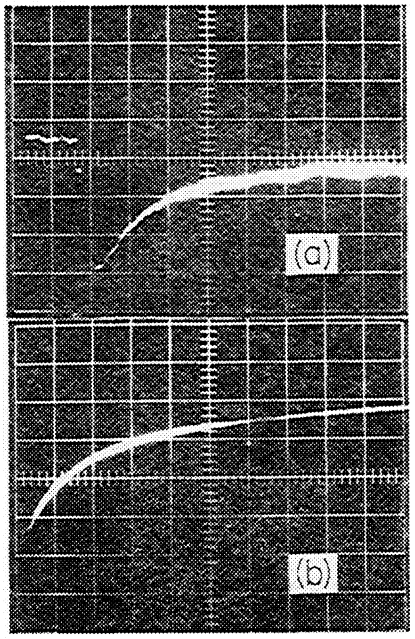

Fig. 9. Typical relaxation curves of the TB-Ch4-S system in 0.015 M phosphate buffer (pH 7.0) at 28 °C: $C_A^\circ = 4\times10^{-5}$ M, $P/D = 5.1$; (a) 550 nm, sweep of 0.2 ms, vertical scale of 0.1 V/div.; (b) 630 nm, sweep of 50 ms, vertical scale of 0.1 V/div.

a polarizer and no relaxation spectrum was detected at any isosbestic point. Furthermore, a more rapid change was observed at 630 nm in the dye solution. The absorbance change, however, was smaller than the fastest one observed at 630 nm in the presence of Ch4-S and was completed within the heating time of the apparatus. This change probably corresponds to a monomer-dimer reaction of the dye which appears under a certain ionic strength ($\mu = 0.06$ in the present work). In the presence of Ch4-S, however, the contribution to the monomer-dimer reaction of free dye was within the experimental error of the spectrum change shown in Fig. 9(b). From the variety of observations described above, the faster relaxation at the metachromatic band was analysed quantitatively as the chemical relaxation due to the equilibrium shift between the monomeric dye and the stacked dye molecules. In the present paper, two slower relaxations at 630 nm which may be interpreted by the conformational changes of TB–Ch4-S complex are not treated; this problem will be reported later.

According to the theory of cooperative binding, the binding of dye to linear polymers consists of the following two elementary steps:[2,4]

$$\text{auu} + \text{A} \underset{k_D}{\overset{k_R}{\rightleftharpoons}} \text{aau} \qquad (k_R/k_D = K), \tag{10}$$

$$\text{auuu} + \text{A}_2 \underset{k_{D'}}{\overset{k_{R'}}{\rightleftharpoons}} \text{aaau} \qquad (k_{R'}/k_{D'} = K^2/K_d). \tag{11}$$

The mean relaxation time τ_b^* of the binding process is[2,4]

$$1/\tau_b^* = 2\tilde{k}_R(g/q^{1/2})\{\bar{\theta}(1-\bar{\theta})\}^{1/2}\cdot p\cdot C_A^\circ, \tag{12}$$

where

$$\tilde{k}_R = \beta k_R + (1-\beta)k_{R'}, \tag{13}$$

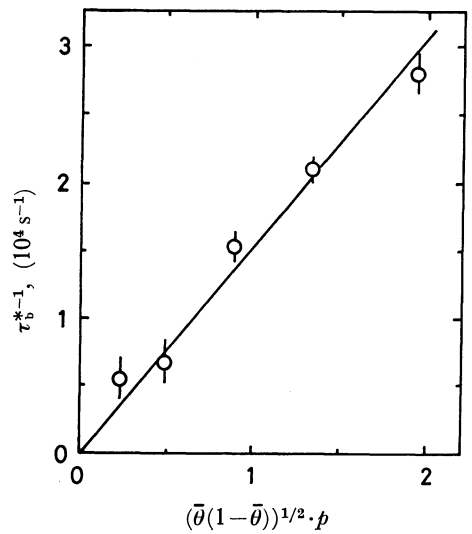

Fig. 10. Plot of the reciprocal mean relaxation time of cooperative binding τ_b^{*-1} against $\{\bar{\theta}(1-\bar{\theta})\}^{1/2}\cdot p$: $C_A^\circ = 4\times10^{-5}$ M.

$$\beta = 1/(1+4K_d\bar{C}_A) \simeq K/(K+4K_d). \quad (14)$$

The plot of τ_b^{*-1} againt $\{\bar{\theta}(1-\bar{\theta})\}^{1/2}\cdot p$ at constant C_A° value is shown in Fig. 10. From the slope of the straight line traversing the origin, $\tilde{k}_R$ was calculated to be 1.6×10^8 M^{-1} s^{-1}. By using the obtained K and K_d values, the β value was found to be approximately 0.8.

Discussion

The nature of the conformations of Ch4-S, which are known to adopt helical states in the solid state,[9,12] has not been estabilshed yet in solution. Chondroitin-6-sulfate, which has two ordered structures containing single helixes,[11,13] forms two states, flexible coil and rigid rod, in solution at a certain ionic strength.[23] As shown in Fig. 3, the titration curve of Ch4-S may imply that Ch4-S has three conformations in solution at least (for example: rigid rod, flexible coil, and random coil). The titration curve in the presence of TB may also imply that the transition between helical conformations (rigid rod⇌flexible coil) of Ch4-S is disturbed by masking the ionizing groups of Ch4-S with TB. Above pH 6, however, the deprotonations of Ch4-S are perhaps completed and the conformation of Ch4-S may be random coil in either the absence or the presence of TB. Therefore, the interaction of Ch4-S with TB at pH 7.0 would be also interpreted by a basic model of the cooperative binding of dye to linear biopolymer.

The number of binding sites per disaccharide repeating unit of Ch4-S was estimated to be $g=2.0$. Apparently the disaccharide repeating unit does not have specific binding sites for TB molecules. The unit of Ch4-S is electrically negative due to the carboxyl and sulfuryl groups and the TB molecule is electrically positive. Starting from the negative charges of the unit, it may be easily explained that the polymer chain only serves as a template favoring the formation of stacked aggregates by reducing the electrostatic repulsion between the positive charges of TB ions. On the other hand, the concentration of $\bar{C}_{st}$ increases with ionizations of sulfuryl and carboxyl groups. From these results, the binding of TB to Ch4-S is considered to be essentially an electrostatic interaction.

The cooperative binding constant K contains the contributions of two binding processes, the nucleation process (denoted by K^*) and the growth process (denoted by q). With a value of $q=270$ the average number of ligands in a stacked aggregate*** is 17.4 at $S=1$ and $\bar{\theta}=1/2$. This value is smaller than the total number of binding sites on one polymer chain (about 100). This may imply that a basic model of binding to a linear polymer can also be applied to a relatively shorter chain such as Ch4-S. On the other hand, the binding constant of the nucleation process K^*, which can be calculated with the relation $K^*=K/q$, is about 380 M^{-1}. The value is the same order of magnitude as K^* in the proflavine–polyglutamic acid system.[5] This means that the binding of isolated (*i.e.*, unstacked) TB molecules to Ch4-S is an electrostatic binding.

The obtained value of the rate constant for the growth process $\tilde{k}_R$ is reasonable considering the ionic strength used in the present work, because it is of the same order of magnitude as those obtained in the systems of acridine orange-polyglutamic acid[4] and proflavine–polyacrylic acid.[5] Since the value of β is about 0.8, it may be suggested qualitatively that the binding of free monomeric dye is approximately 80% in the aggregation process, under the assumption that k_R is the same order of magnitude as $k_{R'}$.

On the basis of the above considerations of the static and kinetic data, it is confirmed that the binding of TB to Ch4-S is essentially an electrostatic interaction but consists of a strong cooperative interaction ($K \gg K^*$), and that the recombination of dye molecules with binding sites in the immediate neighborhood of already occupied sites is a diffusion controlled process.

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*** The average number of ligands in a stacked aggregate is generally calculated by the following relation:[2]

$$m_a^\infty = \lambda_0(g/s)^{1/2}\cdot\{\bar{\theta}/(1-\bar{\theta})\}^{1/2},$$

where

$$\lambda_0 = 1 + (\sigma s)^{1/2}\cdot\{\bar{\theta}/(1-\bar{\theta})^{1/2}, \qquad \sigma = 1/q.$$

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