Competitive Interaction of Polyanions and Anionic Dye with Bovine Serum Albumin

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Synopsis

The binding of anionic dye, *p*-(2-amino-6-sulfonyl-8-naphthylazo)benzene sulfonic acid disodium salt (ASANA) to bovine serum albumin (BSA) at pH 7.5 has been studied by spectrophotometric techniques. The values of the dissociation constants were obtained with the use of the Benesi-Hildebrand equation for ASANA. Competitive binding of polyanions, sodium poly(styrene sulfonate) (PSSNa), potassium poly(vinyl sulfonate) (PVSK), poly(acrylic acid) (PAA), and poly(methacrylic acid) (PMAA) and anionic dye to BSA was evaluated through the variations in the different spectra of BSA-dye-polymer systems.

INTRODUCTION

Many charged small molecules, especially dyes, are known to bind to proteins through electrostatic and hydrophobic interactions.[1] In favorable cases, hydrogen bonding, which is a highly directional force, strengthens the binding. Electrostatic interaction includes an exchange process of the binding molecule with the coexisting anion or cation, Cl^- or K^+ for instance. Since these ions are small compared with the ionic groups on the exchanging molecules, such as sulfonate, carboxylate, and primary amino groups, the electrostatic interaction per se is generally unfavorable for the replacement. It is known that molecular processes that accompany binding assist largely in the free energy gain upon binding. The release of water molecules from around the relevant charged moieties and hydrophobic interactions, for example, have often invoked to explain binding. The latter interaction explains why only a few charged sites out of many on a protein can accomodate the binding molecule. Thus, dyes and their analog bind to specific sites on a protein with the binding constants as high as 10^5. Such binding behavior can easily be studied by methacromasy of the dye upon binding.[2,3] Conversely, if the bound dye state is perturbed by the presence of a third substance, we presume a polyanion, it will provide a means to study the binding stoichiometry and nature of the binding. The studies described in preceding papers[4-8] have revealed that the detection of macromolecular binding to protein in a quanti-

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tative sense is rather difficult. In this paper, we describe the competitive binding to BSA of some anionic polyelectrolytes with anionic dye. Polyelectrolytes were chosen to give some information on the effect on the binding of the hydrophobic moieties in the chain.

EXPERIMENTAL

Materials

p-(2-Amino-6-sulfonyl-8-naphthylazo)benzene sulfonic acid with high purity to give a single spot on paper chromatography was kindly provided by Dr. Nakano in this laboratory.

BSA was purchased from Armour Pharmaceutical Laboratory Co., as fraction V, lot no. V 78003.

Poly(acrylic acid), prepared by radical polymerization, was used. The synthesis and characterization were described in a previous paper.[4,5] The polymer with $M_W = 12 \times 10^4$ was used.

For Poly(methacrylic acid), a 15% solution of distilled methacrylic acid in benzene was polymerized at 70°C for 4 h in the presence of 0.1% benzoyl peroxide. The recovered polymer was purified once by dissolution in methanol and precipitation with ether and dried in vacuo at 40°C. The molecular weight was determined by viscosity measurements through $[\eta] = 6.6 \times 10^4 M$ (0.002 *N* HCl).[9] The polymer with $M_w = 7.1 \times 10^5$ was used.

Poly(styrene sulfonic acid) sodium salt was kindly provided by Dr. Nakano. The M_v was 1.16×10^6 (99% sulfonate).

Poly(vinyl sulfate) potassium salt (PVSK) was purchased from Wako Pure Chemical Co. The polymer was lyophilized once before use. The M_w was 24.3×10^4.

Solutions

All the solutions were prepared in Na_2HPO_4-KH_2PO_4 0.2 *M* phosphate buffer solutions at pH 7.5.

Absorption Spectra

The visible light absorption measurements were carried out with a Hitachi 556 double-wavelength double-beam spectrophotometer equipped with a thermostated cell holder.

Determination of ASANA-BSA Dissociation Constants

Difference spectra for BSA-dye systems were measured in the 350–600 nm region at various temperatures. Spectrophotometric traces were obtained for sample solutions containing BSA and dye measured against reference solutions that were identical except that BSA was omitted. Experiments at a given pH were performed with sample solutions containing a constant concentration of dye and varying concentrations of BSA.

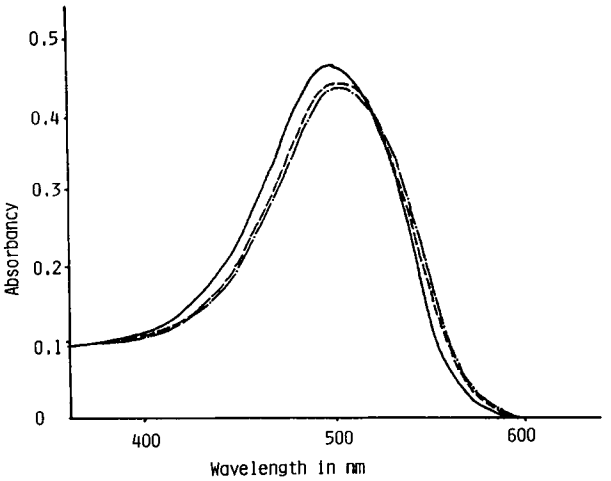

Fig. 1. Spectral change of ASANA in the presence of bovine serum albumin (BSA): (-----) 3×10^{-5} M ASANA; (-·-·-) 3×10^{-5} M ASANA and 6×10^{-5} M BSA; (----) 3×10^{-5} M ASANA, 6×10^{-5} M BSA, and 2×10^{-3} M PAA, in phosphate buffer solution at pH 7.5 ($I = 0.2$) and 25°C.

Competitive Binding of Dye-Polyanion-BSA Systems

Difference spectra were obtained by making measurements with solutions containing BSA and dye in the reference beam and solutions containing polyanions, in addition to the same concentrations of BSA and dye in the sample beam.

RESULTS AND DISCUSSION

Figure 1 shows the spectra change of ASANA upon binding to bovine serum albumin in 0.2 *M* phosphate buffer solution at pH 7.5 and 25°C. Binding of ASANA to BSA causes a remarkable change in the optical absorption of the dye. An ASANA-BSA mixture measured against ASANA exhibits a difference spectrum due to the red shift of the absorbance maximum from 490 nm for the free dye to 510 nm when bound to the BSA. The addition of PAA to solutions of ASANA-BSA produces a change in the difference spectra. The difference spectra are shown in Figure 2. The difference spectra exhibit a large maximum negative deviation at a shorter wavelength of 483 nm, a maximum positive deviation at a longer wavelength of 545 nm, and an isosbestic point at 525 nm. The peak heights of difference spectra increase with an increase in BSA concentration. The dissociation constant for the binding of ASANA to BSA was determined according to the method of Benesi-Hildebrand.[10] Differences in absorbance between solutions of the dye at a constant concentration and BSA with varying concentrations are represented as

$$\Delta A_{545-483} = [D]_0 \epsilon_{MD} - ([D]\epsilon_{MD} + [ED]\epsilon_{MD}) \quad (1)$$

where $[D]_0$ represents the total molar dye concentration and $[D]$ and $[ED]$ the molar concentration of the free dye and of the BSA-dye complex, respectively. ϵ_{MD} and ϵ_{MED} are the molar absorbance coefficients of the dye and of

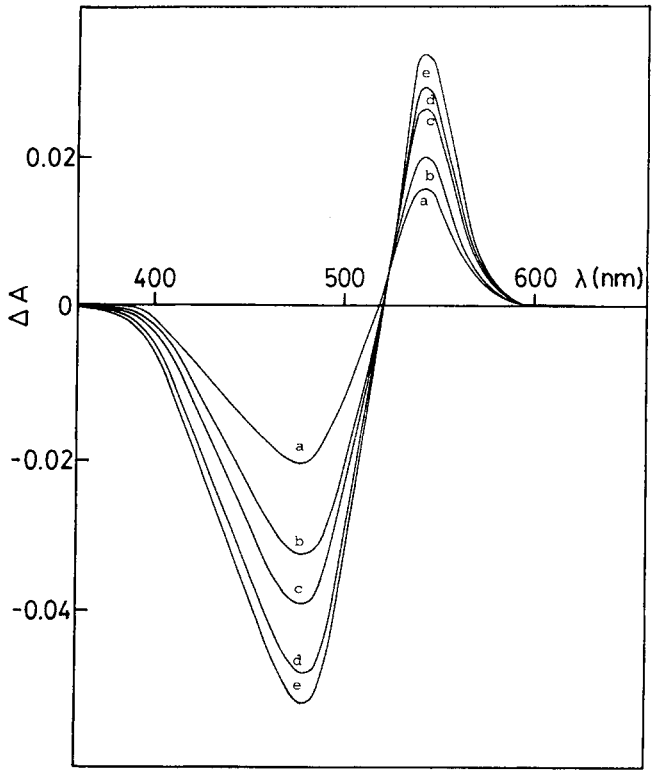

Fig. 2. Difference spectra of ASANA-BSA systems with varying concentrations of BSA in phosphate buffer solution at pH 7.5 ($I = 0.2$); 3×10^{-5} M ASANA: (a) 6×10^{-5} M BSA, (b) 9×10^{-5} M BSA, (c) 12×10^{-5} M BSA, (d) 15×10^{-5} M BSA, (e) 18×10^{-5} M BSA.

the BSA-dye complex at a particular wavelength, $\Delta\epsilon_M = \epsilon_{MD} - \epsilon_{MED}$,

$$[ED] = \Delta A\,[\Delta\epsilon]^{-1} \tag{2}$$

under $[E]_0 \gg [D]_0$, the dissociation constant of the BSA-dye complex is approximated as

$$K_{ED} = \frac{[E]_0([D]_0 - [ED])}{[ED]} \tag{3}$$

$[E]_0$ represents the total molar concentration of BSA. Substitution of eq. (2) into eq. (3) and rearrangement yield a linear relation of eq. (4) on ΔA versus $\Delta A\,[E]_0^{-1}$ from which K_{ED} and $\Delta\epsilon_M$ can be evaluated.

$$\Delta A = \Delta\epsilon_M\,[D]_0 - \frac{\Delta A}{[E]_0}K_{ED} \tag{4}$$

The dissociation constants of the ASANA-BSA complex at various temperatures, calculated from the slopes of the linear lines in Figure 3 range from 1.9 to 3.7×10^{-6}. Figure 4 shows the van't Hoff plots of the dissociation constant

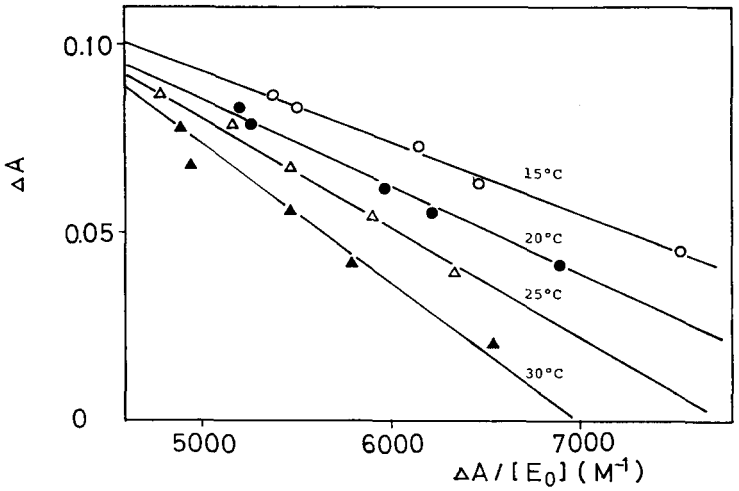

Fig. 3. Benesi-Hildebrand plots for ASANA-BSA systems at pH 7.5 (I = 0.2).

against reciprocal temperature. The dissociation constants and the thermodynamic parameters obtained are given in Table I. The strong binding of ASANA to BSA means that this dye binds to a specific site on BSA. The binding of polyanions with BSA was evaluated spectrophotometrically by utilizing the competition with ASANA. The difference in the absorbances of ASANA-BSA and ASANA solutions was reduced by the addition of some polyanions. Figure 5 shows ΔE values against the concentration of polyanions added to a ASANA-BSA solution at pH 7.5. ΔE is the difference between the maximum and the minimum absorbances in the presence of polyanion. PAA and PMAA scarcely alter the difference spectra of ASANA-BSA systems. The spectral change is not sufficiently large to measure quantitatively the parameter in competitive binding of PAA. For this reason, the measured difference spectra were only as the index of binding.

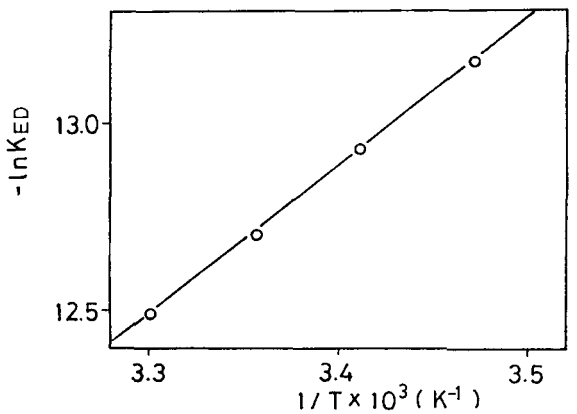

Fig. 4. van't Hoff plots of the dissociation constants for ASANA-BSA complex at pH 7.5 (I = 0.2).

TABLE I
Dissociation Constants and Thermodynamic Parameters for ASANA-BSA at pH 7.5, Ionic Strength 0.2

Temperature (°C)	K_{DE} (mol/L)	ΔH^0 (kJ/mol)	ΔS^0 (J/mol-deg)
15	1.9×10^{-6}	32.2	3.01
20	2.3×10^{-6}		
25	3.0×10^{-6}		
30	3.7×10^{-6}		

An excess amount of BSA over that of ASANA was employed in these experiments to ensure that ASANA was bound only at its strongest binding site: in this way, change in the difference spectrum arizing from the competition among ASANA molecules as competition proceeded was avoided. Figure 5 shows the changes in ΔE for PAA, PMAA, PSSNa and PVSK addition to BSA-ASANA systems. PSSNa gives rise to the strongest decrease in the ΔE of ASANA-BSA systems in comparison with PAA, PMAA and PVSK. This result reflects that PSSNa binds to BSA most strongly in competition with ASANA for the same binding site on BSA. The dissociation constants of ASANA and a polyanion are given as

$$K_d = \frac{[E_0 - ED' - EP][D_0 - ED']}{[ED']} \tag{5}$$

and

$$K_p = \frac{[E_0 - ED' - EP][P_0 - EP]}{[EP]} \tag{6}$$

where $[P_0]$ represents the total molar concentration of polyanion and $[ED']$ and $[EP]$ the molar concentration of BSA-dye complex and BSA-polyanion

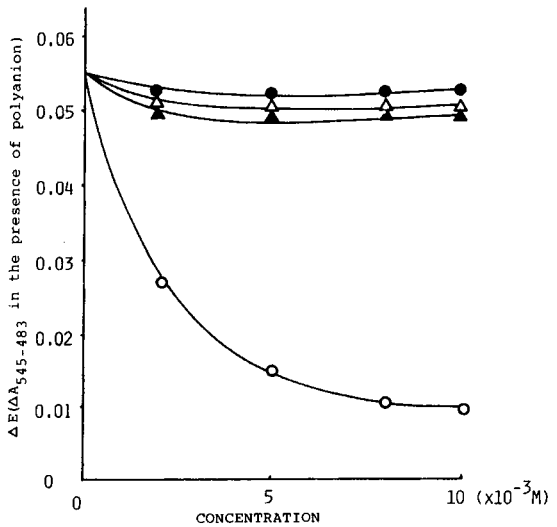

Fig. 5. ΔE of ASANA-BSA solutions with varying concentrations of various polyanions. 3×10^{-4} M BSA and 2×10^{-5} M ASANA with $0\text{–}1 \times 10^{-3}$ residue mole of polyanion. Phosphate buffer (pH 7.5 and $I = 0.2$) at 25°C. (○) PAA; (△) PMAA; (○) PSSNa; (△) PVSK.

TABLE II
Dissociation Constants for ASANA-BSA-Polyanion Systems

Concentration of polyanion (residue mol/L) ($\times 10^{-3}$)	K_p(mol/L)			
	PSSNa ($\times 10^{-6}$)	PAA ($\times 10^{-4}$)	PMAA ($\times 10^{-4}$)	PVSK ($\times 10^{-4}$)
2.0	9.40	5.85	6.86	7.86
5.0	9.50	21.5	21.5	19.1
8.0	10.4	40.6	26.3	18.7
10.0	13.1	103	33.0	100

complex, respectively. From the ΔE changes shown in Figure 5, the dissociation constants K_p of the polyanions to BSA were evaluated. K_p was determined as follows. From eq. (2), $\Delta\epsilon$ was determined and ΔE is given by $\Delta E = \Delta\epsilon[\mathrm{ED}']$. After introducing $[\mathrm{ED}']$ in eqs. (5) and (6) at a constant concentration of BSA and polymer, K_p was determined. The values given in Table II exhibit two different trends in variations with the concentration of PSSNa and PMAA, both of which have hydrophobic moieties nearby. The marked concentration-dependent decrease of K_p values found for PAA and PVSK are worthy of attention. Apparently, dye release from BSA becomes less and less with the concentrations of these polymers. This may occur if the dissociation constants of these polymers become larger with the concentration, which is, however, highly unlikely because of the diversity in the electrolytic nature of the two polymers and because of the constancy of PAA dissociation constants found in preceding papers.[4-6]

We propose a new mechanism in which dye binding is enhanced upon the binding of these polymers to BSA. From the site where the dye is bound when free from these polymers, the dye is released by substitution by the polymer moieties. At some other sites produced by the polymer binding, a new dye-BSA complex is formed. This enhanced dye binding mechanism by cobinding polymers, such as PAA and PVSK, can be interpreted by the deviation of the results in Table II. The balance between these two processes gives rise to the observed insensitivity of the bound dye levels, as we have seen in a previous paper.[7]

References

1. V. P. Schaubhag and C. G. Axelsson, *Eur. J. Biochem.*, **P3**, 363 (1979).
2. J. Reynolds, S. Herbert, and J. Steinhardt, *Biochemistry*, **7**, 1357 (1968).
3. V. P. Schaubhag and C. G. Axelsson, *Eur. J. Biochem.*, **60**, 17 (1975).
4. M. Sakamoto, N. Kuramoto, J. Komiyama, and T. Iijima, *Int. J. Biol. Macromol.*, **4**, 207 (1982).
5. N. Kuramoto, M. Sakamoto, J. Komiyama, and T. Iijima, *Int. J. Biol. Macromol.*, **6**, 69 (1984).
6. N. Kuramoto, M. Sakamoto, J. Komiyama, and T. Iijima, *Makromol. Chem.*, **185**, 1419 (1984).
7. N. Kuramoto, M. Sakamoto, J. Komiyama, and T. Iijima, *Angew. Makromol. Chem.*, **133**, 63 (1985).
8. N. Kuramoto, M. Sakamoto, J. Komiyama, and T. Iijima, *J. Appl. Polym. Sci.*, **30**, 2847 (1985).
9. A. Katchalsky and H. Eisenberg, *J. Polym. Sci.*, **6**, 145 (1951).
10. H. A. Benesi and J. J. Hildebrand, *J. Amer. Chem. Soc.*, **71**, 2703 (1949).

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