Estimation of Interaction Between Polyanions and Bovine Serum Albumin by Means of Affinity Chromatography

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Synopsis

The ligand binding of some polyanions to bovine serum albumin immobilized on Sepharose 4B has been studied by column affinity chromatography. Frontal chromatography using a polyanion of low concentration on an affinity adsorbent gave the dissociation constant K_d of the polyanion-immobilized ligand complex. K_d values determined under various concentrations enabled us to discuss in detail the interactions of bovine serum albumin and polyanions.

INTRODUCTION

Since its introduction in 1910,[1] the technique of affinity chromatography has become an extremely powerful tool in the isolation and purification of biologically active molecules.[2-4] The method is generally based on the original observation that compounds containing free amino groups can be covalently attached to water-insoluble polysaccharides that have been activated by prior treatment with cyanogen bromide.[5] The resourcefulness of this technique in the facile isolation of a variety of biologically active substances has been well documented during the past decade. Affinity chromatography is now becoming a powerful mean in investigations of protein–ligand interactions. As we have discussed in the preceding paper,[6-8] the binding of polymers to proteins is rather difficult to detect. Since the chromatography can give the stoichiometry of polymer–protein binding, the application to the systems of our concern may be fruitful. We have so far studied of interaction of polyanions with proteins to better understand the structure–biological activity relationship between protein and substrate. This paper describes the study of the binding of some polyanions to bovine serum albumin, and the evaluation of dissociation constants by column affinity chromatography. Elution was performed by displacement with pure buffer. The advantage of a chromatographic technique for the study of ligand binding to bovine serum albumin depends on the establishment of a large number of consequtive equilibria between free and bound ligands, thereby increasing the resolution of the binding parameters of different ligands.

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Journal of Applied Polymer Science, Vol. 30, 2847–2852 (1985)
 CCC 0021-8995/85/072847-06$04.00

EXPERIMENTAL

Materials

Bovine serum albumin (BSA) (Armour. Pharm. Co., lot no. V 78003) was immobilized by coupling to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals).[9] After washing out excess albumin, the albumin–Sepharose gel was suspended in a 0.15*M* NaCl and 0.2% sodium azide. This solution was used in all experiments to be described. The gel was filled on columns of a diameter of 1.0 cm.

Poly(acrylic acid) (PAA), poly(methacrylic acid) (PMAA), potassium poly(vinyl sulfate) (PVSK), sodium poly(styrene sulfonate) (PSSNa), and 2-(4′-hydroxyphenylazo) benzoic acid (HABA) were the same as described in the preceding paper.[6-8]

Measurement of Concentration

The measurement of concentration was performed by use of a Refracto Monitor (Mitsumi Scientific Co.) for polyanions. The measurement of HABA concentration was performed with a UV monitor for Shimazu liquid chromatography.

Operation of Frontal Chromatography

Chromatography was carried out in 0.2*M* phosphate buffer at pH 7.5 and 25°C. The column of BSA (4.5 × 1 cm) was previously equilibrated with a 0.2*M* phosphate buffer pH 7.5. After, at the top of the column, solution in the same buffer was applied continuously. The flow rate was about 16 mL/h. Elution of polymer was monitored with the Refracto Monitor.

Regenation of the Column

After the elution had been completed, the columns were regenerated by passing 100 mL of 0.01*N* hydrochloric acid containing 1*M* NaCl, and finally passing 100 mL of 0.2*M* phosphate buffer solution.

RESULTS AND DISCUSSION

When a solution of a substance (concn = $[A]_0$) is applied continuously to the BSA column, a portion of the substance binds to immobilized BSA depending on the equilibrium of this system, and a certain retardation of the substance is observed corresponding to the amount of the bound BSA. However, the concentration of the free substance [A] in the column is maintained at the initial value, because the same amount of free substance relative to the bound BSA is always supplied. The elution volume at which the front of the substance appears is defined as V. The elution volume determined under conditions where the interaction between the substance and the immobilized ligand is completely suppressed is defined as V_0. Then $V - V_0$ corresponds to the amount of the substance which binds to the affinity adsorbent. If the total length of the supporting matrix in the column is assumed to be L, and the density of the substance-immobilized ligand

complex per unit length of the matrix is [AB], the following equation is obtained[10]:

$$[A]_0(V - V_0) = L[AB] \tag{1}$$

If the reaction is

$$A + B \rightleftarrows AB \tag{2}$$

in an equilibrium state, where k_{+1} and k_{-1} are the rate constants of the formation and dissociation of AB, the following equation is obtained[10-12]:

$$K_d = \frac{k_{+1}}{k_{-1}} = \frac{[A]\,[B]}{[AB]} = \frac{[A]_0\,([B]_0 - [AB])}{[AB]}$$

$$= \frac{L[B]_0}{V - V_0} - [A]_0 = \frac{B_t}{V - V_0} - [A]_0 \tag{3}$$

where $[B]_0$ is the density of the total immobilized ligand in the column. Under the condition that $[A]_0$ is negligibly small in comparison with K_d, eq. (3) can be simplified to

$$K_d = \frac{B_t}{V_m - V_0} \tag{4}$$

where V_m is the limiting value of V when $[A]_0$, but analyzed the dependence of V on $[A]_0$. Equation (3) can be rearranged to the following form:

$$[A]_0\,(V - V_0) = \frac{B_t[A]_0}{[A]_0 + K_d} \tag{5}$$

The amount of the substance adsorbed on the immobilized ligand, $[A]_0$ $(V\text{-}V_0)$ is expressed as a function of $[A]_0$. Equation (5) in a double-reciprocal form is

$$\frac{1}{[A]_0\,(V - V_0)} = \frac{K_d}{B_t} \cdot \frac{1}{[A]_0} + \frac{1}{B_t} \tag{6}$$

This equation is analogous to the Lineweaver–Burk equation, in which the terms $[A]_0(V\text{-}V_0)$, B_t, $[A]_0$, and K_d are replaced by v, V_{max}, [S], and K_m, respectively. By plotting $1/[A]_0(V\text{-}V_0)$ vs. $1/[A]_0$, we can determine B_t from the intercept on the ordinate and K_d from that on the abssisa. Thus, frontal chromatography can be treated in the same way as enzyme kinetics. Prior to the frontal chromatography, the elution volume V_0, of the solute under conditions where the specific interactions is completely suppressed, was determined. A column was equilibrated with 0.2M phosphate buffer was applied. The elution volume determined from the elution profile of NaCl was 4.1 mL. Figure 1 shows the elution profile of HABA in (a) $9.6 \times 10^{-5}M$,

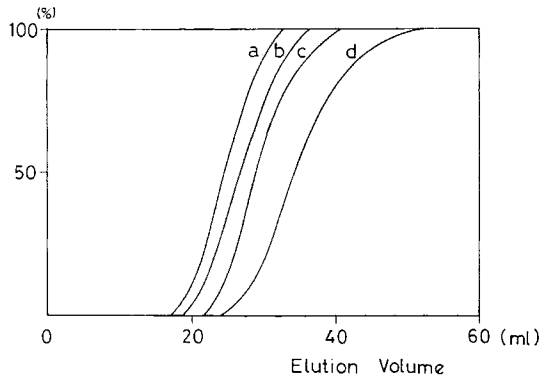

Fig. 1. Elution profiles of HABA. Phosphate buffer (pH = 7.5) at 25°C. $[A]_0$ = concn of HABA ($\times 10^{-5}M$): (a) 9.6; (b) 7.2; (c) 4.8; (d) 2.4.

(b) $7.2 \times 10^{-5}M$, (c) $4.8 \times 10^{-5}M$, and (d) $2.4 \times 10^{-5}M$. The elution volumes determined from the elution front, V, were (a) 28.4 mL, (b) 32.6 mL, (c) 34.9 mL, and (d) 38.1 mL, respectively. Figure 2 shows the plots $1/[A]_0(V\text{-}V_0)$ vs. $1/[A]_0$ for HABA according to eq. (4). The plots of experimental data fitted a straight line well. The values of K_d and B_t were calculated to be $2.0 \times 10^{-5}M$ and $6.6 \times 10^{-7}M$, respectively. Figure 3 shows the plots for PAA and PMAA. The solid circles are the values calculated for PMAA and the open circles are those calculated for PAA. Experiments on PAA and PMAA were performed using a column having a B_t value was somewhat smaller than the capacity for HABA. Such slight inconsistency may be partly attributed to the considerable nonspecific interaction of HABA with the column. The values of K_d calculated by eq. (4) were $9.1 \times 10^{-4}M$ for PAA and $7.1 \times 10^{-4}M$ for PMAA respectively. Experiments on PVSK were performed using a column having a B_t value for PVSK of 5.0×10^{-7} mol. Figure 4 shows the plots according to eq. (6) fitted a straight line. The value of K_d calculated by eq. (4) was $5.4 \times 10^{-4}M$. Figure 5 shows the plots according

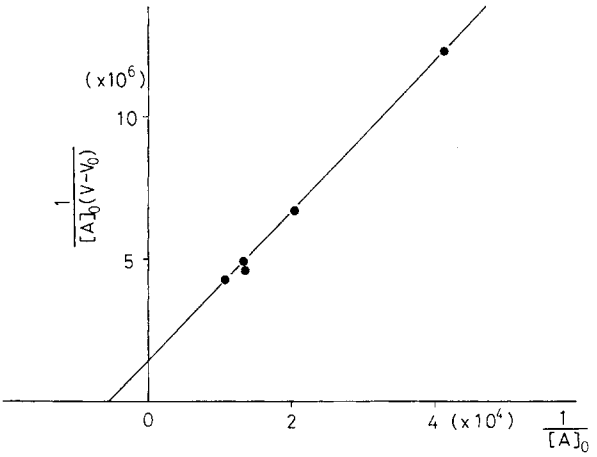

Fig. 2. $1/[A]_0(V\text{-}V_0)$ is plotted against the reciprocal ligand concentration $1/[A]_0$ of HABA dissolved in the eluting buffer. Phosphate buffer (0.2M and pH 7.5) at 25°C.

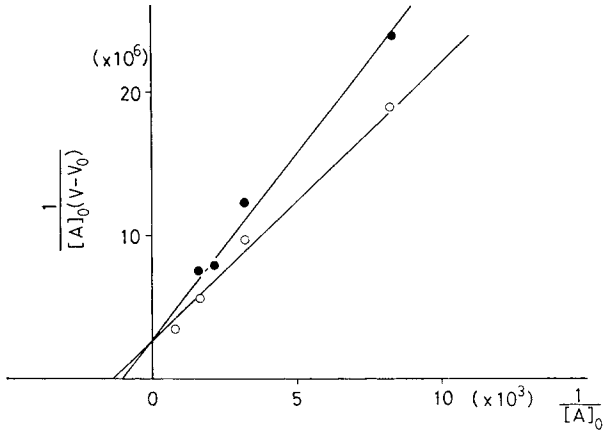

Fig. 3. $1/[A]_0(V-V_0)$ is plotted against the reciprocal ligand concentration $1/[A]_0$ of PMAA (○) and PAA (●) dissolved in the eluting buffer.

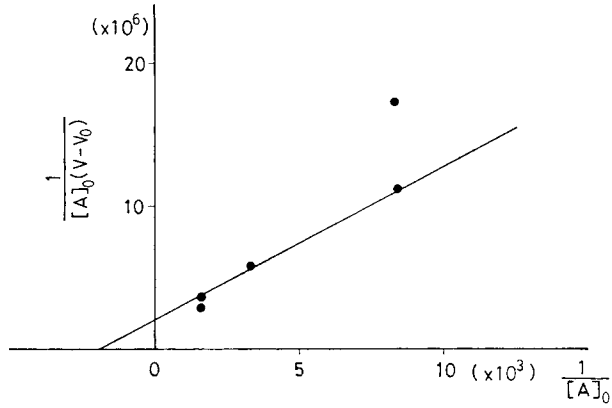

Fig. 4. $1/[A]_0(V-V_0)$ is plotted against the reciprocal ligand concentration $1/[A]_0$ of PVSK dissolved in the eluting buffer.

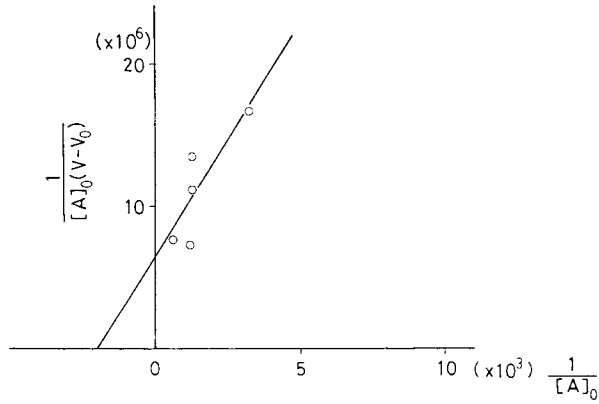

Fig. 5. $1/[A]_0(V-V_0)$ is plotted against the reciprocal ligand concentration $1/[A]_0$ of PSSNa dissolved in the eluting buffer.

TABLE I
The Dissociation Constants K_d Obtained by Frontal Chromatography

Sample	K_d ($\times 10^{-4}$)	M_w ($\times 10^4$)	B_t ($\times 10^{-7}$)
HABA	0.2		6.6
PVSK	5.4	24.3	5.0
PAA	9.1	12.0	3.6
PMAA	7.1	71.0	3.6
PSSNa	5.0	116.0	1.8

to eq. (6) for PSSNa. In this case, the experimental points showed a scatter, but a straight line could well be assumed.

K_d values obtained for all of these substances are summarized in Table I together with the B_t values. It is found that K_d values are in the order PAA > PMAA > PVSK > PSSNa > HABA. The K_d value for HABA corresponds well to the reciprocal binding constant of HABA found in previous paper.[13]. The K_d value for PSSNa is also reasonable since the strongest displacement of bound HABA by PSSNa was found in the same paper. It was found that PAA has the largest K_d values among the polymers investigated. However, PAA has a value of the same order with that for PSSNa, indicating that the binding of these anionic polymers to BSA is largely due to the electrostatic nature of the polymers and not due to the hydrophobic nature of them. In other words, affinity of polymer residue to a specific site or sites on BSA greatly depends on the local hydrophobic nature as found in the previous paper. Yet the overall binding of these polymers is of nonspecific electrostatic nature, as found from the relatively small variation of the K_d values.

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Received September 25, 1984
Accepted October 26, 1984