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BINDING PROTEIN FOR 1-ANILINO-8-NAPHTHALENESULFONATE IN RAT LIVER CYTOPLASM*

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Abstract—The Z-fraction in rat liver cytoplasm, defined first by Levi *et al.* [*J. clin. Invest.* **48**, 2156 (1969)] as an organic anion binding fraction of small molecular weight (10,000–14,000) was further purified by the method of ion-exchange chromatography. The purification was characterized by the use of 1-anilino-8-naphthalenesulfonate (ANS) in sensitively detecting the organic anion binding protein in the Z-fraction. The Z-fraction was found to contain three protein fractions having fairly high binding activities for ANS and sulfobromophthalein (BSP). Among these fractions, the D_2-fraction, which eluted earlier on a DEAE-Sephadex column, had the highest binding affinity for these anions. This D_2-fraction was further purified by CM-Sephadex chromatography. The ANS binding protein, thus purified, was similar in physico-chemical properties to the aminoazodye-binding protein of $S_{20,w}$ 1.6s (Form III) reported by Ketterer *et al.* [*Biochem. J.* **155**, 511 (1976)]. The binding affinities for both ANS and BSP were little influenced by the purification. By the fluorescence method, about 30 per cent of the protein in the Z-fraction was found to be this binding protein. The binding stoichiometries for the high affinity sites were 0.7 for ANS and 0.4 for BSP.

The Z-fraction of hepatic cytoplasm elutes with a molecular weight of about 10,000–14,000 on a Sephadex G-75 column and binds to sulfobromophthalein (BSP), bilirubin and other organic anions [1–3]. A method of isolation of the Z-protein from the Z-fraction, based on both BSP binding activity and radioimmunoassay, was reported by Kamisaka *et al.* [3] and Fleischner *et al.* [4]. The detailed binding characteristics during the isolation, however, have not been reported. Other proteins in the Z-fraction, that have the characteristics of Z-protein, have been implicated in multi-binding activities to xenobiotics and biological substances such as azocarcinogen metabolites [5, 6], cortisol metabolites [7], fatty acid and fatty acid Coenzyme A [8, 9], and thyroid hormone [10]. A fatty acid binding protein [8, 9] that is closely related or identical to the Z-protein in rat liver, is also present in rat small intestine [11]. Recently, Warner and Neims [12] described a purified Z-protein with an affinity for uncharged hexachlorophene. Ketterer *et al.* [13] also purified a protein of $S_{20,w}$ 1.6s and mol. wt 14,000, on the basis of covalent binding to a metabolite of azodye carcinogen.

Our previous report [14] revealed that the fluorescence quantum yield of 1-anilino-8-naphthalenesulfonate (ANS) bound to the Z-fraction is significantly greater than those to the other binding fractions, X and Y. In addition, competition of BSP for the ANS binding sites of the Z-fraction was demonstrated [14]. These findings prompted us to purify the ANS-binding protein and to compare the binding properties for ANS and BSP during the purification.

MATERIALS AND METHODS

Materials

ANS (sodium salt) was purchased from the Tokyo Chemical Industries Co. Ltd., Tokyo, Japan. BSP (in injection form) was purchased from the Daiichi Chemical Industries Co. Ltd., Tokyo, Japan. Standard proteins for the determination of molecular weight were obtained from Böehringer GmbH, Mannheim, West Germany. Sephadex G-75, DEAE-Sephadex A-50 and CM-Sephadex C-50 were purchased from the Pharmacia Fine Chemical Co. Ltd., Uppsala, Sweden. Acrylamide and methylene bis-acrylamide were purchased from the Seikagaku Kogyo Co. Ltd., Tokyo, Japan. All the chemicals were used without further purification. Cells for the equilibrium dialysis method were obtained from the Kokugo Gomu Co. Ltd., Tokyo, Japan. Visking tubing (wall thickness 0.001 inch) was purchased from the Union Carbide Co., Chicago, IL, U.S.A. All other reagents were commercially available and of analytical grade.

Animals

Male Donryu rats (Nihon Rat Co. Ltd., Tokyo, Japan), weighing 270–330 g, were used as the liver source.

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Purification of Z-protein

All procedures were performed in the cold room (4°) or on icc.

Step 1. *Preparation of the supernatant fraction*: A 100,000 *g* supernatant fraction was prepared from a 50% homogenate of 200 g of liver from eighteen rats as described previously [15].

Step 2: *Sephadex G-75 gel filtration (the first time)*: The 140 ml of supernatant fluid from Step 1 was divided into equal-volume fractions (70 ml). Both fractions were chromatographed on a Sephadex G-75 column (5.4 × 85 cm) which was equilibrated with 50 mM Tris–HCl buffer, pH 7.4 (buffer A). Elution was performed with buffer A using a pump driven downward flow (45 ml/hr), and aliquots of 10 ml were collected. The Z-fraction, of which the fraction number was determined preliminarily, was collected.

Step 3: *Sephadex G-75 gel filtration (the second time)*: The Z-fraction from Step 2 (250 ml) was concentrated to 20 ml by lyophilization after dialysis against distilled water. The concentrated Z-fraction was chromatographed on the same Sephadex G-75 column under the same conditions as described in Step 2. Samples around the protein peak at the same fraction numbers as in Step 2 were collected.

Step 4: *DEAE-Sephadex chromatography*: The fraction from Step 3 (120 ml) was concentrated to 20 ml by lyophilization and dialyzed against 10 mM Tris–HCl buffer (pH 8.2) (buffer B) for 24 hr. The dialyzed sample was centrifuged at 10,000 r.p.m. for 30 min; the clear supernatant fraction was placed on a DEAE-Sephadex A-50 column (1.4 × 21 cm) equilibrated with buffer B. After eluting the column with 160 ml of buffer B, a linear gradient composed of 150 ml of buffer B and the same volume of buffer B containing 0.5 M NaCl was used to elute the column. Aliquots of 4 ml were collected at a flow rate of 12 ml/hr. In order of elution, the protein fractions were designated D_1, D_2, D_3, D_4, D_5 and D_6. These fractions were dialyzed against buffer A and diluted with the same buffer to a concentration of 0.13 absorbance units at 280 nm. ANS (0.1 ml, 1 mM) was then added to 3 ml of each fraction; the fluorescence was measured at 480 nm (excited at 400 nm) in a Hitachi MPF-4 fluorospectrometer.

Step 5: *CM-Sephadex chromatography*: The D_2-fraction (25 ml) from Step 4 was concentrated to 8 ml by ultrafiltration, using a Diafilter G-05T membrane, and dialyzed for 24 hr against 50 mM acetate buffer (pH 6.0) (buffer C). The dialyzed sample was placed on a CM-Sephadex C-50 column (1.4 × 15 cm) equilibrated with buffer C. Elution was performed with 200 ml of buffer C at a flow rate of 12 ml/hr. The first protein fraction that eluted was designated as the C_v-protein. The second protein fraction remaining in the column was eluted with buffer C containing 0.5 M NaCl, and was designated as the C_{NaCl}-fraction.

Preliminary identification of the Z-fraction

The peak of the Z-fraction of the gel filtration was preliminarily determined as follows. The supernatant fraction (10 ml) was mixed with BSP (10 μmoles) and the mixture was placed on a Sephadex G-75 column under the same conditions as described in the purification procedure. Three peaks of BSP binding to the proteins were obtained and identified as the X-, Y- and Z-fraction, respectively, following the nomenclature of Levi *et al.* [1].

Protein concentration

The concentration of protein was determined by the method of Lowry *et al.* [16] with bovine serum albumin as the standard. The concentration of bovine serum albumin was determined from the extinction coefficient, $E^{1\%}_{279} = 6.7$.

Electrophoresis

Polyacrylamide electrophoresis (7.5% acrylamide) was carried out by the method of Davis [17] on a running gel at pH 8.9.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis was performed as described by Weber and Osborn [18]. Prior to the electrophoresis, the proteins were incubated with 1% mercaptoethanol for 15–20 hr at room temperature. The gels were fixed with 50% trichloroacetic acid for 1 hr and stained with 0.25% Coomassie brilliant blue–20% trichloroacetic acid.

Molecular weight determination

The molecular weight was estimated by gel filtration on a column (2.8 × 54 cm) of Sephadex G-75 equilibrated with buffer A. The molecular weight of the polypeptide chain was determined by sodium dodecylsulfate–polyacrylamide gel electrophoresis. The proteins used for the calibration of molecular weight were cytochrome *c* (12,500), myoglobin (17,000), chymotrypsinogen (25,000) hen egg albumin (45,000) and bovine serum albumin (67,000), respectively.

Equilibrium dialysis

The protein samples were previously dialyzed against buffer A for 20–30 hr. Equilibrium dialysis was performed using the dialysis cell. The details were described in a previous paper [15].

Determination of binding constant for ANS by fluorescence intensity

Binding studies for ANS were performed in a Hitachi MPF-4 fluorospectrometer at room temperature. To the cuvettes containing 3 ml of protein in buffer A were added small samples (2–10 μl) of ANS stock solution (5 mM), up to a 30 μl total sample volume. The solution was stirred with a glass rod after each addition and the fluorescence was measured at 480 nm (excited at 400 nm). If necessary, the fluorescence intensity was corrected for the 'inner filter' effect by the method of Chignell [19]. The apparent binding constant was calculated from a plot of the reciprocal of the variation in fluorescence against the reciprocal of total ANS concentration; the intercept of the abscissa gives the apparent binding constant for ANS [20]. The reciprocal of the intercept of the ordinate gives the I_{max} value, which is proportional to the number of ANS binding sites per g of protein if the quantum yield of ANS bound is assumed to be constant.

Determination of the stoichiometry for binding of ANS and BSP to C_v-protein

The stoichiometry of the binding of ANS to C_v-

protein was determined from the fluorescence data. The fraction of ANS bound (f_b) was calculated using the following equation,

$$f_b \equiv \frac{C_b}{C_t} = (I_0 - I_f)/(I_b - I_f), \quad (1)$$

where C_t and C_b are the concentrations of total ANS and bound ANS, respectively, and I_0 and I_f are the fluorescence intensities of a given concentration of ANS in the solution with low protein concentration and in the solution without protein, respectively; I_b is the fluorescence intensity of the same concentration of totally bound ANS, i.e. the fluorescence intensity of ANS in the presence of excess C_v-protein. Thus, C_b and free concentration of ANS (C_f) are given, and the stoichiometry is calculated with a Scatchard plot assuming the molecular weight of C_v-protein to be 13,000 (see text).

The stoichiometry of the binding of BSP to C_v-protein was determined by the equilibrium dialysis method. The concentration of C_v-protein was 9.1 μM, and the concentration of BSP in the buffer solution ranged from 2 to 17 μM. The binding parameters were obtained with a non-linear iterative least squares method using a Hitachi 8700/8800 digital computer.

RESULTS

Preparation of ANS binding protein

A summary of the purification steps is presented in Table 1.

The Z-fraction, obtained after gel filtration twice, was placed on a DEAE-Sephadex column. The elution pattern is shown in Fig. 1. Six fractions (D_1, D_2, D_3, D_4, D_5 and D_6), shown by the horizontal bars, were pooled and the binding activities for ANS were determined from the fluorescence intensity. The D_2-, D_3- and D_4-fractions had high binding activities, but other fractions had little activity, as shown in the inset of Fig. 1.

Double-reciprocal plots of ANS binding to these three fractions (D_2, D_3 and D_4), plotted by the method of Wang and Edelman [20], are shown in Fig. 2. Apparent binding constants of $5 \times 10^4\ M^{-1}$, $1.8 \times 10^4\ M^{-1}$ and $1.8 \times 10^4\ M^{-1}$ were obtained for the D_2-, D_3- and D_4-fraction, respectively.

Figure 3 shows the Scatchard plots of BSP binding to these proteins. Binding constants of $1.6 \times 10^6\ M^{-1}$, $0.45 \times 10^6\ M^{-1}$ and $0.2 \times 10^6 M^{-1}$ were obtained for the D_2-, D_3- and D_4-fractions, respectively. Since the D_2-fraction had the highest binding affinity of the three fractions, further purification was performed on a CM-Sephadex column. The protein that eluted on the CM-Sephadex column had a high binding affinity for ANS, whereas the protein that remained in the column (C_{NaCl}-fraction) did not show any binding affinity for ANS.

Binding of ANS to the fractions in the purification process

The binding of ANS to the Z-fraction, the D_2-fraction, and the C_v-protein was determined by the fluorescence method for each purification step. The protein concentration of each fraction or protein was 0.13 absorbance units at 280 nm. The titration curves of these fractions and protein with ANS were plotted by the method of Wang and Edelman [20] as shown in Fig. 4. It is clear that the apparent binding constant was not influenced by the purification, since the intercepts of the abscissa in these plots are the same. On the other hand, the reciprocal of the intercept of the ordinate, i.e. I_{max}, increased with purification. Table 1 summarizes the results of the purification on the basis of the I_{max} value (specific intensity). Consequently, this procedure resulted in a 67-fold purification of ANS binding protein from the liver cytosol.

Electrophoresis of the Z-fraction

The result of polyacrylamide gel electrophoresis of the different samples obtained in the purification process is shown in Fig. 5. The final C_v-protein migrated as a single band. The Z-fraction, after gel filtration twice, contained two major protein bands, namely band 1 and band 2. Band 1 corresponded to the major protein in the D_2-fraction, i.e. C_v-protein, while band 2 corresponded to the major protein in the D_3-fraction.

In the dodecylsulfate-gel electrophoresis, the Z-fraction and the D_2-fraction migrated to the same position as a single band. This finding indicates that the proteins in the Z-fraction may have been of the same molecular weight.

Table 1. Purification table of ANS binding protein in the Z-fraction

Purification step	Total protein (mg)	Specific intensity* (units/mg)	Total intensity* (units)	Yield (%)	Purity (%)
(1) Supernatant fraction	7500	3.6	27,000	100	1.5
(2) Sephadex G-75 gel filtration	172	62	10,664	39	26
(3) Sephadex G-75 gel filtration (Z-fraction)	110	74	8140	30	32
(4) DEAE-Sephadex (D_2-fraction)	3.4	162	551	2	69
(5) CM-Sephadex (C_v-protein)	2.2	234	515	1.9	100

* Arbitrary units.

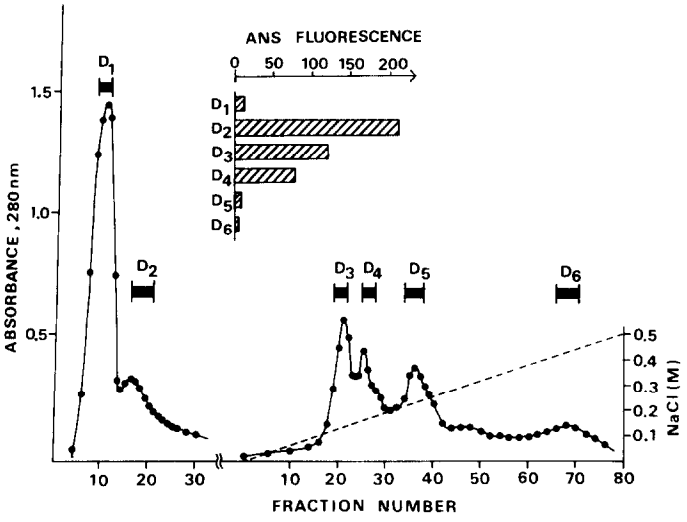

Fig. 1. Elution profile of the Z-fraction on a DEAE-Sephadex A-50 column. Z-fraction, obtained after two gel filtrations (G-75), was placed on a DEAE-Sephadex A-50 column (20 × 1.4 cm) equilibrated with 10 mM Tris–HCl buffer, pH 8.2. Elution was performed with a linear gradient of NaCl at a flow rate of 12 ml/hr, and aliquots of 4 ml were collected. Six fractions, shown by the horizontal bars, were pooled, and binding activities for ANS were determined by the fluorescence enhancement. See text for details.

Molecular weight determination

Molecular weights of purified C_V-protein, estimated by both gel filtration and dodecylsulfate-gel electrophoresis, were 14,000 and 13,000, respectively.

Ultraviolet-absorption spectrum

The ultraviolet-absorption spectrum of the purified C_V-protein in 50 mM Tris–HCl buffer (pH 7.4) revealed an explicit shoulder at 285 nm. This characteristic was reported previously by Ketterer *et al.* [13] in the protein of Form III. The ratio of the absorbance at 280 to that at 260 nm was 1.32. Table 2 summarizes the 280/260 ratio for the various protein fractions in the purification process. The D_2-, D_3- and D_4-fractions, which bind ANS with higher affinity, have similar 280/260 ratios (1.34–1.35), whereas the D_1-fraction, which binds little ANS, has a 280/260 ratio of 1.68.

Stoichiometry of the binding characteristics

The stoichiometry of ANS binding to purified C_V-protein was determined from the *x*-intercept of the slope of a Scatchard plot (Fig. 6), and the value of 0.7 mole of ANS bound per mole of C_V-protein was obtained. The binding constant (K_a) was also determined from the slope; the K_a was $0.4 \times 10^6\ M^{-1}$.

The stoichiometry of BSP binding to the C_V-protein was also determined by the equilibrium dialysis method; a Scatchard plot of the results is shown in

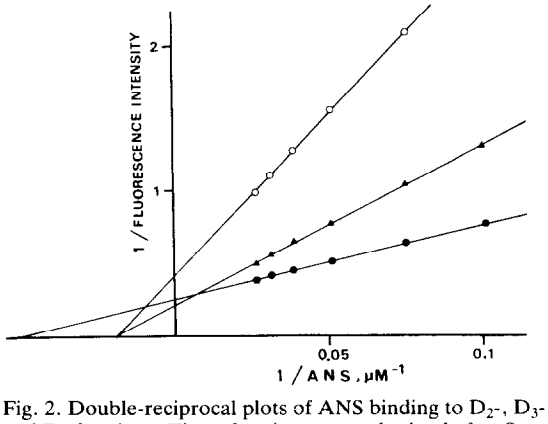

Fig. 2. Double-reciprocal plots of ANS binding to D_2-, D_3- and D_4-fractions. These fractions were obtained after Step 4 (see text for details). The protein concentration of each fraction was 0.13 absorbance units at 280 nm. Key: (●) D_2-fraction; (▲) D_3-fraction, and (○) D_4-fraction.

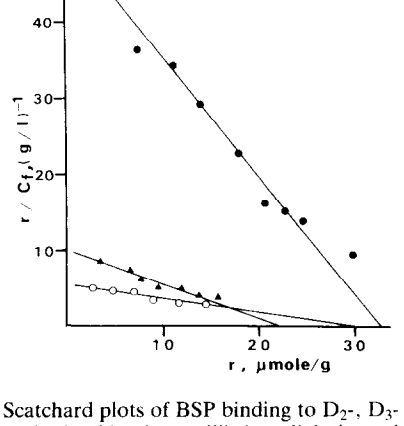

Fig. 3. Scatchard plots of BSP binding to D_2-, D_3- and D_4-fractions obtained by the equilibrium dialysis method. The protein concentrations used were: 350 μg/ml for the D_2-fraction, 400 μg/ml for the D_3-fraction and 410 μg/ml for the D_4-fraction. Key: (●) D_2-fraction, (▲) D_3-fraction. (○) D_4-fraction.

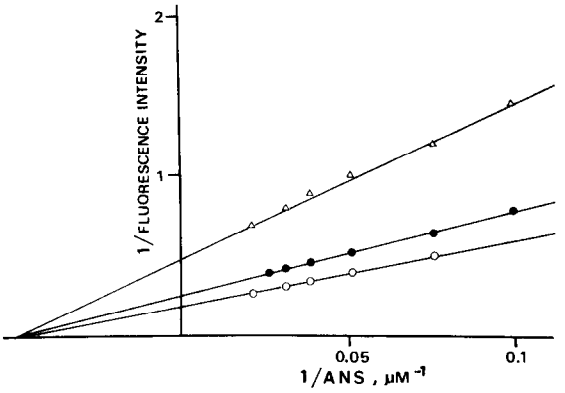

Fig. 4. Double-reciprocal plots of the titration curves for ANS binding to the Z-fraction, the D_2-fraction and C_v-protein. The protein concentration of each fraction and/or protein was 0.13 absorbance units at 280 nm. Key: (△) after Sephadex G-75 gel filtration (second time, Step 3), (●) D_2-fraction after DEAE-Sephadex chromatography (Step 4), and (○) purified C_v-protein after CM-Sephadex chromatography (Step 5).

Table 2. Ultraviolet-absorption spectrum in the purification process

Purification step*	Fraction	Abs(280 nm) / Abs(260 nm)
3	Z-fraction	1.46
4	D_1-fraction	1.68
	D_2-fraction	1.35
	D_3-fraction	1.34
	D_4-fraction	1.34
5	C_v-fraction	1.32

* See details in Materials and Methods.

Fig. 7. The curvature of the plot suggests the existence of at least two classes of binding site. The binding parameters obtained by the least squares method are $n_1 = 0.44$, $n_2 = 2.44$, $K_1 = 2.75 \times 10^6$ M^{-1}. The binding constant for the high affinity site ($2.8 \times 10^6\,M^{-1}$) is in fairly good agreement with the value ($2.5 \times 10^6\,M^{-1}$) as reported by the authors using the crude Z-fraction [14].

DISCUSSION

By the fluorescence method, the Z-fraction, which was homogenous in molecular weight (Fig. 4) was found to contain three protein fractions (D_2, D_3 and D_4) having high binding affinities for ANS. Among these fractions, the D_2-fraction had the highest binding affinity for both ANS and BSP (Figs. 2 and 3). Recently, Ketterer *et al.* [13] isolated three proteins of 1.6s and mol. wt 14,000 with isoelectric points of

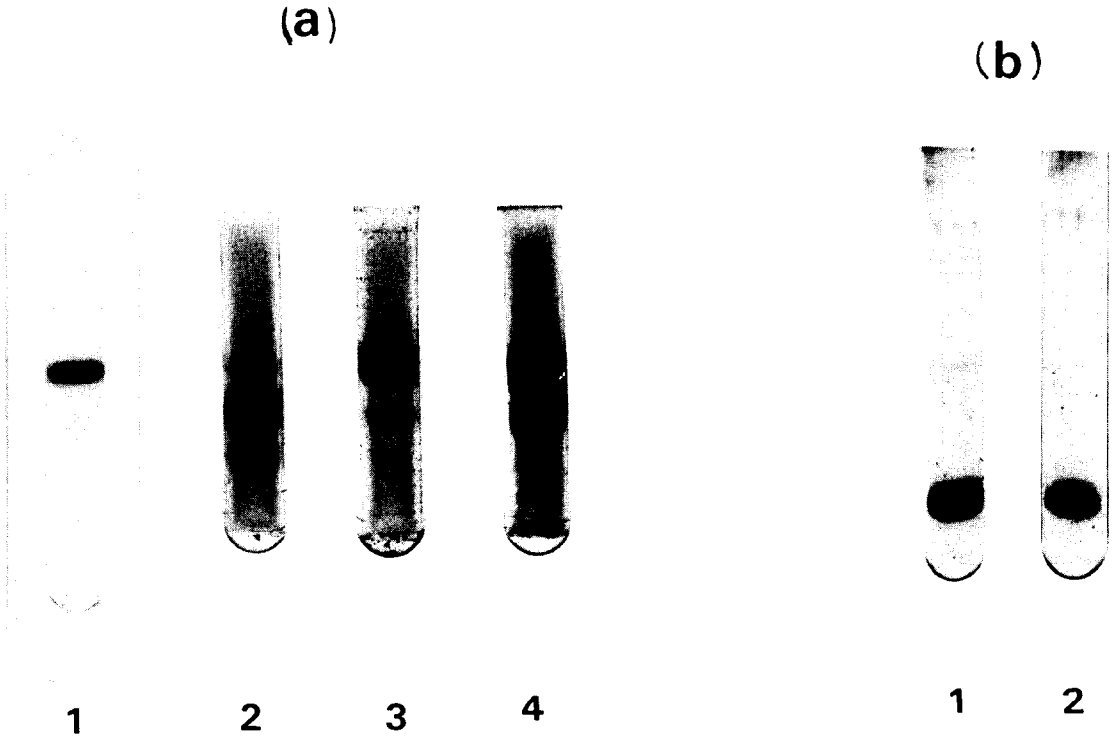

Fig. 5. Panel a: Polyacrylamide disk gel electrophoresis of the various samples obtained in the purification process (7.5% acrylamide at pH 8.9). (1) C_v-protein, (2) D_3-fraction, (3) D_2-fraction, and (4) Z-fraction obtained after gel filtration twice. Electrophoresis of the Z-, D_2- and D_3-fractions was carried out at the same time; the positions of the bands can be compared. Panel b: Dodecylsulfate-gel electrophoresis of Z- and D_2-fractions. Prior to electrophoresis, the proteins were incubated with 1% mercaptoethanol for 15–20 hr at room temperature. (1) D_2-fraction, and (2) Z-fraction after gel filtration twice.

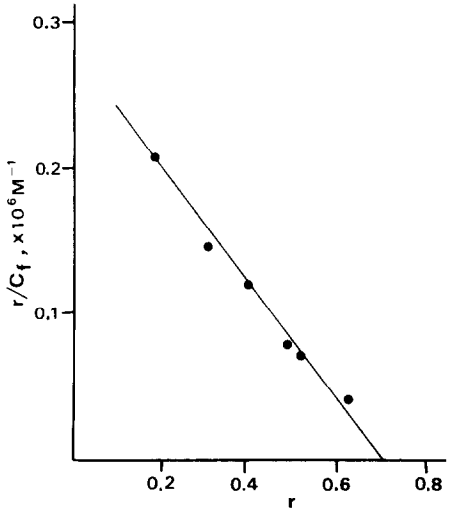

Fig. 6. Scatchard plot of ANS binding to purified C_V-protein, as measured by fluorescence intensity. The sample correlation coefficient r is expressed in moles of ANS bound per mole of C_V-protein, assuming its molecular weight to be 13,000. The concentration range of ANS was 6 to 50 μM, and the concentration of C_V-protein was 25 μM. See text for details.

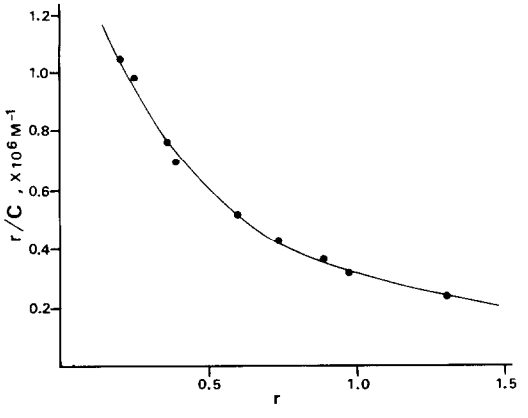

Fig. 7. Scatchard plot of BSP binding to a purified C_V-protein determined by the equilibrium dialysis method. The concentration range of BSP was 2 to 17 μM, and the concentration of C_V-protein was 9.1 μM. The solid line was calculated by a non-linear iterative least squares method using a digital computer, and the binding parameters obtained were: $n_1 = 0.44$, $n_2 = 2.44$, $K_1 = 2.75 \times 10^6\ M^{-1}$, and $K_2 = 1.08 \times 10^5\ M^{-1}$.

5.0 (Form I), 5.9 (Form II) and 7.6 (Form III), respectively, by a method involving isoelectric focusing of rat liver cytosol. They also reported that the basic protein (Form III) had the highest binding affinity for BSP, estrone sulfate and palmitate; the results of the present study support their findings. Our preliminary study by isoelectric focusing of the Z-fraction also suggests that the D_2-fraction corresponds to the fraction with a pI of about 7.5 (unpublished data). On the other hand, Z-protein isolated by Kamisaka *et al.* [3] was reported to be acidic. Furthermore, the fatty acid binding protein, which was isolated by Ockner and Manning [11] from rat intestinal mucosa and appeared to be identical with Z-protein by a comparison of molecular weight, BSP binding activity, and immunological reactivity, had a pI value of 5.55. Accordingly, on the basis of the isoelectric point, the C_V-protein that we purified is similar to the protein reported by Ketterer *et al.* [13], but different from those reported by Kamisaka *et al.* [3] and Ockner and Manning [11]. A possible explanation for these discrepancies is that the Z-protein by Kamisaka *et al.* [3] and Ockner and Manning [11] corresponds to the D_3- or D_4-fraction in the present study and not to the D_2-fraction. A detailed immunochemical study, however, would be necessary for definite identification. Discrepancies in the quantitation of Z-protein by BSP binding and immunological methods were also recently reported by Stein *et al.* [21]. Such diverse findings may result from the multiplicity of the Z-fraction found in the present study.

Previous investigators reported the binding stoichiometry of various substrates to Z-protein, e.g. a molar stoichiometry of 0.5 for bilirubin [3] and of 1 for hexachlorophene [12], BSP, and estrone sulfate [13]. The Scatchard plot of BSP binding showed a curvature, and the best-fitting curve was obtained with an n_1 value of 0.44, using a digital computer (Fig. 7). The number of high affinity binding sites for both ANS and BSP to Z-protein seems to be less than 1. This might have been the result of partial aggregation of Z-protein, as suggested previously [3, 12].

The binding constants for both ANS and BSP do not seem to have been affected by the purification procedures (Fig. 4). On the other hand, Ketterer *et al.* [13, 22] reported that the crude Z-fraction bound various compounds, including BSP, more strongly (6- to 30-fold) than did purified Z-protein, e.g. the binding constants for BSP were $0.25–0.7 \times 10^6\ M^{-1}$ (purified Z-protein) and $2.5–8 \times 10^6\ M^{-1}$ (crude Z-fraction), respectively. However, the C_V-protein, purified in the present study, seems to have retained a fairly high binding affinity for BSP, i.e. $2.5 \times 10^6\ M^{-1}$, which is practically the same value obtained in the crude Z-fraction [14, 15].

Previous experiments in this laboratory [14] revealed that the fluorescence quantum yield of ANS bound to the Z-fraction was greater than that to the Y-fraction, suggesting a higher hydrophobicity of the ANS binding site on the Z-fraction. This ANS technique was used previously by Ketley *et al.* [23] to study the ligand binding properties of ligandin (glutathione *S*-transferase B) isolated from the Y-fraction of rat liver cytosol. In the present study, this sensitive tool using ANS was employed for the detection of the organic anion binding proteins in the Z-fraction of rat liver cytosol.

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