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Protein–ligand interactions studied on bovine serum albumin with free and polymer-bound Cibacron Blue F3G-A as ligand with reference to affinity partitioning

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ABSTRACT

The interactions in aqueous solutions between bovine serum albumin and the textile dye Cibacron Blue F3G-A, in both the free and polymer-bound forms, were studied using difference spectroscopy and gel chromatography. The polymers used as dye carriers were dextran, Ficoll, hydroxypropyl-starch, poly(vinyl alcohol) and poly(ethylene glycol). The addition of poly(ethylene glycol) to the solutions decreases the number of apparent binding sites on the albumin molecule, whereas this does not occur in the presence of dextran. The results were compared with the influence of polymer-bound dye on the partitioning of albumin in aqueous two-phase systems composed of dextran and poly(ethylene glycol). The partitioning of lactate dehydrogenase, which also interacts with this dye, was studied for comparison.

INTRODUCTION

The use of affinity ligands in the purification of proteins has led to rapid and very selective separation methods. The most common procedure is affinity chromatography, in which the affinity ligand is covalently attached to an insoluble matrix. This gives an adsorbent which selectively binds one or several specific proteins. A closely related technique which has advantages for large-scale processes is based on aqueous (liquid–liquid) two-phase systems. These two-phase systems are obtained by dissolving two polymeric substances [*e.g.*, dextran and poly(ethylene glycol) (PEG)] in water [1,2]. An affinity ligand can be localized to one of the phases by attaching the ligand to the polymer concentrated in this phase [3–5]. By introducing the affinity ligand, the partitioning of ligand-binding proteins changes. In some instances, *e.g.*, glucose-6-phosphate dehydrogenase and phosphofructokinase, the change in the partition coefficient of the enzymes has been as large as 10 000-fold [6].

In systems containing affinity ligands a number of interactions can be envisaged. In general, the specific interaction between a ligand and the (target) protein is the basis for the isolation whereas other kinds of interactions (*e.g.*, ligand–ligand and ligand–polymer) might interfere and reduce the separation capacity and the selectivity. The presence of soluble or cross-linked polymers may also influence the binding strength of the ligand to the target protein.

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For both affinity chromatography and affinity partitioning, a useful group of ligands is the textile dyes derived from symmetrical triazine [7]. These dyes can be obtained with a great variety of molecular structures, among which can be found some with a striking affinity for certain enzymes, especially among kinases and dehydrogenases. The interaction between formate dehydrogenase and PEG-bound dyes (Cibacron Blue 3GA and Procion Red HE3B) or NADH was studied by Cordes *et al.* [8] by using ultracentrifugation, fluorescence and affinity partitioning. The dye–polymer–enzyme complexes were shown to have larger dissociation constants in the upper (PEG-rich) phase than in the lower (dextran-rich) phase. In this study, we chose the commonly used ligand Cibacron Blue F3G-A and bovine serum albumin (BSA) as the ligand-binding protein.

EXPERIMENTAL

Chemicals

Dextran T-500 (M_r = 500 000), dextran T-70 (M_r = 70 000) and Ficoll 400 (M_r = 400 000) were purchased from Pharmacia (Uppsala, Sweden), poly(ethylene glycol) (PEG 8000) (M_r = 7000–9000) from Union Carbide (New York, U.S.A.), hydroxypropyl-starch (HPS) (M_r = 35 000) from Perstorp Biolytica (Lund, Sweden) as Aquaphase PPT and poly(vinyl alcohol) (PVA 14) (M_r = 14 000) from BDH (Poole, U.K.). Cibacron Blue F3G-A was purchased from Serva (Heidelberg, F.R.G.). Bovine serum albumin (BSA) and lactate dehydrogenase (LDH) were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade.

Synthesis of dye–polymer derivatives

Cibacron Blue F3G-A polymer derivatives were synthesized as described previously [9,10]. The degrees of substitution (moles of dye per mole of polymer) were 1.0 for PEG, 7.6 for dextran, 1.3 for HPS, 20 for Ficoll and 1.1 for PVA.

Difference spectra

Difference spectra were obtained in the visible range by using a Hitachi 100-60 spectrophotometer equipped with 10- or 2-mm cuvettes at 22°C.

Gel chromatography

A column of Sephadex G-100 (17 × 0.9 cm I.D.) was equilibrated with 305 μM Cibacron Blue F3G-A (deactivated by sodium hydroxide and dialysed) in 25 mM sodium phosphate buffer (pH 7.5). A sample (0.5 ml) containing 100 μM BSA in the above solution was applied. Elution was carried out at 22°C with the equilibration solution. Fractions of 0.6 ml were collected and the concentration of Cibacron Blue F3G-A was determined by absorbance measurement at 615 nm after ninefold dilution.

Two-phase systems

The systems were prepared by weighing out the required amounts of stock solutions of dextran 500 and PEG 8000, together with ligand–polymer, buffer, LDH or BSA and water. All polymer concentrations are given as percentages by weight. The partitioned proteins were desalted before use by dialysis. Cibacron Blue F3G-A was deactivated with sodium hydroxide, neutralized and desalted by gel chromatography

on Sephadex G-25. The systems were equilibrated by careful mixing at 22°C for 30 s and the phases settled within 15 min. Samples were withdrawn from the two phases and analysed. The partition coefficient, K, defined as the ratio of the solute concentrations in the upper and lower phases, was calculated.

Assays

Albumin was measured by the absorbance at 280 nm and Cibacron Blue F3G-A at 615 nm. When partitioned together, albumin was measured at 233 nm and corrections were made for the influence of the dye. The activity of LDH was determined photometrically at 340 nm according to Bergmeyer [11].

RESULTS

The interaction between BSA and the ligand Cibracon Blue F3G-A (Cb) was found to be dependent on whether the dye was free (and deactivated) or bound to a water-soluble polymer. In the latter instance the kind of polymer used as the ligand

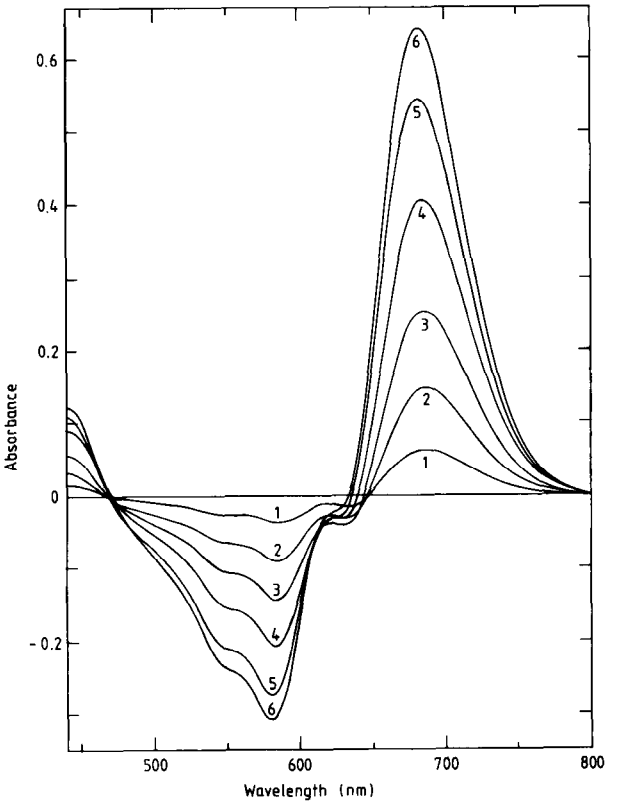

Fig. 1. Difference spectra of Cibacron Blue–PVA (795 μM dye) in the presence of various concentrations of BSA: 1 = 36; 2 = 91; 3 = 181; 4 = 363; 5 = 725; 6 = 1450 μM. The solutions contained 25 mM sodium phosphate buffer (pH 7.5). The measurements were carried out with a 0.2-cm cuvette. Temperature, 22°C.

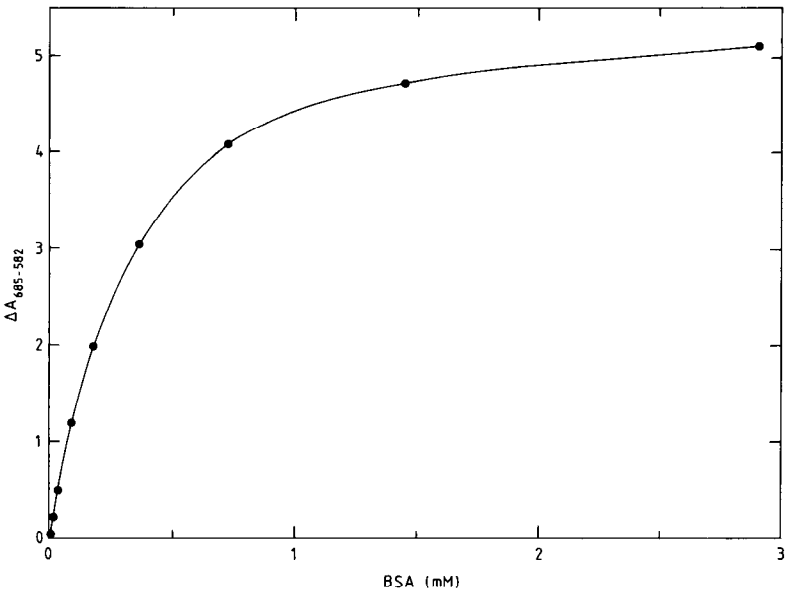

Fig. 2. Difference absorbance of Cibacron Blue PVA with BSA between 685 and 582 nm. The values were recalculated to a 1-cm path length.

carrier was important. This was studied in this work by using difference spectroscopy and also, in a few instances, gel chromatography.

Difference spectra

The type of spectra obtained when the absorbance of mixtures of dye and albumin was measured against a dye solution (of the same concentration) can be seen in Fig. 1. By following the difference in absorbance between two peaks (at two wavelengths) as a function of the concentration of albumin (or other dye-binding substance), curves of the type shown in Fig. 2 were obtained. From the limiting value, obtained by inverse plots [12], which, because of the excess of albumin, are assumed to represent the fully bound dye, the number of moles of dye bound per mole of albumin and the number of moles of free (unbound) dye can be calculated. These relationships for Cb attached to PEG and PVA are shown in Fig. 3. From this kind of experiment, the influence of the carrier molecule on the dye–protein interaction was studied. In the case of (non-polymer-bound) Cb it was difficult to determine the limiting absorbance difference. The apparent maximum number, ν_{max}, of ligand molecules bound per albumin molecule was in the range 3.5–4.5. Both PEG and PVA as ligand carriers gave a distinct value of $\nu_{max} = 2$ (Fig. 3). When the dye was bound to HPS and Ficoll the binding number increased over the whole concentration range studied and values exceeding 3 and 4, respectively, were observed (Table I). Also in the case of Cb–dextran no saturation was observed and the apparent binding number could take values of at least 2 units. The addition of PEG in several instances caused a decrease in the ν_{max} value whereas dextran did not have any noticeable effect (Table I).

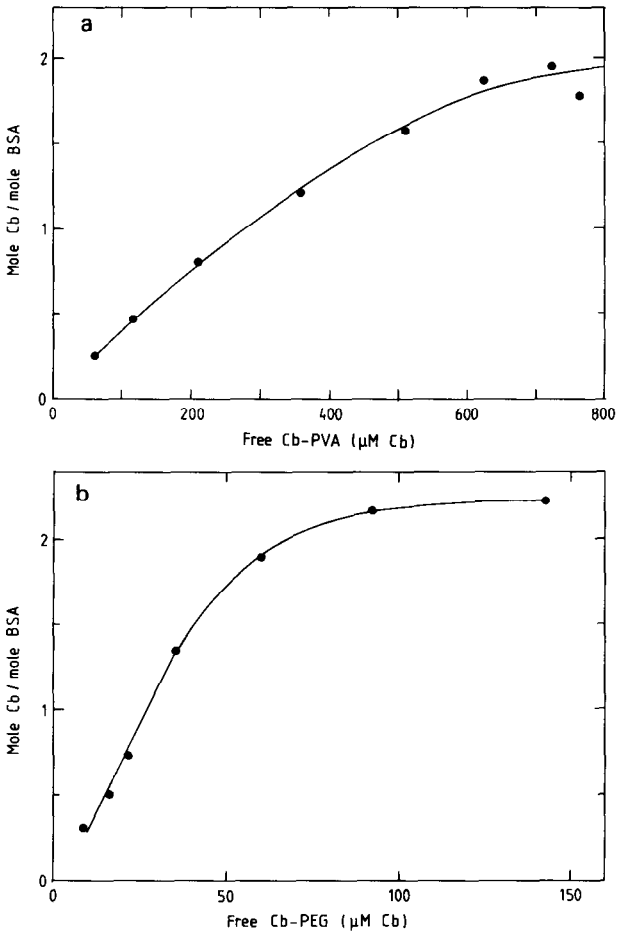

Fig. 3. Molar binding of two Cibacron Blue–polymers to BSA as a function of the concentration of free Cibacron Blue–polymer. (a) Cibacron Blue–PVA; (b) Cibacron Blue–PEG. The solutions contained 25 m*M* sodium phosphate buffer (pH 7.5). Temperature, 22°C.

Relative binding strength

The concentration of free (non-albumin-bound) dye necessary to obtain half saturation was in most instances within the range 20–150 μM. An exception was Cb–PVA with a value of 300 μM (Table I). To determine the relative binding strengths of the first bound ligand molecules, the concentrations of free Cb (deactivated or polymer-bound) in equilibrium with the 1:1 dye–albumin complex were compared. This dye concentration increased in the following order of ligand carriers: PEG < Ficoll = HPS < dextran < PVA, corresponding to a decrease in binding strength in the same order. The respective concentrations of polymer-bound dye were 15, 60, 62, 100 and 125 μM. For the dye itself the value was in the range 4–12 μM. The presence of polymers in some instances affected the binding as determined for Cb and polymer-bound dye. Dextran (10 or 5%) did not change the binding strength noticeably, while it

TABLE I

APPARENT NUMBER OF BINDING SITES, ν_{max}, ON BSA AND THE CONCENTRATION OF FREE Cb–POLYMER, $C_{1/2}$, NECESSARY TO ACHIEVE HALF SATURATION VALUES DETERMINED FROM DIFFERENCE SPECTROSCOPY

Temperature, 22°C.

Ligand	Solvent	ν_{max}	$C_{1/2}$ (μM)
Cb	Water	3.5–4.5	35–85
	10% PEG 8000	2.5	70
	10% dextran 70	3.0–3.5	20
Cb–PEG	Water	2.2	28
	10% PEG 8000	1.1	51
	5% dextran 70	2.1	31
Cb–PVA	Water	2.1	300
Cb–HPS	Water	>3	100–120
Cb–Ficoll	Water	>4	>100
	10% PEG 8000	2	85
Cb–dextran	Water	>2	100–150
	4% PEG 8000	>1	175

was reduced in the presence of PEG (10 or 4%) 2–3-fold (30 μM for the dye and 45 μM for Cb-PEG).

Association of Cb with polymers

The interaction between the dye (in hydroxyl form) and various polymers in aqueous solution could also be studied by difference spectrophotometry at levels up to

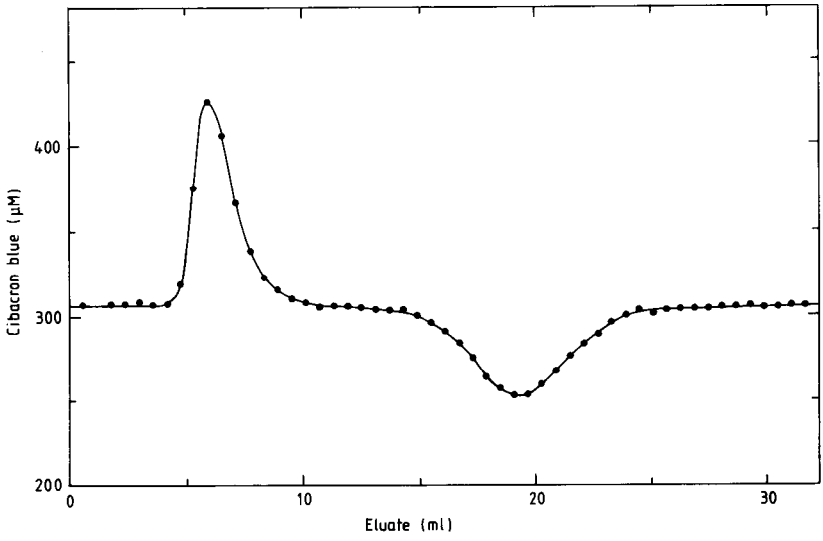

Fig. 4. Gel chromatography of BSA (50 nmol) on Sephadex G-100 equilibrated with 305 μM deactivated Cibacron Blue in 25 mM sodium phosphate buffer (pH 7.5). Temperature, 22°C.

TABLE II

PARTITIONING OF DEACTIVATED CIBACRON BLUE F3G-A (Cb), Cb-PEG AND BSA IN 5.2% DEXTRAN 500, 3.8% PEG 8000 AND 25 mmol SODIUM PHOSPHATE BUFFER PER kg SYSTEM (pH 7.5)

Temperature, 22°C.

Concentration (μmol/kg)	*K*		
	Cb	Cb–PEG	BSA
10	3.17		
50	3.47		
250	3.77		
1050	4.04		
5250	4.54		
15		4.80	
60		5.69	
300		6.83	
600		7.22	
900		7.60	
29			1.10
74			1.05
147			1.01

150 g/l of polymer. The apparent amount of dye bound per gram of polymer was a linear function of the concentration of free dye. The strongest interaction was found for PVA, *i.e.*, 75 μmol of dye per gram of polymer at a 100 μ*M* concentration of free dye. Corresponding values for Ficoll, HPS and PEG were 11, 1.4 and 1.1 μmol/g, respectively. Dextran showed only weak spectral shifts on Cb and no tendency for saturation could be seen.

Gel chromatography

The number of Cb molecules binding to one molecule of albumin was also determined by gel chromatography on Sephadex G-100 (Fig. 4). The value was found to be 5.2 when the concentration of dye was 305 μ*M*. In a similar experiment with Cb–PEG (200 μ*M*) on Sephadex G-200, the positive and negative peaks were not fully separated. A careful estimation gave a binding number of *ca.* 3.

Two-phase partitioning

The partitioning between the two aqueous phases of a PEG–dextran system was studied for BSA, free ligand (Cb) and PEG-bound ligand (Cb–PEG). Table II gives the partitioning of Cb at various concentrations. The Cb favoured the upper, PEG-rich, phase with a partition coefficient $K = 3$–4.5. When the dye was attached to PEG the K value increased by a factor of 1.7 ($K = 4.8$–7.6) (Table II). The partitioning of the BSA was nearly equal between the two phases with $K = 1.0$–1.1 (Table II).

Co-partitioning of albumin with Cb or Cb–PEG

The partitioning of Cb was affected by the presence of BSA in the system (Fig. 5). With an excess of albumin the dye, assumed to be in a 1:1 complex with the protein,

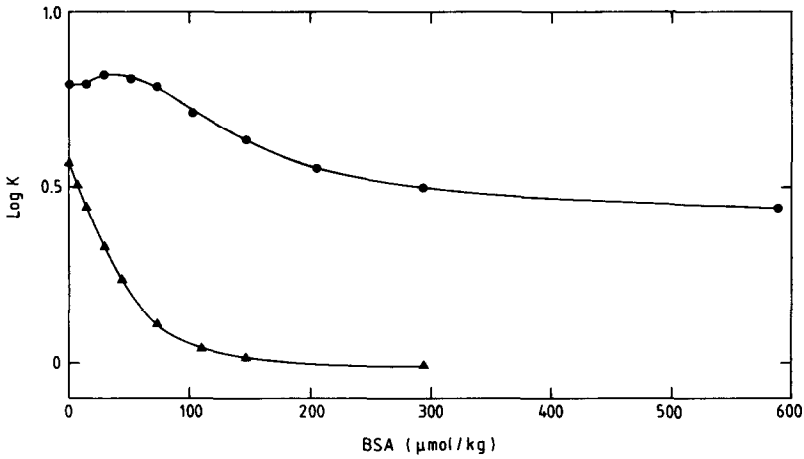

Fig. 5. Partition coefficients, K, of (▲) Cibacron Blue F3G-A (deactivated) and (●) PEG-bound Cibacron Blue as a function of the concentration of BSA in aqueous two-phase systems. The composition of the systems was 5.2% dextran 500, 3.8% PEG 8000, 25 mmol/kg sodium phosphate buffer (pH 7.5), 0.25 mmol/kg Cibacron Blue or Cb–PEG and various amounts of BSA. Temperature, 22°C.

showed the same partition coefficient as the free albumin. On the other hand, when the Cb was bound to PEG (Fig. 5), the 1:1 complex had $K \approx 2.5$ ($\log K = 0.4$). By taking the concentration of albumin necessary to reach half saturation as a measure of the relative binding strength, the curves indicate that the dye itself interacted around five times more strongly with BSA than did the PEG-linked dye.

The extraction curve for albumin (of constant concentration) with increasing

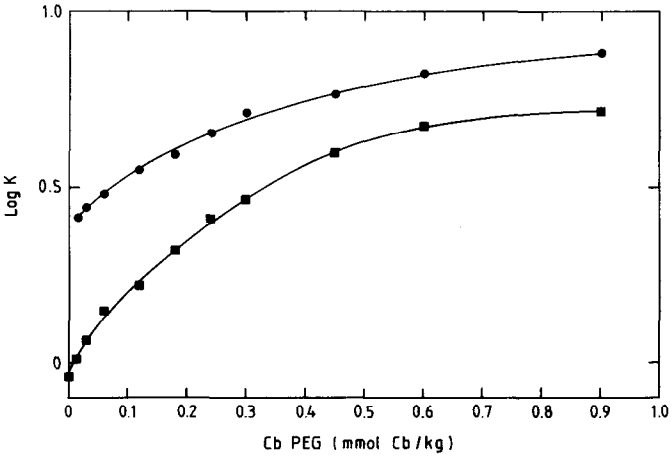

Fig. 6. Partition coefficients, K, of (■) BSA and (●) Cb–PEG when partitioned together in a two-phase system containing 5.2% dextran 500, 3.8% PEG 8000, 25 mmol/kg sodium phosphate buffer (pH 7.5), 147 μmol/kg BSA and various amounts of Cb–PEG. Temperature, 22°C.

TABLE III

PARTITION OF Cb–POLYMERS AND DEACTIVATED Cb WITHOUT AND WITH AN EXCESS OF BOVINE SERUM ALBUMIN

The partition coefficients, K_{Cb}, of Cb (polymer-bound or not) in the presence of excess of BSA are extrapolated values. Two-phase systems as in Fig. 5.

Cb carrier	K_{Cb}	
	Free	In excess of BSA
None	3.17	0.95
PEG	6.83	2.82
PVA	6.87	5.0–6.3
HPS	3.61	1.20
Ficoll	12.0	5.01
Dextran	0.066	0.018

amounts of Cb–PEG is shown in Fig. 6. The K value of albumin approaches 6.3 (log $K = 0.80$), which should be the partition coefficient of the saturated Cb–PEG–protein complex. The co-partitioning of Cb–PEG is also shown in Fig. 6.

Partitioning of various Cb polymers

The partition coefficients of a number of Cb polymers in excess of albumin are summarized in Table III. The K value of the complex was higher for PVA and Ficoll than PEG as ligand carrier. Dextran, giving a complex which favours the lower phase, was more than three times as potent as PEG. While the degree of substitution for Ficoll and dextran is >1 (20 and 7.6, respectively), they may form complexes with several binding points to the same albumin molecule or bridges between several protein molecules.

Partitioning of lactate dehydrogenase (LDH)

Partitioning of LDH from rabbit muscle was studied for comparison (Fig. 7). The most effective extractors (to opposite phases) were Cb–Ficoll and Cb–dextran, respectively. The estimated maximum changes in the logarithm of the partition coefficient, $\Delta \log K_{max}$, are summarized in Table IV. Cb–Ficoll showed the largest shift in log K (0.99 units) for LDH. The power of extraction should, however, be related to the partitioning of the polymer-bound dye. For this comparison we introduce the effectivity number, ε, *i.e.*, the relative value $\Delta \log K_{max,LDH}/\log K_{ligand}$. The ε value ranged from 1.4 to 2.4 with the highest value for dye–HPS (Table IV).

Model for affinity partitioning

The partitioning of a protein between the two aqueous phases containing a polymer-bound ligand can be calculated from the assumed number of binding sites and dissociation constants. We present here an extended version of the well known model for affinity partitioning suggested by Flanagan and Barondes [13], assuming that the protein has two binding sites for the ligand when it is in the upper phase and three binding sites in the lower phase. The dependence of log K on the total concentration of ligand–polymer is derived as follows.

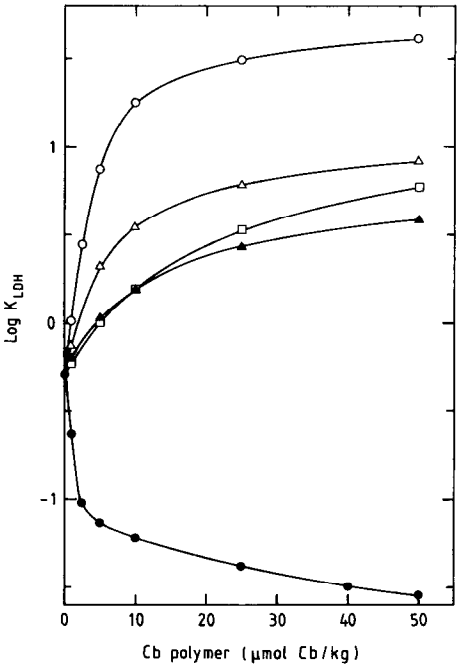

Fig. 7. Partition coefficients, K_{LDH}, of lactate dehydrogenase (12–15 U/kg) in the same system as in Fig. 5 but with various concentrations of Cibacron Blue–polymer derivatives: □ = Cb–PEG; ▲ = Cb–PVA; ○ = Cb–Ficoll; △ = Cb–HPS; ● = Cb–dextran.

With a total concentration C_P of the protein in a system with a volume ratio (top/bottom) V, the free protein has a partition coefficient K_P and the ligand–PEG a partition coefficient K_L. The dissociation constants in the top phase, k_{t1} and k_{t2}, are defined by

$$k_{t1}[PL]_t = [P]_t[L]_t \quad (1)$$

$$k_{t2}[PL_2]_t = [PL]_t[L]_t \quad (2)$$

TABLE IV

PARTITION OF Cb–POLYMERS AND THEIR MAXIMUM EFFECT ON THE PARTITION OF LACTATE DEHYDROGENASE (Δ LOG $K_{LDH,max}$)

Two-phase systems as in Fig. 5. The effectivity number, ε, of polymer molecules bound per enzyme molecule was calculated as ε = log $K_{LDH,max}$/log $K_{Cb-polymer}$.

Cb carrier	Log $K_{Cb-polymer}$	Log $K_{LDH.max}$	ε
PEG	0.88	1.52	1.7
PVA	0.84	1.14	1.4
HPS	0.56	1.34	2.4
Ficoll	1.08	2.07	1.9
Dextran	−1.18	−1.85	1.6

and the three constants in the bottom phase, k_{b1}, k_{b2} and k_{b3}, are defined by

$$k_{b1}[PL]_b = [P]_b[L]_b \tag{3}$$

$$k_{b2}[PL_2]_b = [PL]_b[L]_b \tag{4}$$

$$k_{b3}[PL_3]_b = [PL_2]_b[L]_b \tag{5}$$

where $[P]_i$, $[PL]_i$ and $[PL_2]_i$ are the concentrations of the protein, monoligand protein and diligand protein, respectively, in the top phase (i = t) and bottom phase (i = b). $[PL_3]_b$ is the triligand protein present only in the bottom phase.

The total concentrations of protein, $C_{P,t}$, in the top phase can be written as a function of $[L]_t$:

$$C_{P,t} = [P]_t\left\{1 + \frac{[L]_t}{k_{t1}} + \frac{[L]_t^2}{k_{t1}k_{t2}}\right\} = [P]_tQ \tag{6}$$

and the total protein concentration, $C_{P,b}$, in the bottom phase as

$$C_{P,b} = [P]_b\left\{1 + \frac{[L]_t}{K_Lk_{b1}} + \frac{[L]_t^2}{K_L^2k_{b1}k_{b2}} + \frac{[L]_t^3}{K_L^3k_{b1}k_{b2}k_{b3}}\right\} = [P]_bR \tag{7}$$

The protein concentration in the top phase can also be expressed as the overall concentration of protein:

$$[P]_t = \frac{C_P(1 + V)}{VQ + R/K_P} \tag{8}$$

The overall partition coefficient for the protein, K_{tot}, will then be

$$K_{tot} = \frac{C_{P,t}}{C_{P,b}} = \frac{[P]_t}{[P]_b} \cdot \frac{Q}{R} = K_P \cdot \frac{Q}{R} \tag{9}$$

In the same way, the total concentration of ligand in the top phase, $C_{L,t}$ and in the bottom phase, $C_{L,b}$, can be expressed as functions of $[L]_t$:

$$C_{L,t} = [L]_t + \frac{[P]_t}{k_{t1}} \cdot [L]_t + \frac{2[P]_t}{k_{t1}k_{t2}} \cdot [L]_t^2 \tag{10}$$

$$C_{L,b} = \frac{[L]_t}{K_L} + \frac{[P]_b}{k_{b1}} \cdot \frac{[L]_t}{K_L} + \frac{2[P]_b}{k_{b1}k_{b2}} \cdot \frac{[L]_t^2}{K_L^2} + \frac{3[P]_b}{k_{b1}k_{b2}k_{b3}} \cdot \frac{[L]_t^3}{K_L^3} \tag{11}$$

The overall concentration of ligand, C_L, in the system is then

$$C_L = \frac{1}{1 + V}(VC_{L,t} + C_{L,b}) \tag{12}$$

TABLE V

SETS OF DISSOCIATION CONSTANTS WHICH GIVE GOOD CURVE FITTING FOR THE EXTRACTION CURVES IN FIG. 7

Cb–polymer	Dissociation constant (mM)				
	k_{t1}	k_{b1}	k_{t2}	k_{b2}	k_{b3}
PVA	0.006	0.003	0.02	0.01	0.2
PEG	0.007	0.007	0.035	0.035	3
Ficoll	0.0013	0.0013	0.003	0.003	0.018
HPS	0.003	0.004	0.006	0.008	0.2
Dextran	0.00025	0.00125	0.00025	0.00125	0.1

As the overall partition coefficient, K_{tot}, of protein cannot be expressed explicitly as a function of C_L, both of these values were calculated for a series of values of $[L]_t$ via eqns. 9 and 12. By using $V = 1$, $C_P = 0.15$ mM, $K_L = 6.3$ and $K_P = 1.0$, which correspond to the conditions for the experiment in Fig. 6, a good fit was obtained by using the following values for the dissociation constants: $k_{t1} = 0.025$ mM, $k_{t2} = 0.1$ mM, $k_{b1} = 0.01$ mM, $k_{b2} = 0.04$ mM and $k_{b3} = 10$ mM. Corresponding k values in the top and bottom phases were chosen to be in the ratio 2.5:1. For the case when only one binding site is assumed in the top phase and two in the bottom phase, the same equations can be used if only the $[L]_t^2$ terms in eqns. 6 and 10 and the $[L]_b^3$ terms in eqns. 7 and 11 are omitted. In this event good curve fitting was obtained with $k_{t1} = 0.1$ mM, $k_{b1} = 0.16$ mM and $k_{b2} = 1.2$ mM.

When the above model (with two binding sites in the top phase) was applied to the experimental data for the partitioning of LDH (Fig. 7), excellent fits were obtained by using the sets of dissociation constants summarized in Table V. The values used indicate that the first dissociation constant for LDH–dye–polymer may well be approximately the same in both the bottom and top phases with the exception of the complex with dye–dextran. In the latter instance the curve fitting indicates a fivefold stronger complex in the top (PEG-rich) phase than in the bottom (dextran-rich) phase.

DISCUSSION

The apparent number of binding sites on the albumin molecule for Cb (in water) found in this work (3.5–4.5 spectroscopically, 5.2 by gel filtration) is close to the value of 3.4 found by Antoni *et al.* [14] by using difference spectroscopy but considerably higher than the value of 2.0 reported by Ling and Mattiasson [15] using partitioning in aqueous two-phase systems and equilibrium dialysis. The higher value obtained by using gel filtration could be explained by assuming sandwich binding (one dye molecule associating with one albumin-bound dye molecule), which may not necessarily be reflected in the difference spectrum.

Covalent binding of the dye to the polymers PEG and PVA reduces the number of available binding sites on the albumin molecule. This might be due to unfavourable interaction between the attached polymer chain and that part of the surface of the protein molecule which surrounds one of the binding sites. The effect of polymers is

striking. While addition of bulk dextran had a small influence on the number of bound Cb and Cb–PEG and on the binding strength, both parameters were reduced by the addition of PEG. This indicates that the polymer influences the conformation of the albumin molecule in such way that one of the binding sites drastically lowers its dye-binding ability.

Another possible explanation for the reduced binding could be that one of the elements of the Cb molecule, *e.g.*, the hydrophobic parts, interacts with the polymer chains. If so, contact points (on the dye molecule) for the protein may be masked. Depending on the relative importance of these contact points for the binding strength to the various site on BSA, the weakening of the interaction could be influenced more or less drastically by the presence of polymers such as PEG or PVA.

Polymer-bound triazine dyes have been used as affinity ligands for the isolation of proteins by partitioning between the aqueous phases of dextran–PEG–water two-phase systems. The results obtained here point toward the possibility that proteins, in this instance albumin, do not have to expose the same number of binding sites in the two phases. Earlier models for affinity partition have been based on the assumption of the same v_{max} in both phases. In the model presented above it is assumed that the protein has one more binding site in the bottom than in the top phase. If only one binding site is assumed in the top phase the albumin has to bind Cb–PEG more strongly (1.6-fold) in the top compared with the bottom phase. With two binding sites

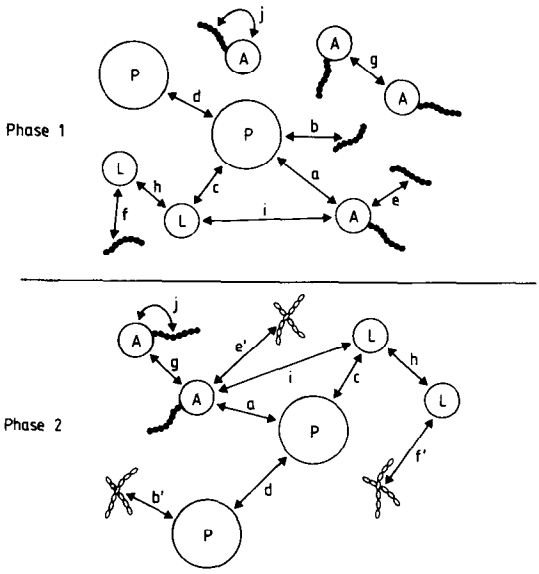

Fig. 8. Scheme of some important interactions which take place in the aqueous two-phase systems containing two polymers mainly concentrated in opposite phases. P, Protein; A, polymer-bound ligand; L, free ligand. The indicated interactions are (a) protein to polymer-bound ligand; (b) and (b′) protein to phase-forming polymer; (c) protein to free ligand; (d) protein to protein; (e) and (e′) polymer-bound ligand to phase-forming polymer; (f) and (f′) free ligand to phase-forming polymer; (g) self-interaction between polymer-bound ligand; (h) self-interaction between free ligand; (i) polymer-bound ligand to free ligand; (j) between polymer bound ligand and its carrier polymer.

in the top phase the binding might be stronger in the bottom phase, as indicated by the binding experiments, by 2.5-fold (for the first constant). In this instance the third binding constant in the bottom phase was without any influence.

The partition experiments also gave the partition coefficients of the minimum complex and of the fully substituted albumin complex (Figs. 5 and 6). As the latter value is greater than the former, it can be concluded that the number of binding sites (in the top phase) must be larger than unity. The two-phase system studied here has phases that contain considerable amounts of both polymers, 5.70% PEG and 1.05% dextran in the top phase and 1.85% PEG and 9.46% dextran in the bottom phase [1] and therefore the "polymer effect" may not be as pronounced as in systems with higher polymer concentrations where the polymers have a more one-sided partition to one phase each.

The curve fitting in Table V indicates that the dye–protein interaction, in the case of LDH, is strongly dependent on the choice of ligand-carrying polymer. The first dissociation constants (in the top and bottom phases) show that the most effective binding is obtained with dextran whereas PEG, which is the most commonly used ligand carrier, gave the lowest binding.

Aqueous two-phase systems applied for affinity partitioning of proteins involve a great number of molecular interactions. The possible 1:1 interactions (between two molecular species) are summarized in Fig. 8. To achieve a good affinity extraction into the top phase, in which the polymer-bound ligand is concentrated, the protein–ligand interaction (a) should be stronger in the top phase and the ligand–ligand (g), ligand–polymer (e) and ligand–carrier polymer (j) interactions should be as small as possible in this phase. These interactions all contribute to the apparent dissociation constants of the protein–ligand–polymer complex. By obtaining a deeper understanding of these factors, more selective and effective separation methods based on affinity ligands may be formulated, *e.g.*, two-phase systems with other polymers than those used today.

CONCLUSIONS

The association of Cibacron Blue F3G-A to serum albumin is influenced both by the binding of the dye to a polymer and by the presence of bulk polymers in an aqueous solution. Both the number of binding sites and the dissociation constants are affected. The kind of polymers used to obtain aqueous polymeric two-phase systems for the affinity partitioning of proteins may therefore be critical.

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