The effect of ligand presaturation on the interaction of serum albumins with an immobilized Cibacron Blue 3G-A studied by affinity gel electrophoresis

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The interaction of the immobilized triazine dye Cibacron Blue 3G-A with rat, rabbit, sheep, goat, bovine and human serum albumins was studied by affinity gel electrophoresis. Dissociation constants were estimated in each instance and showed human serum albumin to have a significantly higher affinity for the dye than did albumin from any other species. Pretreatment of the defatted proteins with bilirubin (3 mol of bilirubin/mol of protein) did not increase the dissociation constants of the serum albumins, whereas pretreatment with palmitate (7 mol of palmitate/mol of protein) increased the dissociation constant in all cases: 3-fold for human serum albumin, 15-fold for other serum albumins. Increasing the bilirubin/albumin ratio (to 7:1) did not affect the dissociation constant of the albumins studied. Decreasing the palmitate/ albumin ratio decreased the dissociation constant for human serum albumin, but did not affect those of bovine and rat albumins. Altering the chain length of the presaturating fatty acid dramatically changed the dissociation constant of both human and bovine serum albumins. Butyrate, hexanoate, octanoate and decanoate did not significantly influence the dissociation constants of bovine and human serum albumins for Cibacron Blue, whereas laurate, myristate and palmitate greatly increased the dissociation constant. These data are discussed in relationship to the behaviour of albumins during dye–agarose column chromatography. In an Addendum the effect of nucleotide presaturation on the interaction between *Bacillus stearothermophilus* 6-phosphogluconate dehydrogenase and the immobilized triazine dyes Cibacron Blue 3G-A and Procion Red HE-3B was examined, and the implications for dye–ligand chromatography are discussed.

The interaction of immobilized Cibacron Blue 3G-A with human serum albumin has been widely applied to the purification of plasma proteins (Travis & Pannell, 1973; Travis *et al.*, 1976; Angal & Dean, 1978; Young & Webb, 1978). However, it has been shown that the capacity of the immobilized dye (defined as weight of albumin bound/ml of affinity gel) for other albumins (mouse and bovine) is lower than for the human protein (Kelleher *et al.*, 1979). We have observed (Leatherbarrow & Dean, 1980) that, although albumins from five different species were bound to Cibacron Blue–Sepharose, the human protein bound to a far greater extent. In addition the bovine, rabbit, sheep and horse serum albumins behaved differently from the human protein on pretreatment with palmitate or bilirubin.

Many of the proteins that have been reported to bind to Cibacron Blue possess a secondary structure known as the dinucleotide fold. Further, Biellmann *et al.* (1979) demonstrated that the bound dye is positioned in the dinucleotide-binding domain of horse liver alcohol dehydrogenase. It has been suggested (Thompson *et al.*, 1975; Wilson, 1976) that the ability of a protein to bind to this dye indicates the presence of the dinucleotide fold. Information relating to the nature of the binding of albumin to Cibacron Blue is thus of interest, as there is no evidence that albumin possesses such a structure (Peters, 1977).

Albumin is known to bind both long-chain fatty acid and bilirubin anions with relatively high affinity (Peters, 1975). Binding of these two ligands occurs primarily at separate sites (Wooley & Hunter, 1970), with fatty acids not interfering with bilirubin binding until the fatty acid/albumin molar ratio rises above

4:1. The effect of presaturation with palmitate and bilirubin on the binding of albumins of different species to Cibacron Blue has been investigated by conventional column chromatography (Leatherbarrow & Dean, 1980). We found that, although bilirubin significantly decreased binding of human serum albumin to immobilized Cibacron Blue, it did not affect dye binding of any of the other species of serum albumin. Conversely, palmitate decreased the binding of the other serum albumins to a much greater extent than that of human serum albumin.

A further investigation of the effect of ligand presaturation on the binding of different species of albumin to Cibacron Blue is described in the present paper, the technique of affinity electrophoresis being used. This technique was initially developed by Takeo & Nakamura (1972), and consists of the electrophoresis of proteins through a matrix containing immobilized ligands with which the protein interacts specifically. By measuring the relative mobility of the protein through gels of different ligand concentration, dissociation constants for the dye–ligand interaction can be measured (Takeo & Nakamura, 1972; Horejsi, 1979). It is claimed by those authors that this technique permits accurate analysis of affinity-chromatographic interactions. This dynamic method has been used to determine the dissociation constant of bovine serum albumin and immobilized Cibacron Blue (Johnson et al., 1980). In the present study, the effect of ligand presaturation on the dissociation constant of the albumin–Cibacron Blue interaction for different albumin species was investigated. The effect of increasing the chain length of the presaturating fatty acid and the ligand/protein ratio on the binding of albumin to Cibacron Blue is also reported.

Experimental

Materials

Cibacron Blue 3G-A (formerly Cibacron Blue F3GA) was obtained from CIBA–GEIGY, Manchester, U.K. Sepharose (6B) was obtained from Pharmacia, Croydon, Surrey, U.K. Rabbit, rat, sheep, horse, goat and bovine serum albumins, as freeze-dried powders (Cohn fraction V), bilirubin, biliverdin, hexanoic acid, myristic acid, tetramethyl-ethylenediamine, ammonium persulphate, Tris and glycine were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Human serum albumin was either obtained from Sigma (Cohn fraction V) or isolated from plasma by using Cibacron Blue 3G-A–Sepharose columns (NaSCN elution). Acrylamide, methylenebisacrylamide, dimethyl sulphoxide, palmitic acid, butyric acid and octanoic acid were obtained from BDH Chemicals, Poole, Dorset, U.K.

Methods

Cibacron Blue 3G-A–Sepharose 6B was prepared by the method of Heyns & De Moor (1974). The concentration of coupled dye was determined by the method of Leatherbarrow & Dean (1980). Gels containing 4% (v/v) Cibacron Blue 3G-A–Sepharose and 5% (w/v) acrylamide were prepared as described by Johnson et al. (1980). The ligand concentration was varied and assessed as described previously. All serum albumins were defatted by the method of Chen (1967). Protein was assayed the absorbance at 280nm corrected for the contribution to the absorbance at 280nm by bilirubin (correction factor of 18% absorbance at 440nm) or by the tannin method of Mejbaum-Katzenellenbogen & Dobryszycka (1959). Bilirubin and biliverdin solutions were freshly prepared by dissolving bilirubin in dimethyl sulphoxide, followed by dilution to the appropriate volume with 25mM-Tris/HCl buffer, pH 8.6. In addition, bilirubin solutions were also prepared by dissolving in the minimum amount of 50mM-KOH followed by immediate dilution with 25mM-Tris/HCl buffer, pH 8.6. Fatty acids were prepared as their potassium salts by the latter method. Bilirubin/albumin solutions and fatty acid/albumin solutions were equilibrated at 4°C for 4h before electrophoresis. The solutions were then mixed with 0.1% (w/v) Bromophenol Blue in 50% (v/v) glycerol as a tracking dye. The final concentration of Bromophenol Blue in these solutions was 0.01%. Preliminary experiments indicated that Bromophenol Blue did not interfere with the estimation of $K_{diss.}$ values: when the tracking dye was omitted, R_F values were obtained from the rate of migration of the tracking dye on a control gel. In these cases, the albumin solution was mixed with a small amount of 50% (v/v) glycerol to increase the viscosity of the applied sample. Samples (10 μl) containing approx. 25 μg of albumin was applied to each blue gel and to the control gel (4% Sepharose, 5% acrylamide, no dye). The gels were (pre-)electrophoresed, stained and destained as described by Johnson et al. (1980).

Columns of Cibacron Blue 3G-A–Sepharose 6B (1 ml) at different ligand concentrations were equilibrated in 25mM-Tris/HCl buffer, pH 8.6. Albumin (10 mg in 450 μl) pre-equilibrated with palmitate or bilirubin was applied. Unbound protein was washed through with equilibration buffer (10 ml). Columns were eluted with 0.5 M-NaSCN in 50mM-Tris/HCl buffer, pH 8.0 (10 ml).

Results and discussion

The dissociation constants of defatted serum albumins for Cibacron Blue 3G-A are shown in Table 1. All albumins tested showed some affinity for the dye, but it is clear that human serum albumin bound

Table 1. *Dissociation constants of defatted albumins for immobilized Cibacron Blue 3G-A measured by affinity electrophoresis*

$K_{diss.}$ values are means $\pm$ s.D. (where appropriate) for the numbers of determinations given in parentheses.

Species	$K_{diss.}$ (μM)	
Human	16 ± 5	(19)
Bovine	196 ± 28	(5)
Rat	80	(2)
Rabbit	150	(1)
Sheep	150	(1)
Goat	150	(1)
Horse	51	(2)

more tightly than any other albumin, having $K_{diss.}$ 16 μM. Furthermore, human serum albumin isolated by plasma fractionation on immobilized Cibacron Blue 3G-A behaved identically with human serum albumin obtained commercially. Bovine, sheep, rabbit and goat serum albumins had dissociation constants one order of magnitude higher (150–190 μM), and the $K_{diss.}$ values for rat and horse serum albumins were intermediate between the two extremes (80 and 50 μM respectively).

The large difference in dissociation constants shown between human serum albumin and all other albumins for the immobilized dye explains the results previously observed for column-binding characteristics of human and other serum albumins (Leatherbarrow & Dean, 1980). These authors found that Cibacron Blue 3G-A had a higher capacity and bound human albumin more tightly than albumins from sheep, bovine, rabbit and horse. The remaining albumins all bound to Cibacron Blue to the same extent.

Fig. 1 shows the relative mobilities of bovine, rat and human serum albumins pretreated with bilirubin (3 mol/mol of protein) or palmitate (7 mol/mol of protein) in affinity gels of different ligand concentration.

The dissociation constant for the human serum albumin–dye interaction obtained on presaturation of the protein with bilirubin is similar to that obtained in the buffer control. Similarly bilirubin did not affect the binding of rat serum albumin to Cibacron Blue 3G-A. Presaturation of bovine serum albumin with bilirubin appeared to tighten binding, giving $K_{diss.}$ 140 μM (although this may not be significantly different from the control, since these values probably lie within the range of experimental error).

Preincubation with palmitate increased the dissociation constant of human serum albumin from 16 μM to 50 μM. Presaturation with this fatty acid dramatically decreased binding of rat and bovine
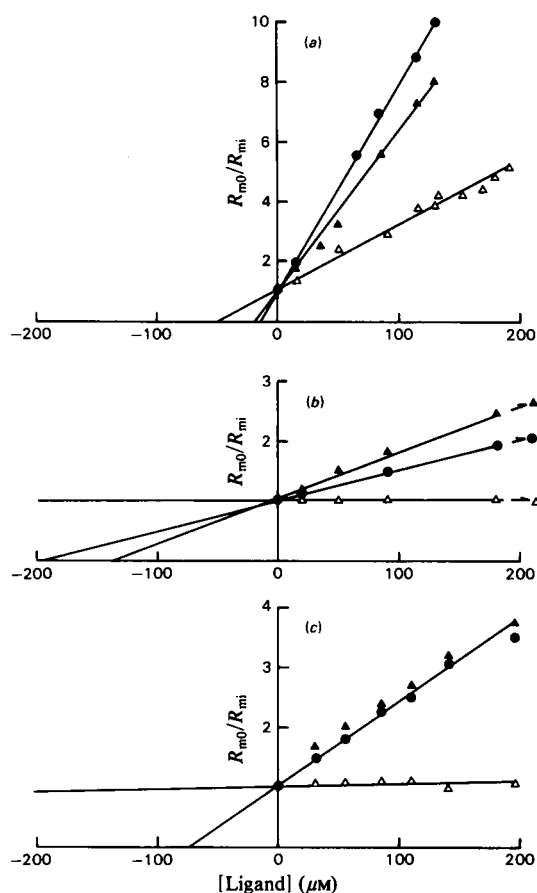

Fig. 1. *Effect of ligand presaturation on the electrophoretic mobility of defatted serum albumins on Cibacron Blue 3G-A–Sepharose/polyacrylamide gels*

(a) Human serum albumin; (b) bovine serum albumin; (c) rat serum albumin. Defatted albumin (25 μg) was electrophoresed on gels of different ligand concentration. Gels were prepared and run as described by Johnson *et al.* (1980). ●, Albumin alone; △, albumin presaturated with palmitate in the molar ratio of 7:1 to protein; ▲, albumin presaturated with bilirubin in the molar ratio of 3:1 to protein. R_{mo}, Distance moved by protein relative to the tracking dye, Bromophenol Blue, in control gels (no immobilized dye); R_{mi}, the same relative distance but in the presence of immobilized dye. $K_{diss.}$ was calculated from the equation:

$$\frac{1}{R_{mi}} = \frac{1}{R_{mo}} \left(1 + \frac{[L]}{K_{diss.}} \right)$$

serum albumins. Indeed, these albumins were not retarded even at the highest ligand concentrations, so that $K_{diss.}$ values cannot be calculated from gels of this concentration range. However, a different

concentration range enabled estimates of $K_{diss.}$ to be made. The large increase in the dissociation constant observed for palmitate-presaturated bovine serum albumin explains the marked decrease in binding to columns of Cibacron Blue 3G-A–Sepharose previously observed for the protein pretreated in such a manner (Leatherbarrow & Dean, 1980). Similarly, the inconsequential increase (3-fold) in the dissociation constant observed on palmitate presaturation of human serum albumin explains the lack of effect on the binding capacity of columns of Cibacron Blue 3G-A–Sepharose reported in the same study.

Table 2 shows the effect of changing the ratio of the presaturating ligand to albumin in the incubation mixture on the dissociation constants. It is clear that presaturation of all serum albumins with bilirubin at ligand/protein molar ratios of 3:1 and 7:1 did not affect the dissociation constant of the dye–albumin complex. Pretreatment of human albumin with palmitate at a ligand/protein ratio of 3:1 did not change the $K_{diss.}$, whereas increasing the ratios to 7:1 decreased binding. In contrast, the binding of bovine and rat serum albumins to Cibacron Blue 3G-A is markedly decreased by pretreatment with palmitate at both ratios.

The work of Leatherbarrow & Dean (1980) predicts that bilirubin should increase the dissociation constant for human serum albumin–Cibacron Blue interaction, but not for bovine serum albumin–Cibacron Blue interaction. This was not observed in the present study. It was suggested that the different method of preparation of the bilirubin stock solutions employed in these studies could explain this anomaly. Consequently the effect of bilirubin prepared in 12% (v/v) dimethyl sulphoxide or 5 mM-KOH on the dissociation constants for human and bovine serum albumins was studied, and is shown in Table 3. Pretreatment with bilirubin did not change the dissociation constants shown for bovine and human serum albumins, regardless of the method of preparation.

In view of the failure to observe any change in the dissociation constant of human serum albumin for Cibacron Blue 3G-A on pretreatment with bilirubin, the capacity for Cibacron Blue 3G-A–Sepharose columns for bilirubin-pretreated human serum albumin was reassessed. The results of this study are shown in Table 4. Bound protein was determined by difference (assayed by the tannin micro method). Pretreatment with bilirubin did not decrease the amount of albumin bound at any ligand concentration. This is in accordance with affinity-electrophoresis data (Fig. 1), where no increase in the dissociation constant was observed on pre-saturation of the protein with bilirubin. In these column experiments, both bound and unbound protein was determined by the absorbance at 280 nm and by the tannin micro method. Absorbance at 280 nm (corrected for the contribution to the absorbance of bilirubin) consistently over-estimated the amount of protein applied to the columns. Protein determined by the tannin method (unbound protein only) was not affected by the presence of bilirubin and thus circumvented this problem. The error involved in protein determination

Table 3. *Effect of dissociation constant on defatted human serum albumin for Cibacron Blue 3G-A pretreated with solutions of bilirubin prepared by different methods*
Bilirubin/protein molar ratio was 3:1; dissociation constants were determined as described in Fig. 1. Solutions of bilirubin were prepared either in 10% (v/v) dimethyl sulphoxide (DMSO) or 5 mM-KOH (see the Experimental section). $K_{diss.}$ values are means ± s.D. (where appropriate) for the numbers of determinations given in parentheses.

	$K_{diss.}$ (μM)		
Albumin	Control	DMSO	KOH
Human	16 ± 5 (19)	17 ± 6 (6)	22 ± 6 (4)
Bovine	196 ± 28 (5)	120 (3)	140 (2)

Table 2. *Dissociation constants of defatted albumins for Cibacron Blue 3G-A pretreated with different amounts of bilirubin and palmitate*
$K_{diss.}$ values are means ± s.D. (where appropriate) for the numbers of determinations given in parentheses.

			$K_{diss.}$ (μM)	
Albumin	Ligand	Ligand/protein molar ratio ...	3:1	7:1
Human	Control		16 ± 5 (19)	16 ± 5 (19)
	Bilirubin		19 ± 7 (8)	16 (2)
	Palmitate		17 (2)	49 ± 1 (5)
Bovine	Control		196 ± 28 (5)	196 ± 28 (5)
	Bilirubin		140 (3)	120 (2)
	Palmitate		1500 (2)	1500 (3)
Rat	Control		80 (2)	80 (2)
	Bilirubin		80 (1)	80 (1)
	Palmitate		1500 (1)	1500 (1)

Table 4. *Effect of bilirubin presaturation on the binding of human serum albumin (HSA) to columns of Cibacron Blue 3G-A–Sepharose 6B*

Columns (1 ml) of Cibacron Blue 3G-A–Sepharose 6B were equilibrated in 25 mM-Tris/HCl buffer, pH 8.6. Defatted albumin (10 mg) presaturated with bilirubin [prepared by the KOH or dimethyl sulphoxide (DMSO) method] in a 3:1 molar ratio to protein was applied (450 μl). Unbound protein was washed through with equilibration buffer (10 ml). The columns were eluted with 0.5 M-NaSCN in 50 mM-Tris/HCl buffer, pH 8.0 (10 ml). All experiments were performed at 4°C with a flow rate of 5 ml/h. Protein was determined by the tannin method (see the Experimental section). Values are means ± S.D. for three determinations.

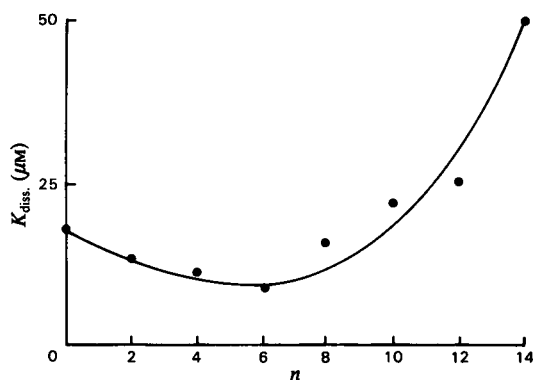| Ligand concentration (μmol/ml of settled gel) | Protein bound (mg) | | |
	HSA alone	HSA + bilirubin (KOH)	HSA + bilirubin (DMSO)
0	0.4 ± 0.3	0.5 ± 0.3	0.6 ± 0.3
0.35	2.2 ± 0.2	1.4 ± 0.6	2.2 ± 1.3
0.62	4.0 ± 0.3	3.0 ± 0.7	3.5 ± 0.8
2.2	6.7 ± 0.9	6.4 ± 1.2	7.3 ± 2.3
6.2	7.1 ± 1.0	6.9 ± 1.6	7.8 ± 2.5

Fig. 2. *Effect of increasing the chain length of the presaturating fatty acid on the interaction of human serum albumin with Cibacron Blue 3G-A*

$K_{diss.}$ values for the protein–dye interaction were assessed by affinity electrophoresis as described in Fig. 1. Albumin (25 μg) was presaturated with fatty acids of increasing chain length, n (where n represents number of methylene groups in the chain, i.e. $CH_3[CH_2]_nCO_2H$), in the ratio of 7 mol of fatty acid/mol of protein.
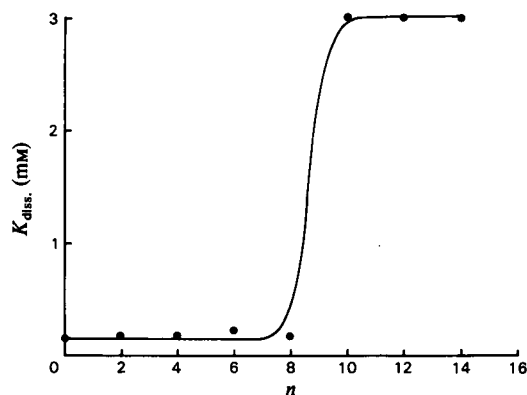

Fig. 3. *Effect of increasing the chain length of the presaturating fatty acid on the interaction of bovine serum albumin with Cibacron Blue 3G-A*
Experimental details are as for Fig. 2.

by absorbance at 280 nm of solutions containing bilirubin probably accounts for the discrepancy between these results and our previous work (Leatherbarrow & Dean, 1980). The tannin procedure seems to us to be less selective than assays based on dye binding, A_{280} or even the Lowry method, and is therefore more appropriate in this instance.

The effect of increasing the chain length of the presaturating fatty acid on the dissociation constant for human serum albumin and Cibacron Blue 3G-A

is shown in Fig. 2. Butyrate, hexanoate and octanoate did not increase the dissociation constant, and may in fact slightly tighten binding. As the chain length increased from $n = 8$ to $n = 16$, the dissociation constant increased. Thus binding was inhibited by presaturation with fatty acids with a chain length equivalent or greater than $n = 8$. The effect of increasing the chain length on the binding of bovine serum albumin to Cibacron Blue 3G-A is shown in Fig. 3. No increase in $K_{diss.}$ was observed after pretreatment with butyrate, hexanoate, octanoate and decanoate. However, pretreatment with a fatty acid of a chain length greater than $n = 8$ dramatically decreased binding. After this sudden increase in the dissociation constant, no further change was observed for a further increase in chain length (up to palmitate).

The large differences in the $K_{diss.}$ values observed for the defatted-albumin–dye interactions confirm and extend the work of Leatherbarrow & Dean (1980) in demonstrating why serum albumins of species other than human fail to bind to columns of Cibacron Blue 3G-A-Sepharose. The relatively small increase in $K_{diss.}$ found for human serum albumin presaturated with palmitate reinforces these data and further emphasizes the unique properties of human serum albumin observed by previous workers.

Furthermore, it is unnecessary to invoke dinucleotide-binding folds in proteins in order to explain the binding of Cibacron Blue 3G-A. The present data clearly show that the occupation of fatty acid-binding sites affects column performance because either (i) the dye occupies fatty acid-binding sites alone or (ii) the occupation of binding sites by fatty acid produces conformational changes that are not observed with bilirubin presaturation.

The presently described electrophoretic method for $K_{diss.}$ determination is clearly a dynamic process. The procedure assumes that the distribution of presaturating ligands is not affected by the process of electrophoresis. The data correlate well with column experiments, which suggests that the electric field does not influence the presaturating ligand concentration. Schroeder (1979) has shown that columns of dyes very similar to Cibacron Blue can be used to remove presaturating ligands such as fatty acids and bilirubin without removing the protein from the column. This suggests that under some chromatographic conditions it is possible to alter presaturating ligand distribution. Other methods such as frontal analysis use dynamic equilibrium processes. In the latter, Dean & Watson (1978) have shown that $K_{diss.}$ alters significantly with changes in ligand concentration.

Despite these criticisms, the value of K_d determined by the electrophoretic method agrees well with results obtained by equilibrium methods.

In conclusion, the dramatic effects of ligand presaturation on the performance of proteins on dye columns are clearly important. Fatty acids and albumins are a good example of this effect. An extension of this work [see the Addenum (Qadri & Dean, 1981)] indicates that presaturation of 6-phosphogluconate dehydrogenase from *Bacillus stearothermophilus* with nucleotide completely abolishes binding to immobilized Cibacron Blue 3G-A; binding

is restored on removal of the presaturating nucleotide. This is an effect predicted by affinity-electrophoresis studies. These data have important practical implications for the use of dye–ligand chromatography in protein purification.

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