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Spectroscopic Studies of the Binding of Bilirubin by Ligandin and Aminoazo-Dye-Binding Protein A

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Ligandin and aminoazo-dye-binding protein A both bind bilirubin at a single site. Quantitative studies of the interactions using difference spectrophotometry show that at pH 7.0, protein A binds the tetrapyrrole with an association constant (K) $\geqslant 2\times10^7$ litre/mol, whereas binding by ligandin is slightly weaker ($K = 7\times10^6$ litre/mol) at this pH. The protein–bilirubin complexes give rise to absorption and fluorescence spectra quite different from those of unbound bilirubin and also to large Cotton effects. It appears that on binding to both proteins, the ligand is forced into a rigid twisted configuration in a hydrophobic environment. Ligandin and protein A resemble serum albumin in their interactions with bilirubin.

The hepatic uptake of bilirubin from the blood and its subsequent intracellular transport and sequestration may involve its binding by ligandin, a protein present in substantial amounts in the soluble cytoplasm of the liver (Ketterer *et al.*, 1967, 1975; Levi *et al.*, 1969; Litwack *et al.*, 1971; Fleischner *et al.*, 1972; Arias, 1972; Meuwissen *et al.*, 1975). Aminoazo-dye-binding protein A (Ketterer *et al.*, 1967, 1976) and Z-protein (Levi *et al.*, 1969; Mishkin *et al.*, 1972), which on the basis of their physical and binding properties appear to be identical, have also been implicated in these processes. Data defining the interactions of bilirubin with these proteins are therefore of considerable interest.

The first quantitative study of the binding of bilirubin by ligandin was made by Meuwissen *et al.* (1972). By using a method based on the partition of the ligand between Sephadex G-10 and the protein in solution, these workers reported an association constant of 1×10^6 litre/mol for binding at a single site. Kamisaka *et al.* (1973) observed large induced Cotton effects associated with bilirubin bound to ligandin, and also concluded that binding takes place at a single site. In a subsequent study (Kamisaka *et al.*, 1975*a*), the induced ellipticities were used to monitor the extent of displacement of bound bilirubin by Indocyanine Green, for which an association constant of 3×10^6 litre/mol had been determined. Calculations based on the results of these competition experiments enabled an association constant for bilirubin of approx. 5×10^7 litre/mol to be derived.

Equilibrium-dialysis studies (Ketterer *et al.*, 1976) showed that bilirubin is able to compete with oestrone sulphate for binding by protein A, but reliable quantitative data could not be obtained by this technique because of aggregation and adsorption of the tetrapyrrole. Kamisaka *et al.* (1975*b*) observed induced Cotton effects associated with bilirubin bound to Z-protein which were similar in magnitude, but opposite in sign, to those of the bilirubin–ligandin complex. In the presence of equimolar amounts of ligandin and Z-protein, the optical activities of bound bilirubin virtually cancelled, and it was concluded that the two proteins bind bilirubin with similar affinities.

In view of the discrepancies in the published binding data for ligandin and of the relative lack of such data for protein A, we considered it important to study the interactions of bilirubin with these proteins in more detail. To avoid first the cumulative errors to which the indirect methods of Kamisaka *et al.* (1975*a,b*) may be subject, and secondly the relatively long equilibration times (2–3h) that are required in the technique of Meuwissen *et al.* (1972), we have used difference spectrophotometry to make direct rapid measurements of binding. We have tried to gain further information on the interactions involved by fluorescence spectroscopy and spectropolarimetry.

Experimental

Materials

Bilirubin IXα, oestrone sulphate, glutathione and bovine serum albumin were purchased from the Sigma Chemical Co., Kingston-upon-Thames, Surrey U.K. Bilirubin was checked for purity by t.l.c. as described by McDonagh & Assisi (1971). Disodium bromosulphophthalein [disodium 3,4-(1′,2′,5′,6′-tetrabromobenz)-5,5-bis-(4-hydroxyphenyl-3-sulphonate)-furan-2-one] was from G. T. Gurr, High Wycombe, Bucks., U.K. Sephadex G-100 and CM-Sephadex C-50 were from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Whatman DEAE-cellulose was from

Hospital and Laboratory Supplies Ltd., London EC1M 6BB, U.K. Ampholines were from LKB Instruments, Croydon, Surrey, U.K. All other chemicals were purchased from BDH Chemicals, Poole, Dorset, U.K.

Methods

Preparation of proteins. Aminoazo-dye-binding protein A, form III, was prepared as previously described (Ketterer *et al.*, 1976). Ligandin was prepared by a modification of the method of Ketterer *et al.* (1967). Homogenates (1:3, w/v) of rat liver in 0.25M-sucrose were centrifuged for 90min at 30000 rev./min in a Beckman 30 rotor, and the supernatant was collected. After dialysis against 1mM-triethanolamine/1.25mM-$CaCl_2$/HCl buffer, pH8.0, this was applied to a column (2.6cm × 30cm) of DEAE-cellulose equilibrated with the same buffer, and the protein passing through the column un-adsorbed was collected. This fraction was made 50mM with respect to NaCl, adjusted to pH5.8 with acetic acid, and applied to a column (2.6cm × 20cm) of CM-Sephadex C-50 equilibrated with 26mM-NaCl/10mM-sodium acetate buffer, pH5.8. The column was washed with buffer and the ligandin was then eluted with 0.2M-NaCl. After dialysis against water, this fraction was subjected to isoelectric focusing on an LKB Uniphor column containing a linear 0–40% (w/w) sucrose density gradient (24 steps of 9.2ml) and 1% (w/v) with respect to Ampholine of pH range 7–10. The separation was carried out at 4°C over 4 days. On the first day the potential difference was 500V, and for the remaining 3 days was increased to 1kV. Fractions (4ml) were collected, and those with 1-chloro-2,4-dinitrobenzene–glutathione transferase activity (EC 2.5.1.18) (Habig *et al.*, 1974) which focused at pH9.0–9.5 were pooled, concentrated by ultrafiltration to approx. 1ml and applied to a column (2.6cm × 90cm) of Sephadex G-100 equilibrated with 0.1M-KCl/0.025M-potassium phosphate buffer, pH7.0. Two protein peaks were eluted, the second of which, corresponding to a mol.wt. of about 46000 (V_e/V_0 1.5–1.6), was purified ligandin.

Preparation of solutions. Protein solutions were made up in 0.1M-KCl/0.025M-potassium phosphate buffer, pH7.0, $I = 0.16$M, or in 0.1M-KCl/0.05M-Tris/HCl buffer, pH8.2, $I = 0.125$M. Concentrations were determined by the u.v.-spectrophotometric methods of Waddell (1956), Tombs *et al.* (1959) and Scopes (1974), and by the colorimetric method of Lowry *et al.* (1951), with bovine serum albumin as standard. Bovine serum albumin concentrations were determined from the extinction coefficient, $E_{279}^{1\%} = 6.7$ (Reynolds *et al.*, 1967). The uncertainty in the protein concentrations determined by these four methods was no greater than ± 5%, and as such had no important effects on the results for either affinities or stoicheiometries of binding.

Stock solutions of bilirubin (about 1mM) were prepared in 10mM-NaOH, and their concentrations determined spectrophotometrically by using the ε_{400} value of 52000 litre·mol^{-1}·cm^{-1} (Blauer & King, 1970). Solutions (1–2mM) of oestrone sulphate and bromosulphophthalein were prepared by dissolution in 0.1M-KCl/0.05M-Tris/HCl buffer, pH8.2.

Spectrophotometry. Measurements were made with a Pye–Unicam SP.1800 double-beam spectrophotometer in cells of 0.1, 0.5, 1.0 and 4.0cm path-length. The temperature of the cell compartment was 24–28°C. Titrations were carried out by adding equal volumes of stock solutions of bilirubin from a microsyringe to both sample and reference cell, the former containing a measured volume of protein solution at pH7.0 or 8.2, the latter an equal volume of buffer (pH8.2). At pH8.2, with total bilirubin concentrations less than 25μM, under which conditions solutions of bilirubin are free from aggregates for up to 40min after preparation (Brodersen & Theilgaard, 1969), complete titrations could be carried out on single protein solutions. At higher concentrations at pH8.2, and in all experiments at pH7.0, a separate solution was used for each measurement. Measurements were made immediately after mixing solutions, and no significant time-effects were observed during the first 10min in any of the solutions investigated.

Fluorescence spectroscopy. Measurements were made with a Perkin–Elmer Hitachi MPF 3L spectrofluorimeter at pH7.0. The cell compartment was maintained at 26°C.

Circular dichroism. Measurements were made with a Cary 61 spectropolarimeter at 24°C.

Results

The binding of bilirubin by both ligandin and protein A is accompanied by a red shift in the absorption maximum, and quite large difference-spectra result (Fig. 1). Fig. 2 shows titrations of the two proteins with bilirubin; although results at only one wavelength are shown in each case, the difference spectra had the same shapes as those in Fig. 1 throughout. Both titration curves level off at bilirubin/protein ratios close to unity, indicating that ligandin and protein A each have a single binding site for the ligand. Because the initial slopes of the titration curves are linear (i.e. $\Delta\varepsilon$ is constant in this region) it can be assumed that the difference spectra shown in Fig. 1, which correspond to the lowest bilirubin protein ratios, represent completely bound ligand. The same values of $\Delta\varepsilon$ (20400 litre·mol^{-1}·cm^{-1} for ligandin, 12900 litre·mol^{-1}·cm^{-1} for protein A) were obtained at pH7.0 and 8.2.

These observations provide a means for the determination of bound and unbound bilirubin and thence the binding parameters for the two bilirubin–protein systems. Concentrations of bound bilirubin are given

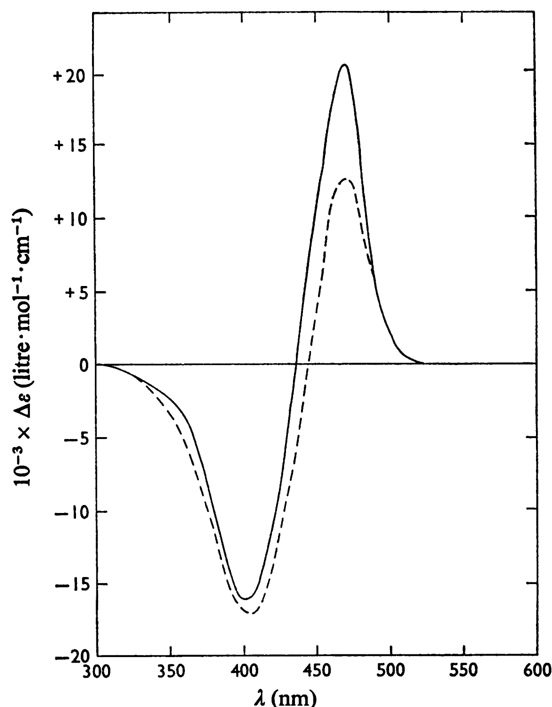

Fig. 1. *Difference spectra resulting from the binding of bilirubin by ligandin (——) and aminoazo-dye-binding protein A (- - - -)*

The sample cell contained 2.51 ml of 5 μM-bilirubin/25 μM-protein solution. The reference cell contained 2.51 ml of 5 μM-bilirubin. The same spectra were obtained at pH 7.0 and 8.2. $\Delta\varepsilon$ refers to total bilirubin.

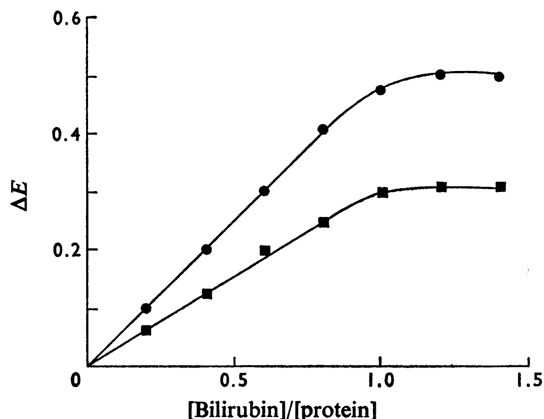

Fig. 2. *Difference spectrophotometric titrations of ligandin (●) and aminoazo-dye-binding protein A (■) with bilirubin, pH 7.0*

The sample cell initially contained 2.5 ml of protein solution (25 μM), pH 7.0, and the reference cell 2.5 ml of buffer, pH 8.2. Equal volumes (10–70 μl) of bilirubin solution (1.25 mM in 10 mM-NaOH) were added to each. Each titration point represents a separate solution. Absorbance differences were measured at 472 nm for ligandin and 474 nm for protein A.

by $\Delta E/\Delta\varepsilon$, where ΔE is the observed difference in absorbance, and unbound concentrations are obtained by subtraction of $\Delta E/\Delta\varepsilon$ from the total bilirubin concentration. To determine binding parameters, we carried out titrations at protein concentrations (1–3 μM) sufficiently low to ensure that significant amounts of bilirubin remained unbound. The results of these experiments were plotted by the method of Scatchard (1949) (Fig. 3).

As expected from the titrations at high protein concentrations, the data from Fig. 3 show that both proteins bind bilirubin at a single site. At pH 8.2 the association constant (K) for ligandin is 1×10^6 litre/mol, whereas at pH 7.0 binding is stronger ($K = 7 \times 10^6$ litre/mol). For protein A at pH 8.2, an association constant of 1.1×10^7 litre/mol is obtained, at pH 7.0 it was not possible to make reliable estimates of unbound bilirubin because binding was so strong at this pH that the difference between bound and total concentrations became too small to determine with precision. These observations suggest that, at pH 7.0, the association constant for protein A is greater than or equal to 2×10^7 litre/mol.

The addition of excess of either bromosulphophthalein or oestrone sulphate to the bilirubin–protein solutions caused marked decreases in the absorbance differences (Fig. 4); bromosulphophthalein was the more effective competitor for both proteins. These results are in qualitative agreement with previous data at pH 7.0 obtained by equilibrium dialysis (Ketterer *et al.*, 1975, 1976).

As well as altering the absorption spectrum of bilirubin, the two proteins also cause changes in its fluorescence spectrum. At a concentration of 2 μM, unbound bilirubin displays a weak fluorescence ($\lambda_{max.}$ = 520 nm). At the same concentration but in the presence of a tenfold molar excess of ligandin, the fluorescence is enhanced approx. threefold ($\lambda_{max.}$ = 519 nm). Protein A causes a sixfold enhancement ($\lambda_{max.}$ = 515 nm) under the same conditions.

Fig. 5 shows another result of the binding of bilirubin by ligandin and protein A, namely the induction of large Cotton effects centred near the absorption maximum of the tetrapyrrole. These have previously been observed for the bilirubin–ligandin complex by Kamisaka *et al.* (1973); our results at pH 8.2 are closely similar to theirs at pH 7.4. The bilirubin–ligandin complex gives rise to two major bands (a maximum at 410 nm and a minimum at 465 nm) and one minor band (a maximum at 515 nm),

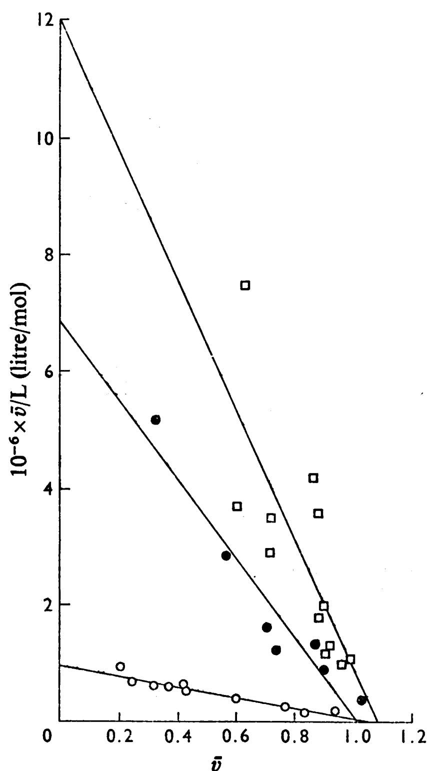

Fig. 3. *Scatchard plots for the binding of bilirubin by ligandin and aminoazo-dye-binding protein A*

□, Protein A, pH 8.2; ●, ligandin, pH 7.0; ○, ligandin, pH 8.2. $\bar{v}$ is the molar ratio of bound bilirubin to total protein; L is the concentration of unbound bilirubin.

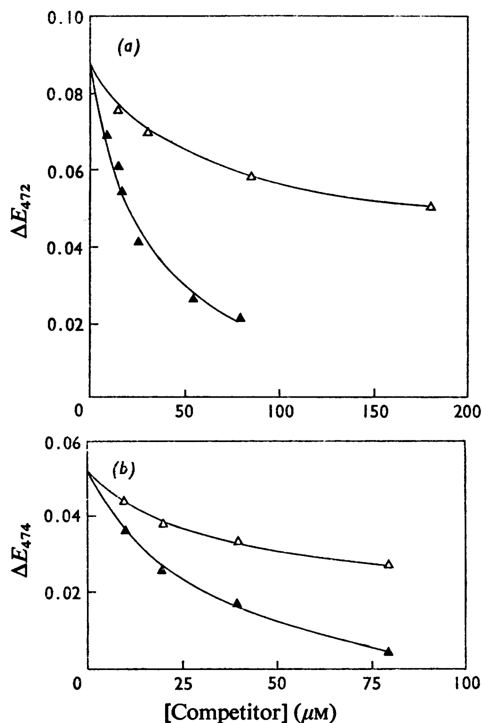

Fig. 4. *Effects of bromosulphophthalein* (▲) *and oestrone sulphate* (△) *on the binding of bilirubin by ligandin and aminoazo-dye-binding protein A, pH 8.2*

(*a*) Ligandin: [protein] = 4.6 μM, [bilirubin] = 8.1 μM; (*b*) protein A: [protein] = 6.6 μM, [bilirubin] = 4.1 μM. For further details, see the text.

whereas the protein A complex is characterized by only two bands (a maximum at 470 nm and a minimum at 410 nm).

Discussion

Our results show that the series of small molecules which are bound with comparable affinities and specificities by ligandin and aminoazo-dye-binding protein A (Ketterer *et al.*, 1975, 1976) can be extended to include bilirubin. In the present study the pronounced spectral changes which arise from the interactions have allowed us to gain insight into the qualitative nature of the binding as well as the energetics; the optical properties of the two protein–bilirubin complexes suggest that they are formed by similar processes.

By difference spectrophotometry we find an association constant of 7×10^6 litre/mol (pH 7.0, I = 0.16 M, 24–28°C) for the binding of bilirubin by ligandin. This result falls between that of 1×10^6 litre/mol (pH 7.4, I = 0.1 M, 4°C) reported by Meuwissen *et al.* (1972) and the value of 5×10^7 litre/mol (pH 7.4, I = 0.02 M, 25°C) derived from competition experiments by Kamisaka *et al.* (1975*a*). The rather wide spread of these results may be due in part to the different conditions used in the three studies and perhaps also to the instability of solutions of bilirubin at neutral pH, which could lead to errors in experiments involving long equilibration times and/or high concentrations of the ligand. The lower association constant (1×10^6 litre/mol) for ligandin at pH 8.2 probably reflects a change in the protein molecule, since the state of ionization of bilirubin is essentially the same at this pH as at pH 7.0.

The association constant at pH 7.0 for protein A falls outside the upper limit of sensitivity of the difference-spectrophotometric technique, and we conclude that the value is at least 2×10^7 litre/mol, but probably no greater than 1×10^8 litre/mol. As in the case of ligandin, we find a lower association constant at pH 8.2; again this presumably reflects a

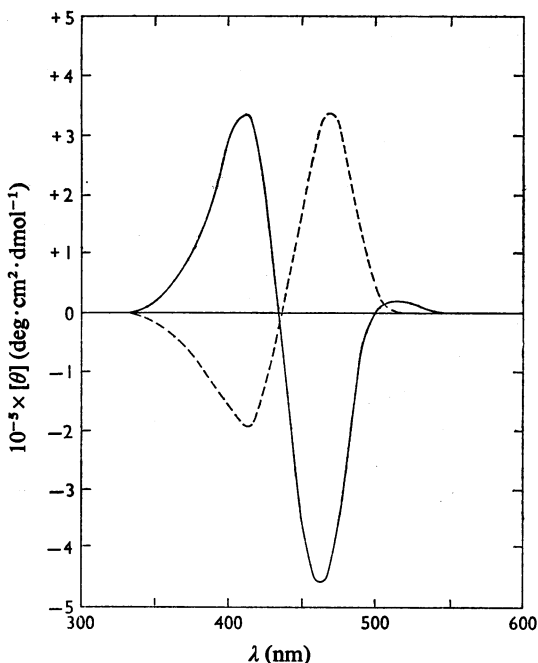

Fig. 5. *Circular dichroism of bilirubin–ligandin* (——) *and bilirubin–aminoazo-dye-binding protein A* (– – – –) *complexes at pH*8.2

The concentration of bilirubin and protein were 2 and 50 μM respectively. Molar ellipticities, [θ], refer to total bilirubin.

change in the protein. Kamisaka *et al.* (1975*b*) reported an association constant of the order of 1×10^8 litre/mol for the binding of bilirubin by Z-protein, and observed Cotton effects for this protein closely similar to those shown for protein A (Fig. 5). These results are further evidence that proteins A and Z are the same, which from a terminological standpoint is somewhat unfortunate.

The red shifts in the absorption maximum of bilirubin which occur on binding resemble those accompanying the transfer of the ligand from aqueous solution to non-polar solvents such as chloroform (see, e.g., McDonagh & Assisi, 1971) and therefore suggest that bound bilirubin is in a largely hydrophobic environment. Red shifts of this kind have also been observed for serum albumins of a number of species (Blauer & King, 1970; Blauer *et al.*, 1970, 1972; Woolley & Hunter, 1970; Harmatz & Blauer, 1975).

The increases in fluorescence associated with binding by ligandin and protein A at pH 7.0 (three- and six-fold respectively) are comparable with the ten-fold increase observed for human serum albumin at pH 8.4 by Beaven *et al.* (1973). These workers found that decreasing the dielectric constant of an aqueous solution of free bilirubin by the addition of dioxan had little effect on the fluorescence, and concluded that the enhanced emission resulting from binding by albumin is caused by immobilization of the ligand rather than by its transfer to a hydrophobic environment. Presumably this is also the case for ligandin and protein A.

The unusually large Cotton effects induced by the binding of bilirubin by ligandin and protein A, and by serum albumin from several species (Blauer & King, 1970; Blauer *et al.*, 1970, 1972; Woolley & Hunter, 1970; Beaven *et al.*, 1973; Harmatz & Blauer, 1975) are similar in magnitude to those for intrinsically dissymmetric chromophores (Moscowitz, 1962) and it appears therefore that the tetrapyrrole is forced into a twisted configuration at the binding sites of these proteins. The generation of the optical activities arising from the dissymmetry has been discussed in considerable detail by Blauer *et al.* (1972), Blauer & Harmatz (1972) and Beaven *et al.* (1973).

Quantitative studies of the binding of bilirubin by serum albumin show that affinities depend considerably on the species from which the albumin is isolated and on the conditions of ionic strength and pH under which binding is measured (Jacobsen, 1969; Beaven *et al.*, 1973; Harmatz & Blauer, 1975). In general, association constants for the primary binding site on the various albumins are between 1×10^6 and 1×10^9 litre/mol at pH 7.0–8.5. Since our results for ligandin and protein A fall within this range, it is clear that these intracellular proteins resemble albumin quantitatively as well as qualitatively with regard to their interactions with bilirubin.

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