Proc. Natl. Acad. Sci. USA
Vol. 75, No. 3, pp. 1213–1216, March 1978
Biochemistry

Ligandin retains and albumin loses bilirubin binding capacity in liver cytosol

(circular dichroism/organic anion uptake/hepatic uptake mechanisms)

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Communicated by Harry Eagle, December 29, 1977

ABSTRACT **Circular dichroism methods were used to detect bilirubin–ligandin interactions in rat liver cytosol and fractions obtained at various stages during purification of ligandin. Ligandin retained its capacity to bind bilirubin in the presence of components of liver supernatant, but albumin, which binds bilirubin in serum, lost the capacity to bind bilirubin in liver supernatant. This was attributed to a greater binding specificity exhibited by ligandin. In their respective physiological milieus, albumin and ligandin are structurally adapted to bind ligands: albumin in serum, and ligandin in the cytosol of the liver cell. These studies are consistent with the hypothesis that the concentration of ligandin within the liver could regulate the net flux of certain organic anions from plasma into the liver.**

Ligandin is an abundant soluble hepatic protein that binds bilirubin and other organic anions *in vitro* and *in vivo* (1–3). The protein is basic (pI = 9.1) (4), has a molecular weight of 46,000, and is a dimer consisting of two different subunits (5–7). Bilirubin–ligandin complexes generate characteristic multiphasic circular dichroic bands in the region of bilirubin absorption (8–10). Circular dichroism has been used to study the binding of bilirubin and other ligands to purified rat ligandin, as well as the exchange of bilirubin between ligandin and albumin (8).

The results of physiologic studies suggest that ligandin may be an important determinant in the uptake, retention, and flux of bilirubin and other organic anions from plasma into the liver (11); however, studies showing that serum albumin has a greater affinity for bilirubin than does purified ligandin challenge this hypothesis (12). Ketterer *et al.* suggest that the affinity of ligandin for bilirubin decreases during purification of the protein (13). Results of the present study indicate that this explanation is probably incorrect and reveal that albumin and ligandin bind best in their respective physiological compartments. Ligandin binds bilirubin most effectively in the cytosol of the liver cell, whereas albumin cannot bind under these conditions.

EXPERIMENTAL SECTION

Stock bilirubin solutions were 1–10 mM in 20 mM NaOH and were stored for no more than 3 hr, in the dark at 0°, prior to use. Absorbancies of bilirubin remained constant. Small aliquots were added to protein-containing samples as indicated, to give the final concentrations noted in the text.

Rat serum albumin was obtained from Sigma Corp., St. Louis, and bilirubin from Eastman Corp. ($\epsilon = 6.0 \times 10^4$ at 450 nm in chloroform). Protein concentrations were determined by the method of Lowry *et al.* (14) or by absorbance at 280 nm for purified albumin or ligandin. Ligandin concentrations in cytosol fractions or during subsequent stages of purification were determined by immunoquantitation procedures described earlier (15).

Liver supernatant fractions that contain little, if any, ligandin were prepared by two procedures. (*i*) Anti-ligandin IgG was added in excess to a rat liver supernatant as described (15). After incubation and stirring at 37°, the immunoprecipitate was removed by centrifugation and the supernatant was studied. (*ii*) After gel filtration of liver supernatant on Sephadex G-75, the ligandin-containing peak was removed and the remaining fractions were combined and concentrated to the original protein concentration.

Circular Dichroism. Spectra were obtained with a Cary model 60 spectropolarimeter with a 6001 CD attachment. The temperature of the cell compartment was 27°, and a cell of 1-cm pathlength was used for all of the measurements. Slit widths were programmed for a spectral band width of 15 Å or less, and absorbancies were always less than 2.0. Data were expressed in terms of observed ellipticities (θ_{obs}) in millidegrees per μM ligandin or albumin.

Preparation of Rat Liver Cytosol and Ligandin. Male Sprague–Dawley rats (200–220 g) were decapitated and the liver was removed and perfused thoroughly with ice-cold 10 mM sodium phosphate, pH 7.4. The liver was homogenized in one volume of 10 mM sodium phosphate, pH 7.4, and centrifuged at 110,000 × *g* for 2 hr. The resulting supernatant was divided into four equal parts. One fraction was used directly for circular dichroic studies (SUP fraction), and a second fraction was dialyzed for 20 hr at 4° against 10 mM sodium phosphate, pH 7.4 (SUP_D fraction). A third fraction was applied to columns containing either Sephadex G-75 or G-100 (2.5 × 100 cm). A fourth fraction was used for purification of ligandin, which involved sequential chromatography on *O*-triethylaminoethyl (TEAE)-cellulose, Sephadex G-75, and QAE-Sephadex A50 columns (16).

Because rat ligandin and serum albumin have secondary bilirubin binding sites (8, 9, 17–19), the stoichiometric ratio of bilirubin to protein was kept at 1:1 unless otherwise indicated. In the presence of excess bilirubin, albumin and ligandin bind bilirubin simultaneously, cancelling effects occur, and the data are too complex to interpret in terms of protein affinities.

RESULTS

Circular dichroism spectra reveal characteristic ligandin–bilirubin ellipticity extrema when bilirubin is added to the supernatant of rat liver homogenates (Fig. 1). The ellipticity band at 515 nm is indicative of binding at a secondary affinity site of purified ligandin (9) and is not observed in the spectra of mixtures of cytosol and bilirubin. These results probably reflect

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Abbreviation: TEAE-cellulose, *O*-triethylaminoethyl-cellulose.

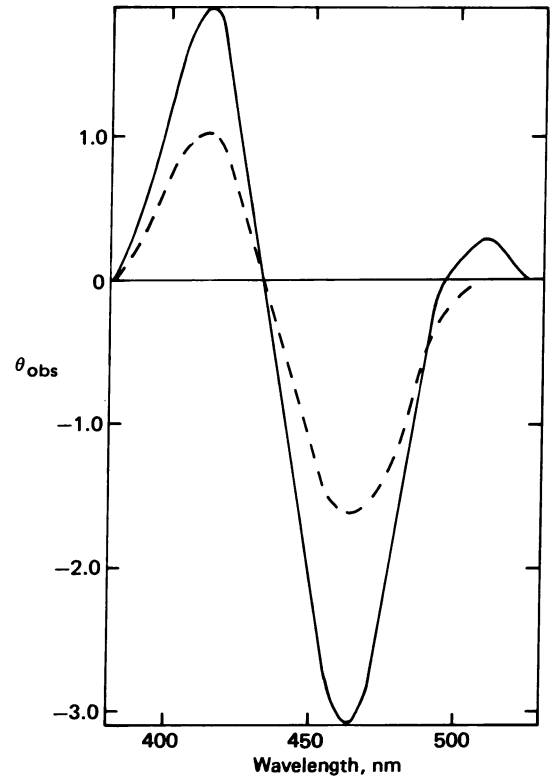

FIG. 1. Circular dichroism spectra of ligandin–bilirubin complexes. The solid line (—) is the spectrum for a pure ligandin–bilirubin 1:1 complex (10 μM in 0.1 M sodium phosphate buffer, pH 7.4). The dashed line (- - - -) represents an average of seven different rat liver supernatant fractions incubated with bilirubin (deviations were less than 10% of the recorded ellipticity values). The data are expressed in terms of the concentration of ligandin in the supernatant, as estimated by immunological methods. θ_{obs} is the observed ellipticity and the data are normalized per μM ligandin.

formation of bilirubin complexes at the primary binding site of ligandin even in the presence of cytosol.

After dialysis of liver supernatant and addition of excess bilirubin, ellipticities reached saturating values, which were about 75% of those expected for a ligandin–bilirubin complex based on circular dichroic data (8) or immunoquantitation of ligandin (15).

Optically active absorption bands were associated exclusively with ligandin-containing fractions. No other protein–bilirubin type of circular dichroism spectra was detected with the remaining proteins after removal of ligandin by fractionation or immunoprecipitation. Quantitation of ligandin by radial immunodiffusion (15) and by circular dichroism (Table 1) was compared. The results indicate that ellipticity magnitudes accurately reflect the concentration of ligandin as determined immunologically at each stage of the purification process. Circular dichroism provides a unique direct spectroscopic means of monitoring purification of the protein.

Albumin–bilirubin complexes from rat serum induce circular dichroic spectra that are virtually mirror images of the ligand–bilirubin spectra, with a positive band at 460 nm and negative band at 410 nm (18). Therefore, circular dichroism spectra may be used to study the exchange of bilirubin between ligandin and albumin. In earlier studies, we showed that, at equimolar concentrations, almost all of the bilirubin bound to ligandin is transferred to rat serum albumin (8, 18). These results imply that albumin has a substantially greater affinity for bilirubin than does purified ligandin. Similar results were also

Table 1. Purification of ligandin as estimated by bilirubin binding

Component*	Total protein, mg/ml	μg ligandin/mg protein: Immunological	μg ligandin/mg protein: CD†
Liver supernatant	29.7	26	15
Dialyzed supernatant	28.0	37	28
Ligandin			
TEAE-cellulose fraction	2.8	135	121
Sephadex G-75 fraction	1.2	752	807
QAE-Sephadex fraction (pure)	0.5	1000	1000

* These experimental components represent fractions obtained during the main procedures commonly used to purify ligandin. The fractions were isolated and circular dichroism and other measurements were made directly as the fractions were eluted from the columns. Total protein concentrations were determined by the method of Lowry *et al.* (14).

† CD, circular dichroism. Ligandin concentrations were based on reported ellipticity magnitudes at 460 and 415 nm of bilirubin–ligandin 1:1 complexes (8). Pure bilirubin–ligandin 1:1 complexes (10 μM) exhibited observed ellipticities of −30.7 millidegrees at 460 nm and +19.1 millidegrees at 415 nm, and concentrations of ligandin in each fraction were estimated from these values.

obtained with ligandin fractions obtained after the TEAE-cellulose chromatographic step and at subsequent stages of purification, where rat albumin quantitatively removed bound bilirubin (Fig. 2). It is pertinent, however, that albumin, even in 100-fold excess, was ineffective in removing bilirubin from ligandin in liver supernatant fractions (Fig. 2). Even after ex-

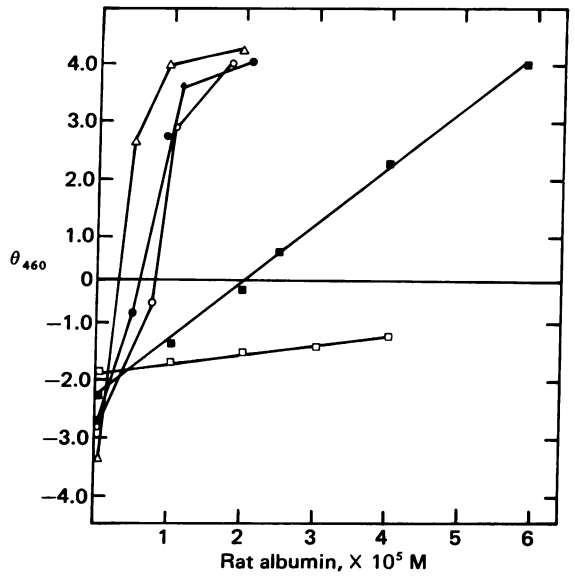

FIG. 2. Transfer of bilirubin from ligandin to rat serum albumin. Ellipticity values at 460 nm, which are positive (+) for the bilirubin–albumin complex and negative (−) for the bilirubin–ligandin complex (8), were used to index the disposition of bilirubin in the mixtures. θ_{460} is the observed ellipticity at 460 nm, expressed in millidegrees per μM protein. Stoichiometric complexes (1:1) of bilirubin–ligandin (10 μM) in each fraction were incubated with the indicated amounts of albumin. (Almost identical results were obtained with protein and bilirubin concentrations of 3 μM.) △, Purified ligandin obtained after QAE-Sephadex gel filtration. ● and ○, Ligandin-containing fractions obtained from the ion exchange (TEAE-cellulose) and gel filtration (Sephadex G-75) purification steps, respectively. Data were obtained with bilirubin complexes with the original supernatant fraction (□) and dialyzed supernatant (■) fractions.

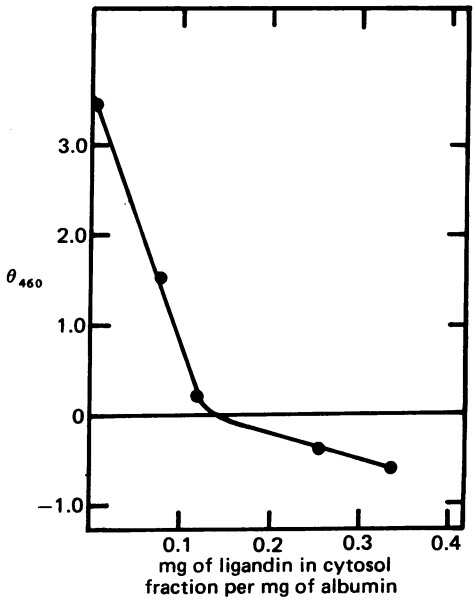

FIG. 3. Bilirubin binding in rat liver cytosol. Displacement of bilirubin from albumin by rat liver supernatant fractions. θ_{460} is the observed ellipticity at 460 nm/μM albumin. Aliquots of supernatant were added to a bilirubin–albumin complex and corrections were made for dilution (less than 10% during these experiments). The concentration of ligandin was estimated by immunological means.

tensive dialysis of the supernatant fraction, the effectiveness of albumin in displacing bilirubin from ligandin was substantially less than that observed with the pure proteins. Tenfold excess of glutathione, a cytosol component that could be a potential factor in influencing removal of bilirubin from albumin, had no effect on bilirubin binding to the purified protein.

Increments of rat liver supernatant fractions were added to complexes of bilirubin and rat serum albumin to reverse the transfer of bilirubin to ligandin. In these studies, cytosol fractions containing less than 0.10 mg of ligandin per mg of albumin abolished the albumin–bilirubin circular dichroism spectrum (Fig. 3). Conversely, addition of supernatant fraction to a purified ligand–bilirubin complex had little effect on the circular dichroic spectrum generated by this complex. Preferential bilirubin binding by albumin and ligandin under various conditions is summarized in Table 2.

Binding experiments with Indocyanine Green were carried out and compared to the phenomena observed with bilirubin.

Table 2. Bilirubin binding by albumin and ligandin in rats

	Albumin	Ligandin
Serum	+	*
Liver supernatant	−	+
Muscle or testes supernatant†	+	+
Equimolar mixture of the two proteins and bilirubin	+	−

The criterion used to determine bilirubin binding to albumin or ligandin (+) was the appearance of circular dichroism extrema characteristic of protein–bilirubin complexes.

* Measurements were not made because of high concentrations of albumin in rat sera.

† Unlike liver supernatant, where both endogenous and exogenous ligandin components generated ellipticity bands, no detectable circular dichroism spectra were obtained after addition of bilirubin to these supernatant functions. Purified ligandin or albumin were thus added to these supernatant components and both proteins exhibited the ellipticity extrema expected for bilirubin complexes under these conditions.

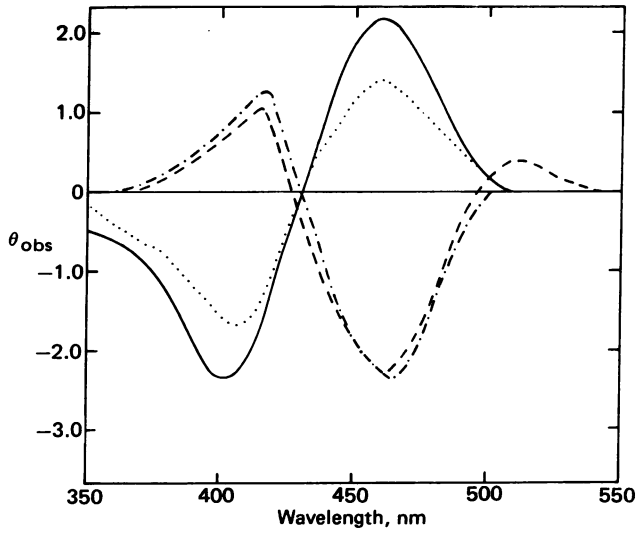

FIG. 4. Transfer of bilirubin from ligandin to rat serum. Circular dichroic spectra for: (- - - - -) a ligandin–bilirubin 1:1 complex containing 20 μM protein and bilirubin; (- · - · -) addition of 50 μl of rat serum per 3 ml to the above; (····) addition of 300 μl of serum; (—) addition of 400 μl of serum. θ_{obs} is the observed ellipticity that was normalized to 1 μM ligandin.

Complexes of Indocyanine Green and rat albumin generate positive ellipticity bands centered near 393 nm. Small amounts of rat liver supernatant fractions also abolished these effects. However, complexes of ligandin and Indocyanine Green, which generate two positive bands at 400 and 340 nm (8), required almost 5-fold higher concentrations of supernatant components to abolish the effects. These data suggest that binding of Indocyanine Green ($K_A = 10^5$ as compared to 5×10^7 for bilirubin) in the supernatant environment also exhibits preferential binding to ligandin as compared to albumin.

Increments of rat serum were added to ligandin, and the results are shown in Fig. 4. At low concentrations of serum, the 515 nm band, which reflects the secondary binding site on ligandin (17–19), was obliterated. Additional rat serum inverted the ellipticity pattern, indicating transfer of bilirubin from ligandin to albumin in rat serum.

DISCUSSION

In the presence of liver cytosol, ligandin binds bilirubin whereas albumin, which binds bilirubin in serum (20), lacks binding capacity and does not promote bilirubin transfer from ligandin. The ineffectiveness of albumin may result from higher affinity binding by ligandin in cytosol as compared to interactions between purified ligandin and bilirubin. Indeed, the transfer of bilirubin from albumin to ligandin as determined by moving boundary sedimentation has been interpreted to indicate higher affinity binding by ligandin in the cytosol (13). This hypothesis implies a loss of binding affinity during purification of ligandin. Results of the present studies, however, indicate that factors other than increased affinity of ligandin contribute to the more effective binding of bilirubin by ligandin than by albumin in the cytosol fraction. Our data show that purified ligandin added to liver supernatant had the same binding properties as endogenous ligandin. The inability of albumin to remove bilirubin from the cytosol fraction cannot be attributed exclusively to competitive binding of bilirubin to ligandin, since cytosol fractions that either lack ligandin or have lower levels of ligandin as compared to albumin prevented bilirubin from binding to albumin. It is likely that liver cytosol components,

such as fatty acids, phospholipids, bile acids, salts, metabolites, or other small molecules, prevent bilirubin binding perhaps by displacing it from its binding site on albumin.

The data suggest that ligandin exhibits a much higher specificity for bilirubin binding than does albumin, even though its affinity for bilirubin is approximately $^1/_{10}$ that of albumin. [Ligandin exhibits a broad specificity and binds a wide variety of ligands, but most are not as tightly bound as bilirubin (8).] The effects of cytosol components on bilirubin binding to ligandin are relatively small; ligandin has a strong capacity for retention of bilirubin in the milieu of the liver cell.

The results of the present study indicate that ligandin in its intracellular environment exhibits a greater tendency to bind bilirubin than does serum albumin. Consequently, bilirubin that has been transferred from plasma into the liver cell is bound to ligandin in a manner that favors its intracellular binding rather than substantial efflux back into the plasma. These studies support the hypothesis that the concentration of ligandin within the liver could regulate the net flux of various organic anions from plasma into the liver.

This work was supported by Grants AM 2019, AM 17702, and HL 11511 from the National Institutes of Health.

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