Giancotti, V., Quadrifoglio, F., and Crescenzi, V. (1973), *Eur. J. Biochem. 35,* 78.
Grizzuti, K., and Perlmann, G. E. (1969), *J. Biol. Chem. 7,* 1764.
Grizzuti, K., and Perlmann, G. E. (1970), *J. Biol. Chem. 10,* 2573.
Grizzuti, K., and Perlmann, G. E. (1973), *Biochemistry 12,* 4399.
Hiller, A., Plazin, J., and van Slyke, D. D. (1948), *J. Biol. Chem. 176,* 1401.
Joubert, F. J., and Cook, W. H. (1958), *Can. J. Biochem. Physiol. 36,* 399.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Snell, D. F., and Snell, T. C. (1949), in Colorimetric Methods of Analysis, Vol. II, New York, N.Y., Van Nostrand, p 396.
Snell, D. F., Snell, T. C., and Snell, A. C. (1959), in Colorimetric Methods of Analysis, Vol. IIA, New York, N.Y., Van Nostrand, p 292.
Taborsky, G. (1974), *Adv. Protein Chem. 28,* 1.

Interactions of Bilirubin and Other Ligands with Ligandin†

Kazuaki Kamisaka,‡ Irving Listowsky,*,§ Zenaida Gatmaitan, and Irwin M. Arias

ABSTRACT: Circular dichroism methods were used to study the structure of rat ligandin and the binding of organic anions to the protein. Ligandin has a highly ordered secondary structure with about 40% α helix, 15% β structure, and 45% random coil. Bilirubin binding occurred primarily at a single high affinity site on the protein. The binding constant for bilirubin ($5 \times 10^7\ M^{-1}$) was the highest among the ligands studied. The bilirubin–ligandin complex exhibited a well-defined circular dichroic spectrum with two major overlapping ellipticity bands of opposite sign in the bilirubin absorption region. This spectrum was virtually a mirror image of that of human or rat serum albumin–bilirubin complexes. Studies on the direct transfer of bilirubin from ligandin to rat serum albumin showed that association constants of bilirubin–ligandin complexes were approximately tenfold less than those of the bilirubin–albumin system. Ligandin exhibited a broad specificity with respect to the type of ligand bound. A series of organic anions including dyes used clinically for liver function tests, fatty acids, hormones, heme derivatives, bile acids, and other ligands that were considered likely to interact with ligandin, were examined. Most induced ellipticity changes consistent with competitive displacement of bilirubin from ligandin and relative affinities of these compounds for ligandin were determined based on their effectiveness in displacing the bilirubin. Some substances such as glutathione, conjugated sulfobromophthaleins and lithocholic acid bound to ligandin but induced anomalous spectral shifts, when added to ligandin–bilirubin complexes. Other compounds, including some that act as substrates for the glutathione transferase activity exhibited by ligandin, revealed no apparent competitive effects with respect to the bilirubin binding site.

Ligandin is an abundant cytoplasmic protein localized mainly in liver cells, proximal tubules of kidney, and nongoblet mucosal cells of the small intestine, in rats, monkey, and man (Levi et al., 1969a,b, Fleischner et al., 1972). Ligandin is a basic protein (pI = 9.1) with a molecular weight of 46,000 and consists of two apparently identical 23,000-dalton subunits (Litwack et al., 1971). It is considered to be a major determinant of the net flux of various organic anions from plasma into the liver (Arias, 1972). These include bilirubin, various dyes, (i.e., sulfobromophthalein, Indocyanine Green, Evans Blue), and metabolites. Ligandin also has glutathione transferase activity for selective substrates and is identical with glutathione transferase B (Habig et al., 1974). Several carcinogen metabolites bind covalently to ligandin (Litwack et al., 1971), whereas interactions with most other ligands are noncovalent.

An objective of the present report was to determine relative affinities of various ligands for ligandin. Competitive binding of these substances relative to bilirubin was analyzed by circular dichroism (CD) methods. Results with sulfobromophthalein using CD were correlated with estimates of affinity constants determined by equilibrium dialysis.

Experimental Section

Materials

Bilirubin was obtained from Eastman Corp., Rochester, N.Y., or Sigma Corp., St. Louis, Mo.; sulfobromophthalein and Indocyanine Green were purchased from Hyson, Westcott-Dunning, Inc., Baltimore, Md.; sodium penicillin and iodipamide were from E. R. Squibb Sons, Inc., New York, N.Y.; chloromycetin sodium succinate was from Parke-Davis Co., Detroit, Mich.; cortisol and oleic acid were from Applied Science Lab., Inc., State College, Pa.; hematoporphyrin, hemin, protoporphyrin, benzpyrene, *p*-aminohippuric acid, probenecid, cholic acid, chenodesoxycholic acid, deoxycholic acid, glycocholic acid, and lithocholic acid were from Sigma Corp., St. Louis, Mo.; glutathione (GSH re-

† From the Liver Research Center, Departments of Medicine (Division of Gastroenterology—Liver Diseases) and Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461. *Received November 13, 1974.* This work was supported by Grants AM 2019, 4465, 4479, and HL-11511 and Liver Center Grant 17702.

‡ Present address: 2nd Department of Internal Medicine, University of Tokyo.
§ Career Development Awardee (GM 19958) from the National Institute of General Medical Sciences, National Institutes of Health.

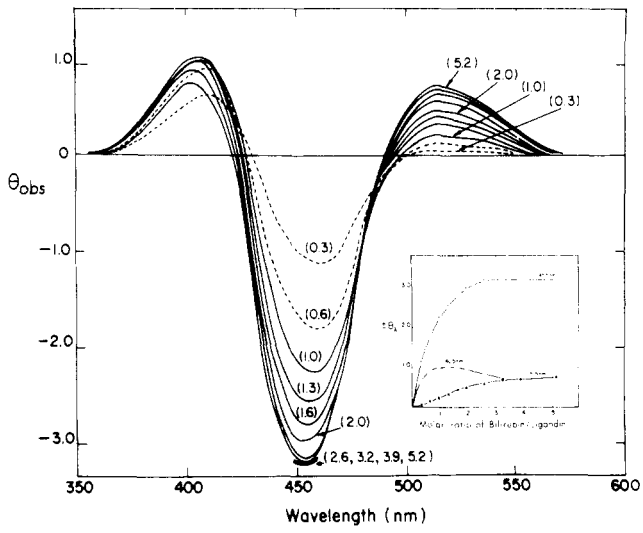

FIGURE 1: Circular dichroism spectrum of the ligandin–bilirubin complex. The increments of bilirubin (moles per mole of protein) are shown in parentheses. Observed ellipticities were calculated on the basis of the observed magnitudes in millidegrees per 10^{-6} *M* ligandin. Actual protein concentrations used were 2–5 × 10^{-5} *M*. Data obtained with less than equimolar amounts of bilirubin are represented at broken curves for clarity. The inset shows a titration of ligandin with bilirubin as reflected by the ellipticity bands at 515, 455, and 405 nm. The relative increase in positive or negative ellipticity ($\pm\theta$) at each wavelength is plotted against the number of moles of bilirubin added per mole of ligandin.

duced and G-S-S-G oxidized) and taurocholic acid were from Nutritional Biochem., Corp., Cleveland, Ohio; thyroxine and triiodothyronine were from Abbott Lab., North Chicago, Ill.; 1-chloro-2,4-dinitrobenzene and Evans Blue (T-1824) were from Eastman Kodak Co., Rochester, N.Y. Human serum albumin and rat serum albumin were purchased from Sigma Corp., St. Louis, or Schwarz/Mann, Orangeburg, N.Y.

Iophenoxic acid and sodium iodopanoate were supplied by Dr. R. Shapiro, Hospital of St. Raphael, New Haven, Conn., propranolol and lidocaine were gifts from Drs. Von Bahr and Sjoqvist, Karolinska Institute, Stockholm, Sweden, and flavaspidic acid–*N*-methylglucaminate was from Dr. Esa Aho, Turku, Finland.

Sulfobromophthalein–glutathione (conjugated sulfobromophthalein) was prepared synthetically by the method of Whelan et al. (1970) and its purity established by paper chromatography according to the method of Carbone et al. (1959).

Chambers for equilibrium dialysis (Karush Type) were obtained from Bellco Glass Inc., Vineland, N.J. Visking tubing (wall thickness: 0.0010 in.) was obtained from Arthur Thomas Co., Philadelphia, Pa., and pretreated with 1% EDTA, 5% $NaHCO_3$, and distilled water.

Methods

Preparation of Ligandin. Rat liver ligandin was purified from Sprague Dawley rats (250–350 g, either sex) as previously described (Kirsch et al., 1975). In brief, a 25% liver homogenate in 0.25 *M* sucrose–0.01 M phosphate buffer (pH 7.4) was centrifuged at 12,000 rpm for 30 min, and then for 90 min, at 100,000*g*. The supernatant was chromatographed on a TEAE column equilibrated in 0.01 *M* Tris buffer (pH 8.8). The protein portion containing ligandin was collected, applied to a Sephadex G-75 column, and eluted with 0.01 *M* phosphate buffer (pH 7.4). The ligandin-containing peak was chromatographed on a QAE-Sephadex column with 0.01 *M* Tris buffer (pH 8.8). The ligandin peak was collected and stored at 4° in the dark. At each step, the protein solution was identified immunologically and by sulfobromophthalein–protein interaction (Fleischner et al., 1972). Protein concentrations were determined by the method of Lowry et al. (1951). Purity of the final protein was at least 95% as determined by electrophoresis in 7.5% polyacrylamide gels (pH 9.5), sodium dodecyl sulfate gel electrophoresis, and immunoprecipitation with a monospecific anti-ligandin IgG.

Preparation of Ligandin–Bilirubin Complexes and Competitive Binding Studies. Protein solutions (10^{-5} *M*) were prepared in phosphate buffer (0.01 *M* total phosphate) (pH 7.4). Bilirubin solutions were freshly prepared in 0.02 *M* NaOH at concentrations of 10^{-2}–10^{-3} *M*. Sulfobromophthalein, Indocyanine Green, sodium penicillin, chloromycetin sodium succinate, iodipamide, *p*-aminohippuric acid, glutathione, taurocholic acid, Evans Blue, lidocaine, and conjugated sulfobromophthalein were dissolved in distilled water, pH 7.0 at concentrations of 10^{-2}–10^{-3} *M*. Throxine and triiodothyronine were dissolved in the distilled water at pH 9.0. Other substances were dissolved in methanol or ethanol at concentrations at 10^{-2}–10^{-4} *M*. At the concentrations used, these organic solvents alone had no detectable effects on the ligandin–bilirubin CD spectra.

Circular Dichroism Measurements. CD studies were performed using a Cary Model 60 spectropolarimeter with a 6001 CD attachment. The optical path lengths of the cells were 1.0 and 0.1 cm. Absorbancies of the solutions were maintained to give readings below 0.6 kV dynode voltage at all wavelengths. A mean residue weight of 110 was used for calculation of molar ellipticity.

Equilibrium Dialysis. Ligandin solutions were 1.8–2.0 × 10^{-5} *M* in 0.01 *M* phosphate buffer (pH 7.4). Dialysis systems utilized 1.5 ml of protein solution on one side of the membrane and protein-free buffer on the other side. Sulfobromophthalein was added to each side in all experiments. The chambers were gently mixed for about 72 hr at room temperature at which time equilibrium was approached. Concentration of the dye was measured spectrophotometrically at 580 nm.

Results

Conformation of Ligandin. The CD spectrum of ligandin is characterized by three ellipticity extrema associated with peptide absorption bands, including a positive band at 192 nm ($[\theta]_{192}$ = +31,000 (deg cm^2)/dmol and negative bands at 208 and 222 nm ($[\theta]_{208}$ = −14,000 and $[\theta]_{222}$ = −13,000 (deg cm^2)/dmol. Relative amounts of the three forms of secondary structure of ligandin were computed from these spectra by methods described earlier (Listowsky et al. 1972; Greenfield and Fasman, 1969) and values of 40% α helix, 15% β structure, and 45% random coil were obtained. No changes in this highly ordered secondary structure were induced by binding of bilirubin or of other ligands used in this study. In the spectral region between 300 and 250 nm, at least six distinct ellipticity extrema were observed (Kamisaka et al. 1973) which were ascribed to transitions of aromatic amino acid residues of the protein.

Ligandin–Bilirubin Complexes. The CD spectra of bilirubin–ligandin complexes featured three ellipticity extrema at wavelengths above 300 nm including positive bands at 515 nm and 405 nm, and a negative peak centered at 455 nm (Figure 1). Bilirubin binding also produced ellipticity

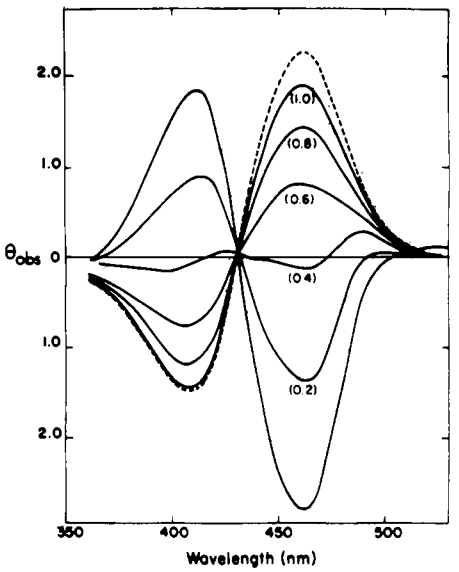

FIGURE 2: Circular dichroism study on the transfer of bilirubin from rat ligandin to rat serum albumin. The solid line is the circular dichroism spectrum of a bilirubin-ligandin 1:1 complex. The numbers in parentheses indicate the number of moles of rat serum albumin added per mole of 1:1 bilirubin-ligandin. The dotted line represents the circular dichroism spectrum of a bilirubin-rat serum albumin 1:1 complex. Ellipticity values shown are observed ellipticities (millidegrees) per 10^{-6} M protein.

changes near 255 nm (Kamisaka et al., 1973) which were probably associated with an optically active transition(s) of the bound bilirubin. The band at 405 nm increased in magnitude with added increments of bilirubin up to a mole ratio of 1:1 bilirubin-ligandin. No further increase was detected at higher bilirubin concentrations. The band at 455 nm attained about 80% of its maximal ellipticity magnitude after addition of 1 mol of bilirubin and no further changes in this band were observed after addition of more than 2 mol of bilirubin. In contrast, the 515-nm band showed major ellipticity increases after addition of 1, 2, or more moles of bilirubin (Figure 1) and ellipticity changes at 255 nm paralleled those at the 515-nm band (Kamisaka et al., 1973). Maximal ellipticity values associated with bilirubin binding to ligandin as well as maximal ordered secondary structure of the protein were observed in the pH range of 7-8 (Kamisaka et al., 1973).

Relative Affinities of Ligandin and Rat Serum Albumin for Bilirubin. The CD spectra of bilirubin-rat serum albumin complexes are characterized by a positive peak at 470 nm and negative band at 405 nm (Figure 2). These spectra are nearly mirror images of the CD spectra of rat ligandin-bilirubin complexes. It was, therefore, possible to study direct transfer of bilirubin from ligandin to rat serum albumin by monitoring changes in the CD profile of mixtures of rat serum albumin, bilirubin, and ligandin. A bilirubin-ligandin 1:1 complex was incubated in the presence of increasing amounts of rat serum albumin. Ellipticity changes shown in Figure 2 indicate that at equimolar concentrations (rat serum albumin-ligandin-bilirubin 1:1:1), over 90% of bilirubin was transferred from ligandin to rat serum albumin. This transfer occurred instantaneously upon addition of rat serum albumin. The association constant at the primary bilirubin binding site on rat serum albumin is, therefore, at least ten times greater than that of ligandin-bilirubin.

The Association Constant of Bilirubin-Ligandin Complexes. Using 10^{-5} M rat ligandin solutions, the magnitude of bilirubin-ligandin ellipticities at 455 nm increased linearly up to molar ratios of 1:1. Since unbound bilirubin was not detected from CD titration curves under these conditions, it may be assumed that the association constant of bilirubin is larger than 10^5 M^{-1} for the primary binding site on ligandin, but cannot be estimated directly from these data. On the other hand, bound bilirubin in a 1:1 ligandin-bilirubin complex was almost completely removed by an equimolar concentration of human serum albumin. This suggests that bilirubin has a greater affinity for human serum albumin than for ligandin. The association constant of a ligandin-bilirubin complex must therefore be less than 1.4×10^8 M^{-1} which is the association constant of human serum albumin-bilirubin (Jacobsen, 1969).

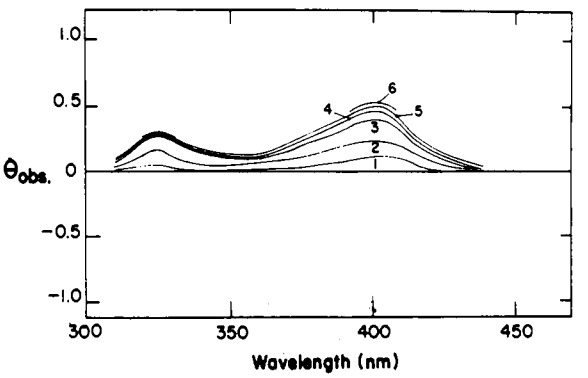

FIGURE 3: Circular dichroism spectra of Indocyanine Green-ligandin complexes. The numbers 1-6 indicate the number of moles of Indocyanine Green added per mole of ligandin. Data are expressed in terms of observed ellipticities (θ_{obs}) per 10^{-5} M protein.

Indocyanine Green generated optically active bands in 400-nm region upon binding to ligandin (Figure 3) and has a lower affinity than bilirubin for ligandin. Binding constants were directly obtained from the CD data. Based on CD titration curves of ligandin with Indocyanine Green, the approximate association constant was 3×10^6 M^{-1}. These data were used to calculate the association constant for bilirubin using the following relationship derived earlier (Kamisaka et al., 1974), and based on competitive binding data,

$$\bar{\nu}_B = \frac{K_B[B]}{1 + K_B[B] + K_I[I]} \quad (1)$$

where ν_B is the ratio of moles of bilirubin bound to total moles of ligandin, [I] and [B] represent concentrations of free Indocyanine Green and bilirubin, respectively, and K_B and K_I are association constants for bilirubin- and Indocyanine Green-ligandin complexes, respectively. Based on studies of the displacement of bilirubin by Indocyanine Green, K_B was 5×10^7 M^{-1}. Inherent in the quantitation of this approach are assumptions that (1) binding of a competing molecule occurs exclusively at the primary bilirubin site, (2) binding occurs in a simple noncooperative fashion, and (3) there are no conformational alterations of the bilirubin-ligandin complex rather than displacement of bilirubin from the protein.

Competitive Binding Studies. Various low molecular weight ligands were added to solutions of ligandin-bilirubin 1:1 complexes and CD spectra examined. In the presence of most of these substances, ellipticities associated with bilirubin-ligandin complexes decreased but no changes in the CD profile with respect to crossover points or location of maxima were observed. Diminution of ellipticities associated

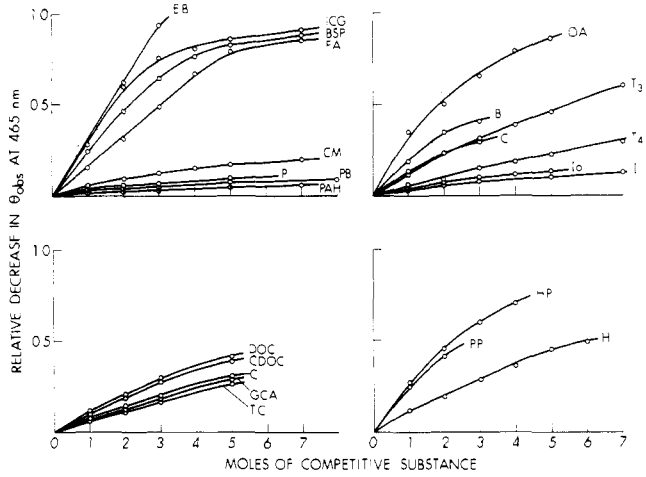

FIGURE 4: Competitive binding of various organic anions, dyes, hormones, bile acids, and heme derivatives. Displacement of bilirubin was monitored by the decrease in ellipticity magnitude of the band at 465 nm. Data are expressed in terms of moles of competitive substance per mole of the bilirubin-ligandin complex. Abbreviations used are: EB, Evans Blue; ICG, Indocyanine Green; BSP, sulfobromophthalein; FA, flavaspidic acid; CM, chloromycetin, P, penicillin; PB, probenecid; PAH, p-aminohippuric acid; OA, oleic acid; B, benzpyrene, T3, triiodothyronine; T4, thyroxine; C, cortisol, Io, Iophenoxic acid; I, iodipamide, DOC, deoxycholic acid: CDOC, chenodesoxycholic acid, C, cholic acid, GCA, glyocholic acid, TC, taurochlolic acid, HP, hematoporphyrin; PP, protoporphyrin; H, hemin.

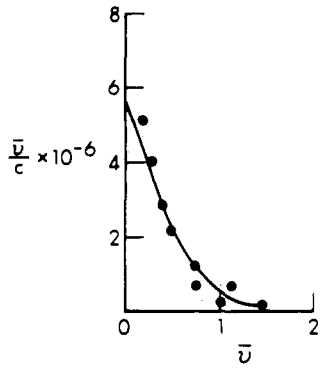

FIGURE 5: A Scatchard plot of the binding of sulfobromophthalein to ligandin. Data were obtained by equilibrium dialysis experiments according to the procedure outlined in the text. $\bar{\nu}$ represents the number of moles of sulfobromophthalein bound per mole of protein and C is the concentration of unbound (free) sulfobromophthalein.

with the bilirubin-ligandin complex probably reflect displacement of bilirubin from the primary binding site on the protein. Figure 4 shows competitive effects of several categories of these substances on the bilirubin-ligandin complex.

Approximate association constants for these compounds calculated using eq 1 were: oleic acid, Indocyanine Green, sulfobromophthalein, hematoporphyrin, and Evans Blue, $10^6\ M^{-1}$, benzpyrene, cortisol, hemin, and l-triiodothyronine had affinity constants of about $10^5\ M^{-1}$, and l-thyroxine and chloromycetin had binding constants of $10^4\ M^{-1}$ Iodipamide, penicillin, *p*-aminohippuric acid, and probenecid had constants of $10^3\ M^{-1}$ and iophenoxic acid interacted with binding constants of less than $10^3\ M^{-1}$. Propranolol, lidocaine, iodomethane, and 1-chloro-2,4-dinitrobenzene had no detectable competitive binding effects. Equilibrium dialysis of sulfobromophthalein and ligandin yielded an association constant of $6 \times 10^6\ M^{-1}$. A plot of these data according to Scatchard is shown in Figure 5.

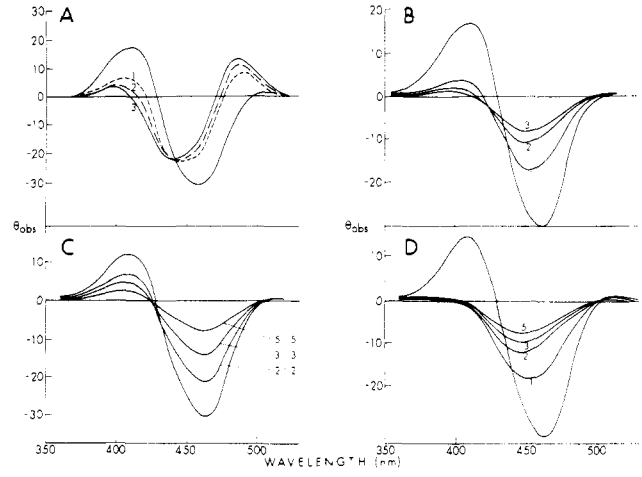

FIGURE 6: The effects of glutathione, conjugated sulfobromophthalein, and lithocholic acid on the bilirubin-ligandin complex. The unlabeled curves in each figure represent a bilirubin-ligandin 1:1 complex. θ_{obs} (millidegrees) is expressed per $10^{-5}\ M$ protein. (A) Effect of glutathione; (B) effect of conjugated sulfobromophthalein; (C) effect of simultaneous addition of sulfobromophthalein and glutathione; (D) effect of lithocholic acid. The numbers indicated are the number of moles of each substance added per mole of protein.

Glutathione, conjugated sulfobromophthalein, and lithocholic acid exhibited anomalous effects on bilirubin-ligandin CD spectra. Glutathione changed the CD pattern of the ligandin-bilirubin complex with new peaks appearing at 490, 449, and 400 nm (Figure 6A). The phenomenon appeared to be specific for the ligandin system and was not observed with albumin-bilirubin complexes. Oxidized glutathione had no detectable effect on CD spectra of bilirubin-ligandin or Indocyanine Green-ligandin complexes. Addition of conjugated sulfobromophthalein to ligandin-bilirubin complexes also promoted drastic changes in shape of the CD spectra (Figure 6B). Mixtures of these compounds, however, yielded simple displacement phenomena (Figure 6C). Spectral shifts were also induced by lithocholic acid (Figure 6D). With increased amounts of conjugated sulfobromophthalein or lithocholic acid, the ellipticity bands diminished.

Discussion

The CD data suggest that ligandin, which consists of two subunits, has only a single high affinity site for binding bilirubin. The CD spectrum of a bilirubin-ligandin 1:1 complex exhibits two major overlapping ellipticity bands of opposite signs in the visible spectral region and is thus comparable to the spectra of bilirubin-albumin complexes. Blauer and coworkers explained these overlapping bands and large rotational strengths associated with them, in terms of exciton coupling of the transition moments of the two dipyrromethene chromophores of a bilirubin molecule in a folded conformation (Blauer et al., 1972). The three-dimensional structure of bilirubin bound to ligandin may be compared to that of bilirubin in albumin complexes. With ligandin, a third band near 515 nm was observed which was of lower intensity than the two major bands but was more pronounced than the corresponding bands of the albumins (Kamisaka et al., 1973). Since the 515-nm ellipticity band showed major increases after addition of more than 1 mol of bilirubin, it may reflect binding at a secondary site of lesser affinity or stacking of bilirubin molecules at the primary

binding site (Lee and Gartner, unpublished). Increases in the 515-nm band are paralleled by changes at 255 nm which may involve additional optically active transitions of bilirubin bound to protein that are obscured by prominent peptide ellipticities in this spectral region.

The other potential ligands studied were considered in terms of interactions at the primary bilirubin binding site on ligandin. Ellipticities associated with the bilirubin-ligandin complexes were diminished by these compounds but the shapes, crossover points and wavelengths of extrema were not altered by most of the competing molecules. Relative affinities of the different classes of compounds were therefore derived from CD competitive binding data. These assessments appear to be reasonable because affinity constants of the sulfobromophthalein determined by equilibrium dialysis and of Indocyanine Green determined by inhibition of glutathione transferase activity of the protein (Habig et al., 1974) were in good agreement with results obtained from the competitive binding data.

Glutathione affects the CD spectrum of bilirubin-ligandin complexes in a unique manner when compared with other potential ligands and produced spectral shifts rather than diminution in ellipticity bands. Gel filtration and equilibrium dialysis data reveal glutathione is bound to ligandin in the absence of bilirubin (Kamisaka, unpublished observation). The observed spectral changes imply that glutathione is bound at a site adjacent, or in close proximity, to the bilirubin binding site. Alternatively, glutathione may interact with bilirubin itself, but if such interaction occurs, it is of specific nature since these effects were not observed with oxidized glutathione-bilirubin complexes or with bilirubin bound to albumin, and occur exclusively with bilirubin bound to ligandin. Conjugated sulfobromophthalein and lithocholic acid, but not other structurally related bile acids, also induce spectral shifts in the CD pattern of bilirubin-ligandin complexes. The binding of these substances cannot be explained by simple competitive binding mechanisms such as those proposed for other organic anions, and the exact nature of these interactions has not been determined.

Organic anions which bind to ligandin in vitro are transferred from plasma into the liver in vivo where, when specifically studied, they were bound to ligandin with varied affinity. This binding is probably at the same site as bilirubin is bound. Lidocaine, an organic cation, and propranolol, a neutral compound, are also rapidly excreted by the liver but did not inhibit binding of bilirubin to ligandin. Other cytoplasmic proteins may be involved in binding these and related substances.

Ligandin is identical with glutathione transferase B (Habig et al., 1974) on the basis of immunologic studies, molecular weight, isoelectric point, amino acid composition, and glutathione transferase activities with iodomethane, chlorodinitrobenzene, ethacrynic acid, and other substances as substrates. The present report shows that glutathione is bound to ligandin but the binding mechanism is different from that of other substrates studied. Bilirubin, bile acids, cortisol, fatty acids, and other ligands which are not conjugated with glutathione in vivo are also firmly bound to ligandin. Iodomethane and 1-chloro-2,4-dinitrobenzene, which are substrates for the catalytic activity of ligandin, were ineffective in competitive displacement of bilirubin.

Based on tissue localization and quantitation, phyllogenetic and ontogenetic development, pharmacologic induction, and competitive studies in vivo and binding in vitro, ligandin was postulated to function in regulating the net flux of various organic anions from plasma into the liver (Levi et al., 1969; Fleischner et al., 1972; Levi et al., 1969b; Reye et al., 1971). Transfer of these anions across the plasma membrane of the liver cell is believed to occur by nonionic diffusion and/or carrier-mediated transport. The results of the present study may predict competitive relationships in vivo for net hepatic uptake of various ligands. For example, bilirubin reduces hepatic uptake of sulfobromophthalein, Indocyanine Green, triiodothyronine, and penicillin (Scharschmidt et al, 1974; Lichter and Arias, 1975; Arias, 1972). Although Evans Blue (T-1824) is poorly transferred from plasma into the liver, it binds readily to ligandin in vitro (Levi et al., 1969). Sodium taurocholate does not readily compete with sulfobromophthalein or Indocyanine Green for hepatic uptake (Paumgartner and Reichen, 1974; Glasinovic et al., 1974). Competition in vivo has not been systematically studied using other ligands.

The association constant of the bilirubin-ligandin complex was approximately ten times less than that of the bilirubin-rat serum albumin complex and, at equimolar concentrations, transfer of bilirubin from ligandin to albumin was detected. These observations suggest that net bilirubin transfer from plasma into liver is probably not solely regulated by intracellular ligandin. The relative abundance of ligandin in the liver cell may partially account for the transfer. To quantitate the role of ligandin in organic anion uptake by liver, it is necessary to develop techniques for determination of the concentration of free and bound ligands within the parenchymal liver cell.

References

Arias, I. M. (1972), *Semin. Hematol., 9,* 55.

Blauer, G., Harmatz, D., and Snir, J. (1972), *Biochim. Biophys. Acta 278,* 68.

Carbone, J. V., Grodsky, G. M., and Hjelte, V. (1959), *J. Clin. Invest. 38,* 1989.

Chen, Y. H., Yang, J. T., and Martinez, H. M. (1972), *Biochemistry 11,* 4120.

Fleischner, G., Robbins, J., and Arias, I. M. (1972), *J. Clin. Invest. 5,* 677.

Glasinovic, J. C., Dumont, M., Duval, M., and Erlinger, S. (1974), *Gastroenterology 67,* 819.

Greenfield, M., and Fasman, G. D. (1969), *Biochemistry 8,* 4108.

Habig, W. H., Pabst, M. J., Fleischner, G., Gatmaitan, Z., Arias, I. M., and Jakoby, W. B. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3879.

Jacobsen, J. (1969), *FEBS Lett. 5,* 112.

Kamisaka, K., Listowsky, I. and Arias, I. M. (1973), *Ann. N.Y. Acad. Sci. 266,* 148.

Kamisaka, K., Listowsky, I., Betheil, J. J., and Arias, I. M. (1974), *Biochim. Biophys. Acta 365,* 169.

Kirsch, R., Kamisaka, K., Fleischner, G., and Arias, I. M. (1975), *J. Clin. Invest.* (in press).

Levi, A. J., Gatmaitan, Z., and Arias, I. M. (1969a), *J. Clin. Invest. 48,* 2156.

Levi, A. J., Gatmaitan, Z., and Arias, I. M. (1969b), *Lancet 2,* 139.

Lichter, M., and Arias, I. M. (1975), *Am. J. Physiol.* (in press).

Listowsky, I., Blauer, G., Englard, S., and Betheil, J. J. (1972), *Biochemistry 11,* 2176.

Litwack, G., Ketterer, B., and Arias, I. M. (1971), *Nature (London) 234,* 466.

Lowry, O. H., Rosebrough, N. J., Farr, A. J., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Paumgartner, G., and Reichen, J. (1974), *Gastroenterology 67,* 818.
Reye, H., Levi, A. J., and Arias, I. M. (1971), *J. Clin. Invest. 50,* 2242.
Scharschmidt, B. F., Waggoner, J. G., Vergalla, J., and Berk, P. D. (1974), *Gastroenterology 67,* 827.
Whelan, G., Hoch, J., and Coombs, B. (1970), *J. Lab. Clin. Med. 75,* 542.

Correlation between Quaternary Structure and Ligand Dissociation Kinetics for Fully Liganded Hemoglobin†

J. M. Salhany,*‡ S. Ogawa, and R. G. Shulman

ABSTRACT: The quaternary structures of fully liganded adult hemoglobin and hemoglobin Kansas ($\alpha_2\beta_2$ 102 Asn → Thr) bound by carbon monoxide or nitric oxide were spectroscopically characterized using high-resolution nuclear magnetic resonance (NMR) and ultraviolet circular dichroism (CD). The spectral markers used for the quaternary transition were the line in the NMR spectrum in H_2O −14 ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate and the negative peak at 285 nm in the ultraviolet CD spectrum. In the nitrosyl derivatives, these two structural markers were compared with the electron paramagnetic resonance (EPR) spectrum at room temperature for the purpose of correlating structural changes in the protein with changes at the heme. The kinetic properties of these hemoglobins were investigated by measuring the rate of CO dissociation by replacement with NO. The results indicated that fully saturated adult nitrosyl hemoglobin can be switched from the high ligand affinity (or R) quaternary structure to the low ligand affinity (or T) quaternary structure by the addition of inositol hexaphosphate (IHP). The fully saturated carbon monoxide form of adult hemoglobin is in the R state in the absence or presence of IHP. In stripped (phosphate-free) hemoglobin Kansas, simply replacing CO by NO produced a switch in the quaternary structure from the R state (for the CO form) to the T state (for the NO form). It was previously demonstrated that the addition of IHP to the carbon monoxide form of hemoglobin Kansas caused the quaternary structure to switch from R to T (Ogawa, S., Mayer, A., and Shulman, R. G. (1972), *Biochem. Biophys. Res. Commun. 49,* 1485-1491). Two types of room temperature EPR spectra were found which could be correlated with these changes in the quaternary structure of the protein. Stripped adult nitrosyl hemoglobin, which is in the R state according to the NMR and CD measurements, showed a symmetric room temperature EPR spectrum. Stripped nitrosyl hemoglobin Kansas and adult nitrosyl hemoglobin +IHP showed strongly anisotropic room temperature EPR spectra. These anisotropic room temperature EPR spectra were assigned to the T state based on the NMR and CD results. The kinetic properties followed these changes in the quaternary structure. The structural results shown above indicate that the CO replacement reaction by NO *will not always* be a replacement reaction within a single quaternary structure. This was found to be the case kinetically. Stripped adult hemoglobin is in the R state when both CO and NO are the heme ligands. The kinetics showed a slow, wavelength independent rate of CO dissociation. Both adult hemoglobin + IHP and stripped hemoglobin Kansas start in the R state with CO as the ligand and end in the T state with NO as the ligand. At pH 7.0, and appropriate protein concentrations, both of these conditions presented cooperative CO dissociation kinetics reflecting the switch in the quaternary structure during the reaction. Hemoglobin Kansas + IHP is in the T state with both CO and NO as heme ligands at appropriate solution conditions. Under these circumstances, the rate of CO dissociation was not cooperative and was an order of magnitude faster than the value in the R state. These results are discussed in terms of the two-state model of Monod et al. (Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 88-118).

The possibility that *fully* liganded hemoglobin ordinarily in the high affinity R structure can be switched to the low affinity or T quaternary structure is a consequence of the model of Monod et al. (1965). This switch was first accomplished in the deoxygenated mixed valency hybrids, where hemoglobin could be maintained in solution with two ligands per tetramer (Ogawa and Shulman, 1971). In this way the energies of the R and T manifolds were closer to equality than they were with zero ligands, where the T state dominated, or with four ligands, where the R state dominated. With near equality there was enough energy in the preferential binding of organic phosphates like IHP[1] to the T

† From the Department of Biophysics and Theoretical Biology, University of Chicago, Chicago, Illinois 60637 (J.M.S.) and Bell Laboratories, Murray Hill, New Jersey 07974 (J.M.S., S.O., R.G.S.). *Received October 21, 1974.*

‡ Present address: Bell Laboratories, Murray Hill, N.J. 07974.

[1] Abbreviations used are: bis-tris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; IHP, inositol hexaphosphate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Nes-des-Arg hemoglobin, hemoglobin allowed to react with *N*-ethylmaleimide and carboxypeptidase B; NMR, high-resolution nuclear magnetic resonance; EPR, electron paramagnetic resonance; CD, circular dichroism; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; DPG, 2,3-diphosphoglycerate; Tris, tris(hydroxymethyl)aminomethane. The phrase "stripped hemoglobin" is used in this paper to refer to organic phosphate free hemoglobin.