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BILIRUBIN AND BILIVERDIN BINDING TO RAT Y PROTEIN (LIGANDIN)*

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SUMMARY

The binding of bilirubin to poly(L-lysine) produces an optically active complex at pH 10.1. Circular dichroism spectra of these complexes are distinguishable from those generated by binding of bilirubin to the high affinity sites on albumin. Comparison of the circular dichroism spectra of bilirubin bound to the hepatic protein ligandin with those of bilirubin complexed with albumin or polylysine indicates that binding of bilirubin to ligandin occurs at two types of sites. These are distinguishable on the basis of their spectral properties, one resembling the high affinity site of bovine serum albumin and the other resembling polylysine. Complexes of biliverdin with albumin and ligandin bear similarities to the bilirubin-protein complexes. The native protein itself has an ordered structure which consists of 41% α -helix and is not altered by the binding of bilirubin.

INTRODUCTION

Studies of the interaction of unconjugated bilirubin with serum albumin have shown that binding of the chromophore to protein generates an intense, biphasic circular dichroism spectrum in the region of the visible bilirubin absorption bands [1-5]. The high molar ellipticity values observed ($>10^5$ deg·cm²/dmol) have been attributed to fixation of the bilirubin molecule at the protein binding site in a configuration which is helical and inherently dyssymmetric. More recently, the name ligandin has been applied to a protein of the liver cytosol that binds bilirubin and other anions [6]. The identity of this protein with azo-dye carcinogen binding protein and corticosteroid I binding protein [7] and with glutathione *S*-transferase B [8] has been established. Circular dichroism data for the interaction of bilirubin with ligandin have been reported [9, 10]. Among the findings was the observation that the bilirubin-

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ligandin CD spectrum differs qualitatively from that of bilirubin-albumin by the presence of a band in the 500–580 nm region. This band increases in intensity after the addition of more than 1 mol of bilirubin per mol of protein. Its origin has not been explained.

We have investigated the optical properties of bilirubin bound to various model compounds such as synthetic amino acid polymers, as well as to proteins such as albumin and ligandin. The present study describes spectral data for the binding of bilirubin to the synthetic polymers poly(L-lysine) and poly(L-arginine) and to ligandin. The results indicate that the binding of the chromophore to polylysine generates a circular dichroism spectrum which is distinguishable from that produced by binding to the high affinity site of bovine serum albumin. Comparison with the ligandin case indicates that the CD spectrum of the bilirubin-ligandin complex consists of contributions from bilirubin bound at two types of sites. One of these resembles bilirubin-bovine serum albumin and one resembles bilirubin-polylysine in optical properties.

MATERIALS AND METHODS

Bilirubin ($\epsilon_{mM, 450\text{ nm}} = 62$ in chloroform) and biliverdin ($\epsilon_{mM, 375\text{ nm}} = 25$ in methanol) were obtained from Sigma Chemical Corp. Poly(L-lysine) ($M_r = 30\,800$) and poly(L-arginine) ($M_r = 13\,900$) were obtained from Miles Laboratories.

Rat ligandin was prepared by ion exchange chromatography and G75 Sephadex chromatography of rat liver supernatant [6] and stored frozen in 0.01 M Tris, pH 8.1. Purity was established by gel electrophoresis and by precipitation with monospecific antibody. A molecular weight of 40 000 was assumed for the protein. Bovine and human serum albumin were the same preparations as previously described [2].

Spectral measurements were carried out in 0.1 M sodium phosphate buffer and all solutions were protected from light. Solutions of bilirubin and biliverdin were made up by first dissolving the pigments in small quantities of 0.1 M NaOH and then diluting with buffer. Absorption measurements were carried out on a Cary 15 recording spectrophotometer and circular dichroism measurements on a Jasco UV-5 recording spectropolarimeter equipped with a Sproul Scientific SS-20 CD attachment.

Circular dichroism results for the native protein are expressed in terms of $[\theta]$, the mean residue ellipticity, using the value of $n = 355$ for the number of amino acid residues in the protein. The α -helix content of the native protein was calculated using the approximation of Greenfield and Fasman [11].

RESULTS

Absorption spectra of bilirubin-polymer complexes

Fig. 1 shows the absorption spectra of bilirubin bound to poly(L-lysine) and poly(L-arginine). In each case the effect of binding is to produce hypochromism in the bilirubin absorption with broadening of the spectrum on the long wavelength side. The composite nature of each spectrum is evident. The bilirubin-polylysine complex shows a maximum at 408 nm and broad shoulder in the 480 nm region, while the bilirubin-polyarginine complex shows a maximum at 420 nm and a broad shoulder in the 480 nm region. There is a distinct effect of pH on the absorption spectrum of bilirubin-polylysine. Full titration of the polymer to the uncharged state at pH 12

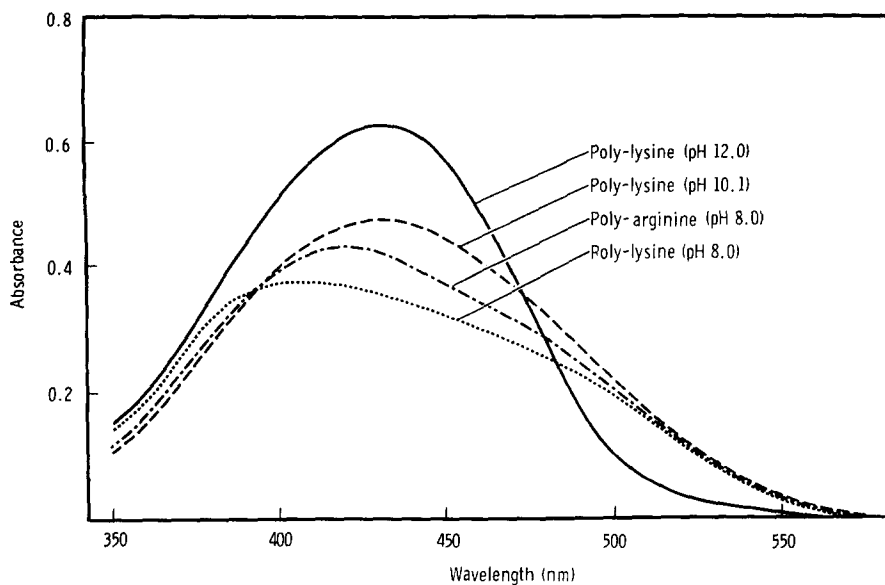


Fig. 1. Absorption spectra of bilirubin-amino acid polymer complexes. The absorption spectrum of bilirubin-polylysine at pH 12 is identical to that of free bilirubin at the same pH.

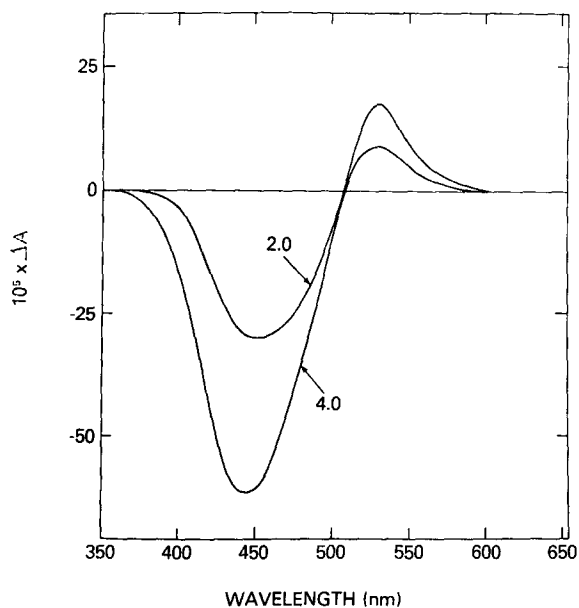


Fig. 2. Circular dichroism spectra of bilirubin-polylysine, pH 10.1 at molar ratios of bilirubin to polymer of 2.0 and 4.0 mol/mol.

results in reversion of the bilirubin absorption spectrum to that of free bilirubin at the same pH. The spectrum at pH 10.1 is intermediate between the bound and free forms.

CD spectra of bilirubin-polylysine and bilirubin-polyarginine

Bilirubin-polylysine complexes are not optically active at pH 7.4 and at this pH the CD spectrum in the ultraviolet shows no formation of α -helix by the polymer. At pH 10.1 there is α -helical structure to the polymer, as judged by the CD spectrum in the far ultraviolet, and the bilirubin absorption bands become optically active with the appearance of a positive band at 525 nm and a negative band at 444 nm (Fig. 2). Loss of charge on the polymer at pH 12 results in loss of optical activity despite the persistence of α -helical structure. This correlates with the absorption spectrum at this pH, which is that of free bilirubin. Thus bilirubin-polylysine complexes show optical activity at pH 10.1, where the polylysine exists as a partially charged α -helix. Optical activity was best demonstrated at a bilirubin/polymer ratio of 4.0–8.0 mol/mol, based on the molecular weight of 30 800 for the entire polymer. However, optical activity was clearly demonstrable at a bilirubin/polymer ratio of 0.7, indicating that bilirubin-bilirubin stacking interactions are not essential to the generation of optical activity.

Bilirubin-polyarginine complexes were not optically active in this pH range, as the polymers did not form α -helical structures between pH 7 and pH 12.

CD spectra of bilirubin-ligandin complexes

The CD spectra at various molar ratios of bilirubin to ligandin are shown in Fig. 3. At bilirubin/protein ratios of 1.0 or less, the spectrum is very similar to that of bilirubin-bovine serum albumin. Above 1 mol bilirubin/mol protein a new band appears with a maximum at 515 nm and in the region of 1–4 mol of bilirubin/mol protein the changes in the spectrum are dominated by the increases in the size of this band. The maximum intensities at 408 nm and 465 nm are almost achieved by the addition of one mole of bilirubin per mol of protein, while the band at 515 nm continues to increase up to 4 mol bilirubin/mol protein.

Certain other features of these CD spectra are of importance. Accompanying the increase in ellipticity at 515 nm, there is a small increase in the magnitude of the large negative band, which is also shifted 10 nm from 465 nm to 455 nm. At the same time, the band at 405 nm decreases in magnitude. These latter changes could be accounted for by the presence of another negative band between 405 nm and 465 nm which increases in magnitude simultaneously with the positive band at 515 nm. In this respect, the results of a computer analysis of the bilirubin-ligandin CD spectrum are of interest. A curve-fitting program (Modelaide) written by Richard I. Shrager of the NIH Division of Computer Resources and Technology was utilized to assign the best fit of Gaussian curves to the spectrum. Although the assumption of three Gaussian components provided good fit to the data, an improved fit was obtained by allowing for the presence of a fourth band, negative in sign, and positioned at 437 nm (Fig. 4).

The CD spectrum of native ligandin was in agreement with that previously reported [9, 10]. In the far ultraviolet the calculated mean residue ellipticity per peptide bond was 15 800 deg·cm²/dmol at 208 nm, from which the α -helix content of the

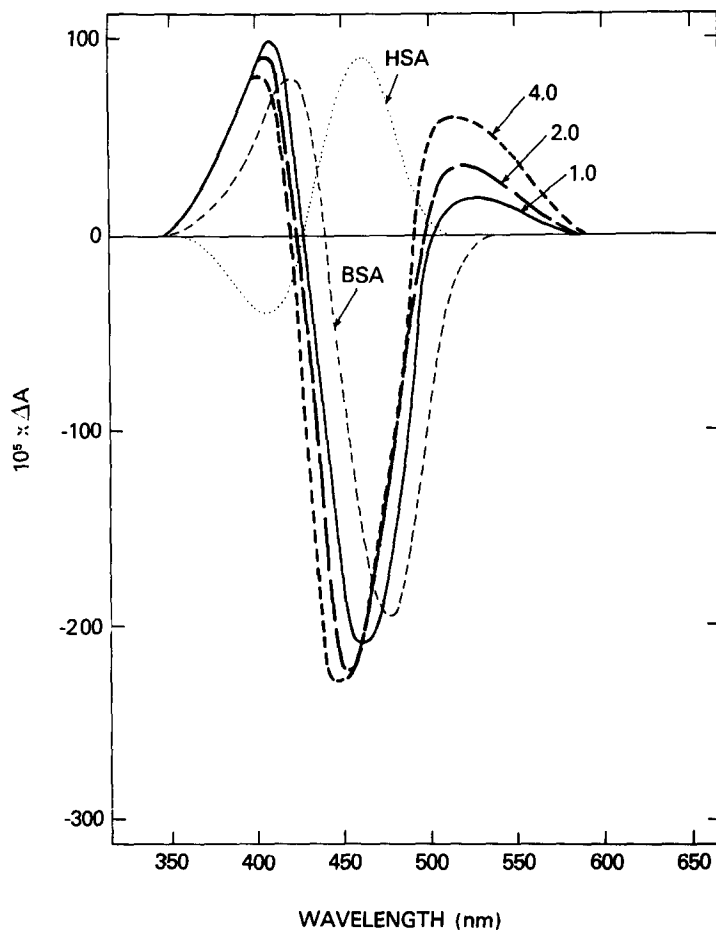


Fig. 3. Circular dichroism spectra of bilirubin-ligandin, pH 7.4, at molar ratios of bilirubin to protein of 1.0, 2.0, and 4.0 mol/mol. For comparison, the CD spectra of 1:1 complexes of bilirubin-bovine serum albumin and bilirubin-human serum albumin are also shown.

protein was estimated at 41%. This agrees well with other independent measurements of this value [9, 10]. The binding of bilirubin to the protein did not alter this content of α -helix. In the near ultraviolet the spectrum is complex, indicating the presence of several oriented chromophores. The salient features are a negative band at 296 nm, positive bands at 291 nm and 284 nm and negative bands at 268 nm and 261 nm. A broad shoulder between 270 nm and 280 nm contains at least one additional positive band. Upon binding of bilirubin, a new positive band appears at 252 nm. Although an oriented disulphide bond could produce a transition in this spectral region, it is more likely that this represents the shoulder of an optically active bilirubin absorption band at 220 nm.

Absorption and CD spectra of biliverdin binding to protein

The binding of biliverdin to albumin and to ligandin was investigated. The absorption spectrum is shown in Fig. 5 and consists of two bands in the visible region.

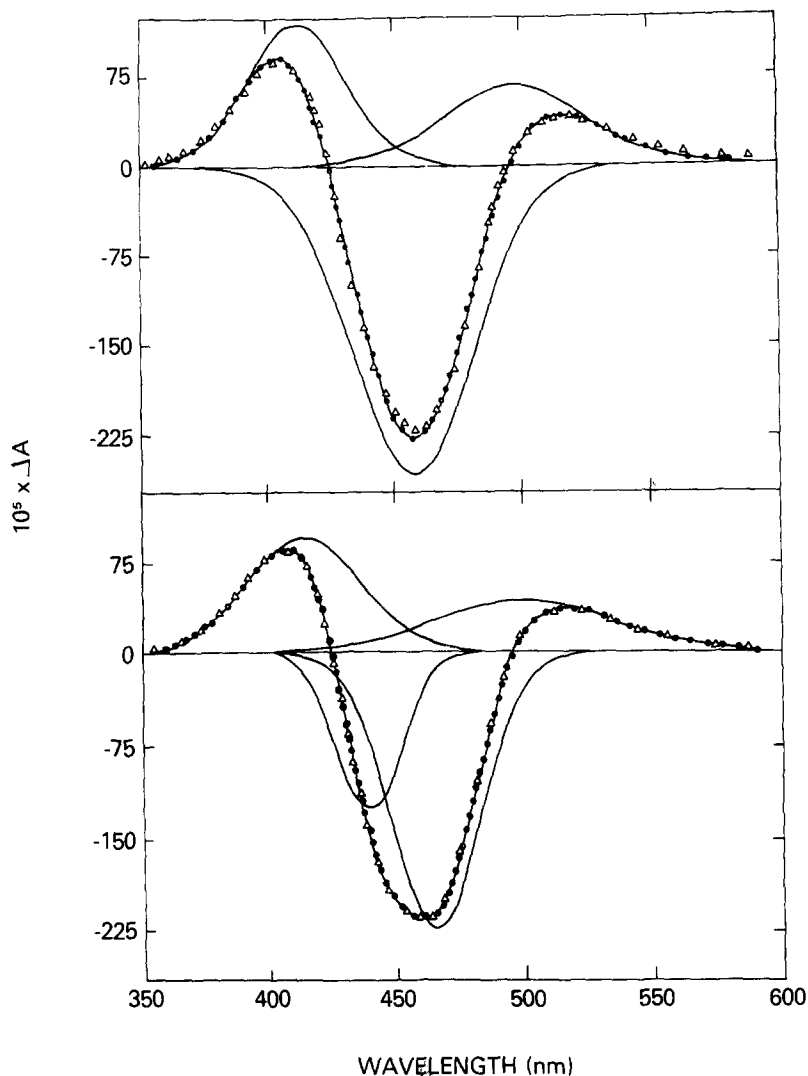


Fig. 4. Computer-fitted representation of the bilirubin-ligandin CD spectrum as the sum of three and of four Gaussian components. ●, experimental points; △, fitted points.

Unlike the bilirubin absorption spectrum which shows strong dependence upon solvent, the biliverdin spectrum was rather insensitive to solvent changes. The peak at 374 nm in aqueous solution was shifted to 379 nm in chloroform with no change in extinction. Binding to protein also produced slight shifts in the 374 nm peak, to 379 nm upon binding to human serum albumin and to 383 nm for binding to ligandin, without change in extinction. The long wavelength band at 640 nm showed little or no change in position or intensity as the result of binding to protein.

Circular dichroism spectra for the binding to human serum albumin and ligandin are shown in figure 5. Optical activity of both visible bands occurs, the two CD bands in each case being of opposite sign. Like the bilirubin-protein complexes,

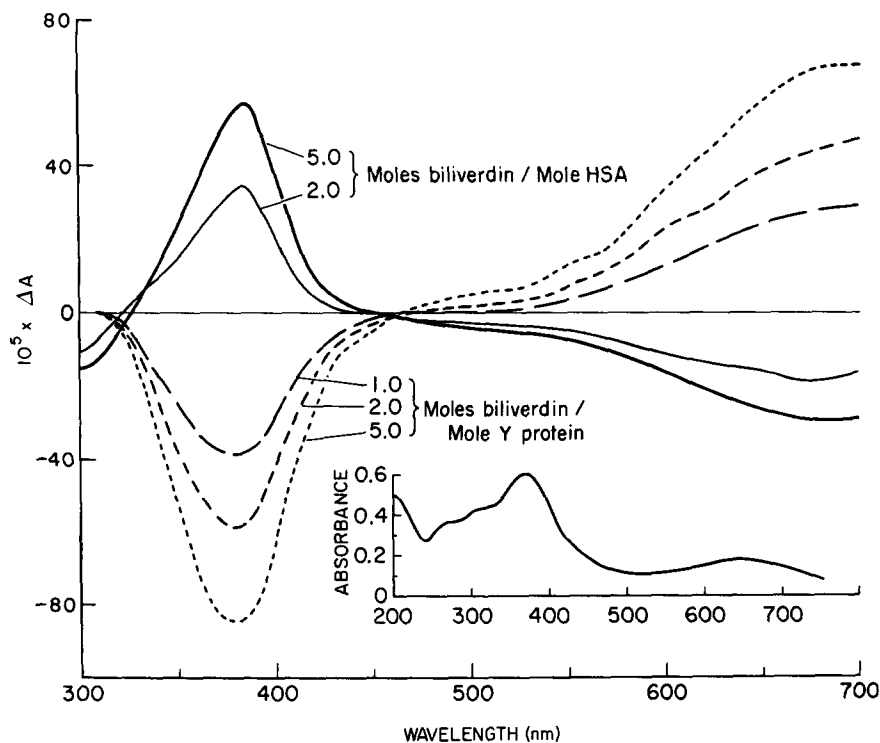


Fig. 5. CD spectra of biliverdin-human serum albumin and biliverdin-ligandin. Inset shows absorption spectrum of free biliverdin.

biliverdin-bovine serum albumin and biliverdin-ligandin were of the same chirality and were opposite to that of the biliverdin-human serum albumin complex. Spectrally distinct binding sites were not observed. Biliverdin was able to displace bilirubin from its high affinity site on ligandin, in that a 10 molar excess of biliverdin reduced the intensity of the 460 nm CD band of a 1:1 bilirubin-ligandin complex by about 65%.

DISCUSSION

Circular dichroism data for bilirubin-protein complexes have been reported by numerous authors [1-5, 9, 10, 13]. Binding of bilirubin to the first high affinity site on albumin produces a spectrum that consists of two intense ellipticity bands of opposite sign with maxima located in the 405-415 nm region and the 455-470 nm region. Although marked changes in these spectra may accompany changes in pH [3], salt concentration [4], or molar ratio of bilirubin to protein [2, 4], or the addition of competing anions [2], the occurrence of a distinct CD band beyond 500 nm is not observed. The interaction of one mole of bilirubin with ligandin produces a CD spectrum that is qualitatively similar to that of a 1:1 complex of bilirubin-bovine serum albumin. However, further increase of the molar ratio of bilirubin to ligandin produces increased ellipticity in the 500-580 nm region and slight blue shift of the 465 nm band. These phenomena indicate that the long wavelength band results from the binding of bili-

rubin to additional sites (s) that are of lower affinity than the first and are also of different spectral properties. The finding that optically active complexes of bilirubin-polylysine at pH 10 exhibit ellipticity in the 500–580 nm region provides an explanation for the behavior of the bilirubin-ligandin complexes. Such an interpretation would require the presence of a second negative band in the bilirubin-ligandin spectrum, and the behavior of this spectrum is consistent with the existence of such a band. Furthermore, computer resolution of the spectrum yields an improved fit to the data when a negative band at 437 nm is included. While such a computer resolution does not constitute proof of the existence of a band, the results are consistent with that interpretation.

Bilirubin-polyarginine complexes were not optically active, but their absorption spectra were quite like those of bilirubin-polylysine. It thus seems probable that binding of bilirubin to either arginine or lysine residues within the oriented structure of a protein could give rise to CD spectra of the same general character as those of bilirubin-polylysine.

It is thus possible to distinguish two types of protein binding sites for bilirubin on the basis of circular dichroism properties. Kuenzle [12] has presented evidence that free bilirubin in solution exists in the form of an internally stabilized resonance hybrid in which the propionic acid carboxyl groups are hydrogen-bonded to the pyrrole nitrogens and to the pyrrolenone oxygen atoms on the outer rings. The disruption of this structure upon binding to polylysine may be in part responsible for the observed spectral changes. In addition, the absorption spectrum of bilirubin is rather strongly dependent upon solvent and the distinction between the two sites of binding to ligandin may be also due to differences in the hydrophobic character of the first site relative to the second.

The CD data for biliverdin-ligandin complexes provide useful comparison with the bilirubin case. Previous descriptions [14–16] have indicated that both bovine and human serum albumin complexes of biliverdin are optically active. The configurations of these spectra show rather marked dependence upon pH and upon the specific binding protein. Blauer [16] has reaffirmed the importance of a highly specific mode of binding between pigment and protein with respect to the generation of circular dichroism spectra, and has indicated that the geometry of the bilirubin and biliverdin molecules may be correlated with the observed spectral data. Our data for the biliverdin-human serum albumin complex at pH 7.4 were in agreement with those described by Blauer [14, 16]. The data for the biliverdin-ligandin complexes showed the occurrence of well defined CD bands which were of opposite chirality to the biliverdin-human serum albumin complexes. Spectra of biliverdin-bovine serum albumin complexes at pH 7.4 showed that under these circumstances, the chirality was the same as that of biliverdin-ligandin. This appears to agree with the observations of Lee and Cowger [15] for biliverdin-bovine serum albumin at this pH. Thus for both bilirubin and biliverdin, the complexes with ligandin and bovine serum albumin are of like chirality and are opposite to those of the human serum albumin complexes. This indicates the role of these proteins in fixing the two chromophores into specific twisted conformations.

These data provide further indications of the utility of circular dichroism as a tool for the study of the interactions of bile pigments with protein. Such considerations may be applicable to other systems such as the basic myelin protein of Gurba

and Zand [13] which binds bilirubin and upon doing so exhibits CD in the 500–600 nm region. It is also possible to consider the theoretical situation of a CD spectrum derived from one protein binding site of the bilirubin-human serum albumin chirality plus an additional site of the bilirubin-polylysine character. Such a combination does not appear to have been observed in practice but may be encountered with further studies.

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