CIRCULAR DICHROISM OF BILIRUBIN-HUMAN SERUM ALBUMIN COMPLEXES IN AQUEOUS SOLUTION

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1. Introduction

The physiologically important complex bilirubin-serum albumin has been investigated by various chemical and physical methods (for a compilation of literature, see [1]). ORD* spectra of the system bilirubin-BSA measured under various conditions, have recently been reported [2, 3]. At pH 5, a negative Cotton effect curve was observed in the visible region for the bilirubin-BSA complex. Calculated on a bilirubin basis, the molar amplitude of this complex Cotton effect was about 1.5×10^6 deg. cm^2 per decimole. The amplitude decreased markedly at both lower and higher pH values without a change in the sign of the Cotton effect curve. Preliminary CD measurements in the visible region showed both negative (longer wavelengths) and positive bands at pH 5. At pH 7.3 to 7.4, the negative band was smaller and the positive band diminished to a fraction of its magnitude at pH 5. In the absence of protein, dissolved bilirubin did not show measurable optical rotation at either pH 5 or 7.5, in the range of 220 to 600 nm and under the conditions used [3].

To the best of our knowledge, no similar Cotton effects have been characterized for the system bilirubin-HSA, although some measurements of optical rotation at various wavelengths have been reported [4]. A preliminary report of CD data obtained for the bilirubin-HSA complex in the range of 330 to 550 nm is now presented. A striking dependence on pH of the visible-range CD bands is observed. Also, at constant pH, the optical rotatory properties of the bilirubin-HSA complex are markedly different from those of the bilirubin-BSA complex.

* *Abbreviations:*
ORD: optical rotatory dispersion
CD : circular dichroism
HSA : human serum albumin
BSA : bovine serum albumin.

2. Materials and methods

HSA (4 × crystallized) and bilirubin were obtained from Nutritional Biochemicals and were used without further purification. The molecular weight of HSA was taken as 68,000.

CD and ORD were measured on a Cary Model 60 Recording Spectropolarimeter using a Model 6002 accessory for CD measurements. The slit width was programmed for a band width of 15 Å. Absorption spectra were measured on a Cary Model 14 Recording Spectrophotometer.

3. Results and discussion

Two dichroic bands of opposite sign are measured in the visible region at pH 4.8 for the bilirubin-HSA complex at a mole ratio of 1:2 (fig. 1). At pH 7.3, the same system also has two main bands of opposite sign; however, they seem inverted with respect to those obtained at pH 4.8. In addition to the possible complexity of all these bands, there is one smaller negative band on the long-wavelength side and another small positive band on the short-wavelength end at pH 7.3. The light-absorption spectra (fig. 1) show com-

posite bands which are apparently related to the main CD-bands observed. The corresponding ORD difference-spectra between the complex and the protein show an inversion of the sign of the Cotton effect curves between pH 4.8 and 7.3, as would be expected from the CD profiles. By plotting the amplitudes as a function of pH, and considering the sign of the Cotton effects, the inversion of sign occurs at about pH 5.0–5.2. At pH 4.8, the amplitudes are one order of magnitude lower than those of the bilirubin-BSA complex [2, 3].

The general pattern of both CD and ORD data obtained for the bilirubin-HSA complex may suggest exciton splitting (e.g., [5–7] and refs. cited therein) involving the bilirubin chromophores. By interpretation of the Cotton effects observed for the bilirubin-BSA complex at pH 5 it has been suggested [2, 3] that a dissymmetric conformation of the bilirubin is formed upon specific binding to the protein. In this conformation, dipole-dipole coupling between the transition moments of the juxtaposed dipyrrylmethene chromophores should contribute to the observed rotations, in addition to possible extrinsic effects. In the present case, besides the latter effects, the possibility of juxtaposed transition dipole moments at larger distances from each other or at different angles than in the case of BSA should be considered. The observed inversion of sign with pH of the main CD-bands may then be due to a change in the dissymmetric mode of binding of the bilirubin by HSA, resulting a different (opposite) relative orientation between the transition dipole moments of the dipyrrylmethene chromophores. These changes with pH could involve both ionizable groups participating in the binding and conformational changes in the protein. The optical rotatory spectra in the far ultraviolet region of HSA alone and of the bilirubin-HSA complex remain practically invariable in the pH range considered.

It should be noted that spectrophotometric titrations near pH 5 or 7.4 indicate the formation, at excess protein, of a bilirubin-protein complex at a mole ratio of 1:1 for both BSA [2, 3] and HSA, at least near pH 5. At this mole ratio no significant association

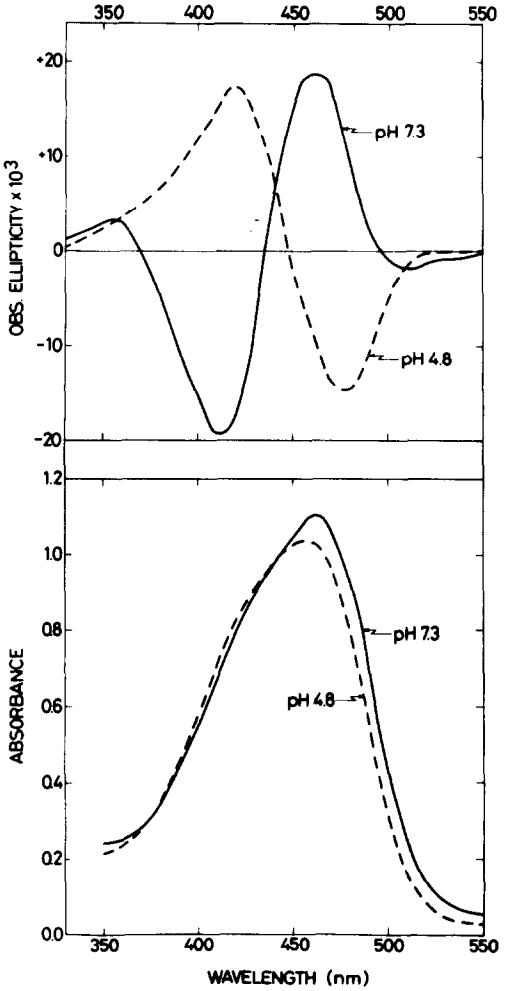

Fig. 1. CD- and light-absorption spectra of the bilirubin-HSA complex in aqueous solution at two different pH values. Bilirubin, 2.5×10^{-5} M; HSA, 5.0×10^{-5} M; temp., $28 \pm 1°$; low ionic strength.

Upper part: observed ellipticity in degrees (optical path 1.0 cm), measured after constant values were reached (about 3 hr from preparation of the complex at pH 4.8 ± 0.05, and about one hr at pH 7.3 ± 0.1).

Lower part: absorbance per cm, measured at similar time intervals as for ellipticity.

The ellipticity of the protein was practically zero under the experimental conditions used and in the wavelength range given. Calculated on a bilirubin basis, the molar ellipticities of the main band maxima are in the range of $(6–8) \times 10^4$ deg. cm^2 per decimole. The light-absorption spectrum of bilirubin in the absence of HSA has been measured in apparently supersaturated aqueous solutions at pH 5 or 7.5. In both cases there is a maximum at about 440 nm with an extinction coefficient of 30 to 40 mM^{-1} cm^{-1} (see ref. 2). Water is the reference solvent in all cases. The light absorption of HSA is negligible over the wavelength range given.

occurred at either pH 5 or pH 7.4, as judged by measurements in the analytical ultracentrifuge.

In contrast to light absorption of the bilirubin-HSA complex, ORD and particularly CD are very sensitive tools to discriminate between differences in binding (fig. 1). This is evident from both the pH-dependence observed and from comparison of the present data with those obtained from the bilirubin-BSA complex [2, 3]. While HSA and BSA show similarities in amino acid composition and molecular weight (e.g., [8]), the widely different ORD or CD spectra observed for the complexes of these proteins with bilirubin indicate structural differences affecting the respective binding sites and allow easy differentiation between the two serum albumins.

Further work on the bilirubin-HSA system and on other bile pigment-protein complexes is in progress and will be reported elsewhere.

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