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CIRCULARLY POLARISED LUMINESCENCE OF BILIRUBIN BOUND TO HUMAN SERUM ALBUMIN

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SUMMARY

Circular dichroism and circularly polarised luminescence are used to study the ground and excited state conformation of bilirubin when bound to human serum albumin at various pH and sodium chloride concentrations. The resultant dihedral angle between the dipyrromethene moieties of bilirubin bound to human serum albumin is shown to be greater in the excited state than in the ground state at pH 4.8, 7.4 and 9.0. At pH 4.1, bilirubin transforms from a right handed helical conformation in the ground state into a left handed conformation in the excited state. The excited state conformation of bound bilirubin unlike the ground state conformation is unaffected by NaCl concentration at pH 7.4.

INTRODUCTION

Bilirubin (BR) is an important agent in neonatal jaundice (1). Phototherapy has proved to be a successful treatment of this malady and a study of its excited state geometry, particularly bound to proteins, such as human serum albumin (HSA) should provide information about the mechanism of this treatment.

In its free state, BR is not optically active although when bound to albumins it does become so and the observed circular dichroism (CD) has already been used to probe (2,3) its bound ground state conformation (2, 3,4). It has been found (3) that the ground state conformation of BR when bound to HSA depends on pH and on electrolyte concentration although there is little information concerning the corresponding excited state conformations of BR. This latter information can be obtained by studying

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Abbreviations used: BR: bilirubin, HSA: human serum albumin, CD: circular dichroism, CPL: circularly polarised luminescence.

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the circular polarization of the fluorescence of BR which becomes fluorescent when bound to albumin (5,6). Using CD and circularly polarised luminescence (CPL) spectroscopy, a comparative study of the ground and excited state chiralities of BR bound to HSA is reported at various pH values (between 4 and 9) and various NaCl concentrations.

MATERIALS AND METHODS

Bilirubin (Sigma) was used as received since its extinction coefficient in chloroform at 453 nm was 6.0×10^{4} M^{-1} cm^{-1} in agreement with the accepted value for the pure pigment (7). A stock solution of BR, prepared in the dark just before each experiment, was used immediately due to the instability of BR. This stock solution (1×10^{-4} M BR) was made by dissolving BR in water with a few drops of 0.1N NaOH.

Essentially fatty acid free HSA, obtained from Sigma (lot 70F - 9310), was used as received. Molar protein concentrations were based on weighing and a molecular weight of 66000 (3). BR bound to HSA solutions were prepared by adding the stock solution of BR to a dilute protein solution. Acetate buffer (pH 4.0 and 4.8), phosphate buffer (pH 7.4) or borate buffer (pH 9.0) were then added and the solutions were made up to a known volume to give 5×10^{-5} M BR, 5×10^{-5} M HSA and 5×10^{-3} M buffer. The final solution was adjusted to the required pH by the addition of 0.1N HCl or NaOH and then kept in the dark for two hours to reach equilibrium before measurement began.

Details of the absorption, fluorescence, CD and CPL instrumentation are given in references (8,9). Absorption (g_{abs}) and emission (g_{em}) dissymmetry factors are defined as:

$$g_{abs} = \frac{(OD_L - OD_R)}{OD} \quad ; \quad g_{em} = \frac{(I_L - I_R)}{I}$$

where $(OD_L - OD)_R$ is the differential optical density for left and right circularly polarised light of a solution having an isotropic optical density OD, emitting a total isotropic fluorescence I and a differential circularly polarised fluorescence $(I_L - I_R)$.

To minimise photolysis, the fluorescence spectra were run as quickly as possible within the time constant restrictions of the experiment, and to reduce circular polarisation artifacts from Rayleigh scattering the excitation wavelength was set as low as the noise level would permit. The latter expedient was particularly necessary with the NaCl solutions when protein solubility is limited.

RESULTS AND DISCUSSION

The chiroptical parameters (g_{abs} and g_{em}) of 5×10^{-5} M BR, 5×10^{-5} M HSA in 5×10^{-3} M buffer at pH 4.1, 4.8 and 7.4 and 9.0 are shown in Fig. 1. The absorption dissymmetry factors are generally in agreement with those derived from the reports of Blauer et al (2) and

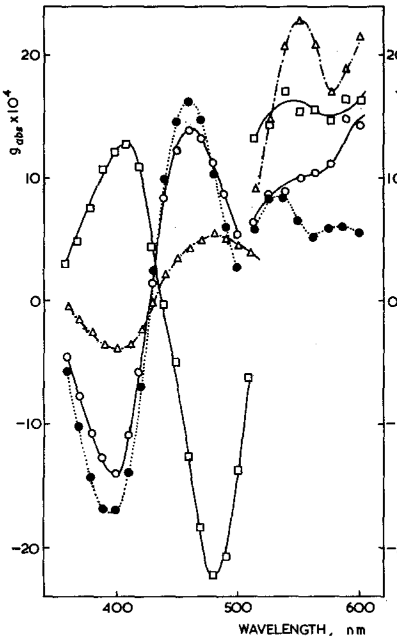

Figure 1: Absorption (g_{abs}) and emission (g_{em}) dissymmetry factors of 5 x 10^{-5} M BR bound to 5 x 10^{-5} M HSA in 5 x 10^{-3} M buffer at pH 4.1 (□); 4.8 (Δ; 7.4 (O) and 9.0 (●).

Beavan et al (3), with a general decrease from 9.0 to pH 7.4 and pH 4.8 and a sign reversal as the pH changed from 4.8 to 4.1. The bisignate nature of the CD has been reasonably explained (4) as deriving from a particular dihedral angle between the two dipyrromethene moieties of the bound BR while the sign change at pH 4.1 has been said (3) to be connected with the protein N-F transition at pH 2.0 although under these conditions BR may also dimerise.

It was suggested, in a previous communication (8) based on a CPL and fluorescence decay study of BR in HSA, bovine serum albumin and dioctadecyldimethylammonium chloride vesicles, that the species emitting below 530 nm are of the Z - Z form of BR whilst above 530 nm the Z - E, E - Z and E - E isomers contribute to the fluorescence. Up to 530 nm g_{em} mirrors g_{abs} but from 530 nm to 600 nm it is sufficiently different to confirm the idea that there is more than one emitting species.

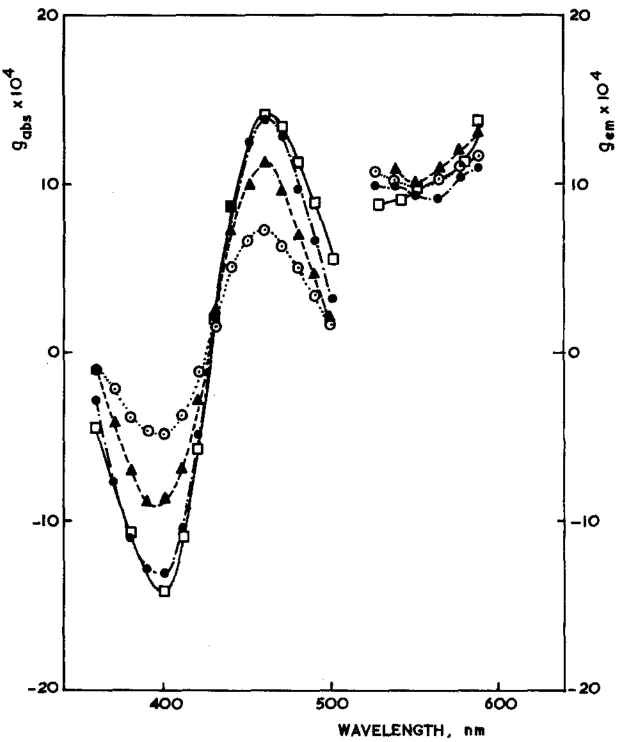

Figure 2: Absorption (g_{abs}) and emission (g_{em}) dissymmetry factor of 5×10^{-5} M BR bound to 5×10^{-5} M HSA in 5×10^{-3} M phosphate buffer at pH 7.4 without NaCl (□); with 0.01 M NaCl (●); 0.1 M Nacl (▲); and 1.0 M NaCl (O).

The pH dependence of g_{em} is opposite to that of g_{abs}. As the pH decreases from 9.0 to 4.8 so that g_{em} increases although at pH 4.1 the trend is reversed. At pH 7.4 whilst the g_{em} is independent of NaCl concentration the g_{abs} decreases with increasing NaCl concentration (Fig. 2).

In the ideal case where there is no change of geometry in the excited state compared to the ground state, or any change in the electronic transition dipole moment direction between emission and absorption, then for a particular transition $g_{abs} = g_{em}$ (10). The sign and magnitude of the lowest energy absorption CD band can be correlated (4) with the molecular chirality of BR bound to HSA or, more particularly, with the resultant dihedral angle between the two dipyrromethene residues. Therefore any differences in g_{em}, specifically with respect to g_{abs}, can also indicate changes in this dihedral angle provided there is no photoisomerisation. The pH dependency of g_{em} may thus result from a change in geometry upon excitation. With reference to the work of Blauer and Wagniere (4) it is concluded that the resultant dihedral angle between the dipyrromethene moieties of BR bound to HSA is much greater in the excited state than in the ground state at pH 4.8, 7.4 and 9.0; in other words, upon excitation, bound BR prefers to adopt a more extended structure.

At pH 4.1, BR bound to HSA takes up a right-handed helical conformation in the ground state but transforms into a left-handed conformation in the excited state. To the best of our knowledge, this is the first observation of a complete inversion of configuration between ground and excited states. It is not, however, unreasonable since the energy barrier associated with the ground state racemization of BR is estimated to be 74.9 ± 2 kJ/mole (1) which is comparable to the rotation barrier (72.8 kJ/mole) for 2,2-dimethyl-biphenyl (1). The energy barrier for excited state racemization is probably smaller than in the ground state as in the case with binaphthyl (11).

At wavelengths longer than 530 nm the fluorescence is complicated by emission from isomeric species (8). The direction of the transition electric dipole moment in these geometric isomers will necessarily be different to that in the parent ground state Z - Z isomer and the observed dissymmetry factors will be accordingly different. All that can be reasonably suggested at this stage is that the variation of g_{em} with wavelength confirms the existence of these isomers in the excited state and that they appear to adopt an excited state conformation that is associated with a positive dissymmetry factor. The pH dependence of g_{em} in this wavelength region can be interpreted as previously involving changes in the molecular conformations although now a change in the relative amounts of various geometric isomer may also be important.

The variation in g_{abs} with NaCl concentration is evidence for the presence of multiple binding sites for BR on HSA. At a BR:HSA ratio of 1:1 there are two binding sites (3); the stronger one has a weaker CD and stronger fluorescence (i.e. smaller g_{em} assuming no change in geometry in the excited state) while the weaker one is associated with stronger CD and weaker fluorescence (i.e. larger g_{em} with no change in geometry in the excited state) (3). Increasing NaCl concentration results in a decrease in g_{abs} (Fig. 2) since BR is driven from the weaker binding site to the stronger one. This ground state movement is feasible since the weaker binding site is affected by electrolyte (3). Excited state migration of this form to explain differences in g_{abs} and g_{em} can be excluded since it would require BR to be capable of migrating from one site to another during its excited state lifetime (c.a. 40 p sec) (12) which is to short to allow such movement. As g_{em} appears to be independent of NaCl concentration at pH 7.4 then it can be said that the resultant excited state conformation derived from the two unequally populated ground sites is, surprisingly, also independent of NaCl concentration.

Evidence has been presented in the present work for differences in ground and excited state conformations of BR bound to HSA. At pH 4.8, 7.4 and 9.0 bound BR prefers to adopt a more extended structure upon excitation whilst at pH 4.1 it transforms from a right-handed helical conformation in the ground state into a left-handed one in the excited state. Lightner has suggested (1) that the reason for the success of phototherapy in the treatment of neonatal jaundice is the production of the various geometric tensors of BR upon excitation. These isomers are more water soluble than the Z - Z isomer and can, therefore, be rejected from the body more readily. It has been confirmed that these isomers are generated upon excitation and that the dominant species are those whose excited state conformation is one which has an associated positive dissymmetry factor.

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