On the Structure of Albumin-bound Bilirubin

SELECTIVE BINDING OF INTRAMOLECULARLY HYDROGEN-BONDED CONFORMATIONAL ENANTIOMERS*

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The intramolecularly hydrogen-bonded bichromophoric tetrapyrrole pigments, bilirubin-IXα and mesobilirubin-XIIIα, adopt either of two folded, intramolecularly hydrogen-bonded, enantiomeric conformations which are in dynamic equilibrium in solution. Added human serum albumin binds preferentially, although not necessarily exclusively, to one conformational enantiomer, and the solutions exhibit bisignate circular dichroism Cotton effects in the region of the pigment's long wavelength electronic transition. In contrast, the bichromophoric tetrapyrrole pigment mesobilirubin-IVα, which is incapable of adopting intramolecularly hydrogen-bonded folded conformations, and the monochromophoric pyrromethenone, xanthobilirubinic acid, show only monosignate induced circular dichroism Cotton effects under the same conditions. Application of exciton coupling theory indicates a preference for complexation of the right-handed (or positive) chirality conformational enantiomer of bilirubin-IXα or mesobilirubin-XIIIα to human serum albumin at physiologic pH.

(4Z,15Z)-Bilirubin-IXα (BR-IX), the yellow-orange cytotoxic pigment of jaundice, is produced in abundant quantities by heme catabolism in mammals and is transported as a noncovalent bonded association complex with albumin to the liver for glucuronidation and subsequent excretion (1). However, when the conjugation apparatus in the liver is not yet functional, as in the neonate, ready excretion of the toxic pigment is thwarted, and the albumin serves as a biologic buffer against bilirubin encephalopathy and other tissue damage. Consequently, a knowledge of the binding capacity of the protein and the structure of its noncovalent bonded association complex with bilirubin have attracted considerable interest. Much of this interest has focused on analyses by spectroscopic methods, of which circular dichroism (CD) spectroscopy has proved both useful and promising because the (otherwise optically inactive) bilirubin pigment exhibits CD in the presence of albumin (2) and therefore serves as a powerful probe of association parameters and of the complex structure. Although the induced optical activity of bilirubin complexed to albumins and other proteins has been investigated for nearly 20 years (2)—from both the view of protein (3) and pigment structure (4) and affinity constants (2, 3)—the nature of the protein binding site remains incompletely understood, as does the exact structure of the bound bilirubin. Previous studies (5) have shown a common feature to the CD spectra of bilirubin-albumin solutions: bisignate CD Cotton effects (CEs). Thus, dilute solutions of BR-IX in pH 4.0–9.7 buffered human serum albumin (HSA) all gave bisignate CD CEs associated with the pigment's intense UV-visible absorption near 450 nm, but the CEs were not identical over the range of pH. In particular, the signed order of the CEs seen at pH 4.0 with HSA was opposite to that found for solutions of pH between 6.0 and 9.7 (6), and the signed order at a given pH depended even on the species of albumin to which the bilirubin was bound (2, 5, 7). The origin of the induced optical activity of achiral BR-IX is clear: the pigment must be strongly perturbed by association with the (chiral) protein. However, the origin of the bisignate nature of the long wavelength CD CE is less clear and has been interpreted variously as being due to exciton coupling in a skewed bilirubin conformation, coupling of bilirubin transition moments with those of the protein, or the presence of two different (chiral) bilirubin species (2). Yet, in none of these mechanisms has a unique chiral structure been proposed for the protein-bound pigment.

In the present work, we extend the BR-IX + HSA CD studies to include the symmetric bilirubin derivatives, mesobilirubin-XIIIα (MBR-XIII) and mesobilirubin-IVα (MBR-IV) as well as to their pyrromethenone analog, xanthobilirubinic acid (XBR). Through these studies, we provide an explanation for the induced bisignate CD spectra in terms of enantiomeric complexation by HSA to intramolecularly hydrogen-bonded conformers of bilirubin.

MATERIALS AND METHODS

Bilirubin-IXα (Sigma) was purified by crystallization as described previously (8). Mesobilirubins-XIIIα and IVα and xanthobilirubinic acid were prepared by total synthesis (9, 10). Buffer solutions, 0.1 mM Tris (Trizma, (Tris base), from Sigma), were prepared as described earlier with and without added human serum albumin (Sigma, defatted) in argon-saturated distilled water and protected from light (11).

All circular dichroism spectra were recorded on a JASCO J-40 spectropolarimeter equipped with a photoelastic modulator, and all UV-visible spectra were measured on a Cary 219 spectrophotometer.

RESULTS AND DISCUSSION

Albumin is an optically active, chiral protein that binds to BR-IX and a multitude of other substances. The affinity of HSA for BR-IX is rapid, reversible, and strong (12), with an association constant of the order of 10⁷–10⁹ for the first bilirubin and only about an order of magnitude less for a second (5). The 1:1 stoichiometric complex in pH 7.3 aqueous
buffer exhibits an intense, nonsymmetric band for the pigment long wavelength electronic transition at 457 nm, a transition which is strongly red-shifted from that ($\lambda_{\text{max}}$ = 411 nm) of the uncomplexed pigment (Fig. 1). As noted previously (2, 4–7), this complex shows an intense bisignate CD DE for the long wavelength transition with a (+) maximum at 460 nm and a (−) maximum at 407 nm.

Albumin also binds to xanthobilirubic acid (XBR), a pyrromethene model for one-half of a bilirubin. XBR complexes noncovalently with HSA at the same binding site as does BR-IX, but less strongly (13). The monochromophoric XBR-HSA complex exhibits an induced CD, which unlike that of bichromophoric HSA-BR-IX complex, has only a monosignate CE ((+) at 426 nm) in pH 7.3 aqueous buffer (Fig. 2). Like that of the HSA-BR-IX complex, however, the HSA-XBR complex has a red-shifted pigment long wavelength electronic transition at 423 nm versus that of the uncomplexed pigment at 411 nm. The shifts in $\lambda_{\text{max}}$ are characteristic of binding to the protein and have been used to determine the association constants (13). Clearly, as judged from both the UV-visible spectral shift and the induced CD spectrum, XBR binds to HSA at pH 7.3. It also binds to bovine serum albumin in pH 7.3 buffer, and that complex also exhibits a monosignate, but (−) CD CE ($\Delta$s = −3.4). At pH 5, however, where more XBR is complexed to albumin, the CE of the XBR-bovine serum albumin complex becomes bisignate ($\Delta$s = −4, $\Delta$C = +3). Bisignate CEs thus seem to be characteristic of either a bichromophoric pigment complexed to the protein, or a protein complex containing two chromophores which are not covalently linked to each other

![Diagram](image)

**Fig. 1.** Circular dichroism (---) and UV-visible (---) spectra of 2.58 × 10⁻⁴ M bilirubin-IXα in pH 7.32 argon-saturated 0.05 M Tris buffer in the presence of 5.07 × 10⁻⁵ M human serum albumin at 22 °C. The spectra were recorded within 15 min after preparation of the solution and remained essentially invariant for hours at 22 °C. A CD spectrum of the same concentration of BR-IX without added HSA falls on the $\Delta$ = 0 line; a UV-vis spectrum on the line (O⋯⋯O).

![Diagram](image)

**Fig. 2.** Circular dichroism (---) and UV-visible (---) spectra of 2.58 × 10⁻⁴ M xanthobilirubic acid in pH 7.33 argon-saturated 0.05 M argon-saturated Tris buffer in the presence of 5.29 × 10⁻⁵ M human serum albumin at 22 °C. The spectra were recorded within 15 min after preparation of the solution and remained essentially invariant for hours at 22 °C. A CD spectrum of the same concentration of XBR without added HSA falls on the $\Delta$ = 0 line; a UV-vis spectrum on the line (O⋯⋯O). When the XBR concentration is increased to 5.70 × 10⁻⁴ M in the presence of 5.29 × 10⁻⁵ M HSA, $\Delta$s falls slightly to +9.7, but the UV-visible spectrum is unchanged.

but which presumably bear a proximal relationship.

In keeping with this concept, the bichromophoric tetrupyrrole pigment, mesobilirubin-XIIIα (MBR-XIII), which has a symmetric ordering of the pyrrole β-substituents, reveals a strong bisignate CD CE in pH 7.3 aqueous HSA solution of the same signed order as that of the HSA BR-IX complex (Fig. 3). It also exhibits UV-visible spectral shifts akin to those of BR-IX when complexed with HSA. In marked contrast, however, the isomeric bichromophoric tetrupyrrole pigment mesobilirubin-IVα (MBR-IV) shows only a (+) mono-
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FIG. 3. Circular dichroism (---) and UV-visible (---) spectra of 2.42 × 10⁻⁸ M mesobilirubin-XIIIa in pH 7.33 argon-saturated 0.05 M Tris buffer in the presence of 5.07 × 10⁻⁷ M human serum albumin at 22 °C. The spectra were recorded within 15 min after preparation of the solution and remained essentially invariant for hours at 22 °C. A CD spectrum of the same concentration of MBR-XIII without added HSA falls on the Δε = 0 line; a UV-vis spectrum on the line (O· · · O).

FIG. 4. Circular dichroism (---) and UV-visible (---) spectra of 2.83 × 10⁻⁸ M mesobilirubin-IVa in pH 7.29 argon-saturated 0.05 M Tris buffer in the presence of 4.00 × 10⁻⁷ M human serum albumin at 22 °C. The spectra were recorded within 15 min after preparation of the solution and remained essentially invariant for hours at 22 °C. A CD spectrum of the same concentration of MBR-IV without added HSA falls on the Δε = 0 line; a UV-vis spectrum on the line (O· · · O).

FIG. 5. Upper, interconverting, intramolecularly hydrogen-bonded enantiomeric conformers of bichromophoric (4Z,15Z)-bilirubin-IXa (R¹ = R² = R³ = CH₃, R⁴ = CHH₃) and (4Z,15Z)-mesobilirubin-XIIIa (R¹ = R² = CH₃CH₃, R³ = CH₃). Lower, the relative orientations of the electric dipole transition moments (viewed at C₁₉) of the pyromethene chromophores of A (left) and B (right). The A enantiomers have left-handed (−) chirality of the twin pyromethene chromophores; the B enantiomers have right-handed (+) chirality.

Association with the protein is indicated by UV-visible spectral shifts typical of those seen for BR-IX, MBR-XIII, and even XBR, but the CD spectrum is more like that of the HSA-XBR complex than that of the HSA-MBR-XIII complex. These data indicate that bisignate CD CEs are not uniquely associated with the bichromophoric structure of the pigment but must also be governed by at least one additional important structural characteristic, which is shared by BR-IX and MBR-XIII, but not by either MBR-IV or XBR.

Perhaps the most important aspect of the three-dimensional structure of BR-IX—an aspect with important implications for biological function—is its ability and marked tendency to form intramolecular hydrogen bonds and thereby control its conformation and polarity (1). The key structural features which collectively govern the shape of BR-IX include: (i) syn-periplanar conformations of the two pyrromethenone chromophores possessing Z-configuration carbon-carbon double bonds at C₄ and C₁₅, (ii) two propionic acid groups located at C₅ and C₁₁, each capable of forming intramolecular hydrogen bonds with the opposing pyrromethene lactam C=O/NH and pyrrole NH groups, and (iii) an sp² carbon at C₁₀ which keeps the two pyrromethene chromophores ~109° apart. These structural elements permit and almost constrain BR-IX to adopt either of the two enantiomeric conformations (A) and (B) shown in Fig. 5, which are stabilized by six intramolecular hydrogen bonds. These enantiomeric conformations have been found in crystalline bilirubin (13, 14) and appear to be equally preferred in solutions of bilirubin in achiral organic solvents (15, 16). Remarkably, even when ionization of the propionic acid reduces the number of hydrogen bonds (but probably increases the strength of the remainder) conformers like A and B are found in crystals of BR-IX bis-isopropylammonium salt (17) and also in solutions of BR-IX bis-tetra-n-butylammonium salt (18). This marked preference for (enantiomeric) conformers in which polar groups are intramolecularly hydrogen-bonded explains the lipophilic character of bilirubin—that property which prevents its ready excretion across the liver into bile (1). It also explains why isomers of bilirubin with vinyl groups reduced to ethyl (mesobilirubins), e.g. MBR-XIII, or with vinyl and methyl groups interchanged at C₅/C₁₁ or C₁₇/C₁₅, e.g. symmetrically substituted bilirubins-IIIa and XIIIa, all exhibit similar solubility properties. However, isomers which have an E-configuration carbon-carbon double bond at C₄ or C₁₅, or isomers that do...
not have their propionic acid groups positioned at C_9 or C_13, e.g. MBR-IV, exhibit markedly different chemical and biological properties because they cannot withstand the intramolecular hydrogen bonding expressed in Fig. 5.

The enantiomeric conformers (A and B) of BR-IX and MBR-XIII are in dynamic equilibrium (15, 16). They interconvert (A ≈ B) by breaking and remaking all six H-bonds— an enantiomeric equilibration process which has potential importance in biological reactions involving chiral recognition. In the absence of special effects, equal amounts of A and B are expected, with solutions of BR-IX and MBR-XIII exhibiting no optical activity. However, if the dynamic, interconverting racemic mixture can be shifted away from 50% A + 50% B to, e.g. 40% A and 60% B, the pigment will no longer be racemic, and its solutions will exhibit optical activity. The shift from a racemic mixture to one with an enantiomeric excess might be achieved in a chiral solvent or by converting racemic mixture can be shifted away from 50% B to, e.g. 40% B and 60% A, the pigment will no longer be racemic, and its solutions will exhibit optical activity. The shift from a racemic mixture to one with an enantiomeric excess might be achieved in a chiral solvent or by selective interaction with a chiral solute. For example, aqueous solutions of BR-IX and MBR-XIII containing sodium deoxycholate (19) exhibit CD associated with the pigment chromophore, and induced CD has even been observed for BR-IX dimethyl ester in ethyl (S)-(−)-lactate and (R,R)-2,3-butandiol solutions (20). We believe that the optical activity and bisignate CD spectra of albumin (or other proteins) complexed to BR-IX and MBR-XIII can be explained by a displacement toward predominantly A or B of the conformational enantiomeric equilibrium represented in Fig.

Among the various explanations accounting for the induced optical activity of BR-IX, Blauer and others (2-7) have suggested that the pigment is held by the protein in a (skewed) chiral conformation. The CD of BR-IX and MBR-XIII bound to HSA (Figs. 1 and 3), with their bisignate CEs (BR-IX: $\Delta_{499}^{408} = +49$, $\Delta_{267}^{408} = -26$; MBR-XIII: $\Delta_{442}^{408} = +45$, $\Delta_{305}^{408} = -43$), implicate two proximal, electronically interacting chromophores held in a chiral conformation. Such conformations might be adopted by other tetrapyrrole pigments, such as MBR-IV, but the latter, which cannot adopt the intramolecularly hydrogen-bonded conformations shown in Fig. 5, gives only a slightly less intense monosignate CD CE ($\Delta_{408}^{442} = +28.9$ (Figure 4)). The protein apparently does not select a single chiral conformation of MBR-IV, or, if it does, the pyrromethenone chromophores lie essentially in the same plane. The CD behavior is thus similar to that of a single pyrromethene complex, as in the XHR-HSA chromophore (Fig. 2), where the monosignate $\Delta_{499}^{442} = +12.7$ CE is approximately one-half the magnitude of HSA-MBR-IV CE. These data support the notion that HSA complexes selectively, although not necessarily exclusively, to one of the chiral, intramolecularly H-bonded conformations of BR-IX and MBR-XIII (Fig. 5).

The observed bisignate CD CEs (Figs. 1 and 3), whose $\lambda_{\text{max}}$ flank but do not coincide with the UV-Vis $\lambda_{\text{max}}$ (BR-IX: $\lambda_{\text{max}}^{442} = 49$, $\lambda_{\text{max}}^{408} = 500$; MBR-XIII: $\lambda_{\text{max}}^{408} = 46,500$) are characteristic of chromophore-chromophore interaction of the locally excited states of the twin pyrromethenone chromophores, i.e. exciton splitting (21). This splitting, which generates two transitions, one higher in energy and one lower in energy than that for the isolated pyrromethenone chromophore, depends on a number of factors. It is especially dependent on the magnitudes and directions of the two electronic transition moments involved (21, 22), and it is expected to be largest for molecular geometries of the bichromophoric tetrapyrole pigments where the pyrromethenone transition moments (4) make a skew angle of ~90° (21). The two transitions are not widely separated and show considerable overlap in the UV-visible spectrum, where electronic transitions always have the same sign. This explains the broad, nonsymmetric structure of the relevant UV-visible bands (Figs. 1, 3, and 4) of bichromophoric BR-IX, MBR-XIII, and also MBR-IV—either complexed with albumin or alone—even when the pigment has two identical pyrromethenone chromophores. In the CD spectra, however, where exciton splitting leads to two transitions with oppositely signed CEs, the observed spectra (Figs. 1 and 3) have widely separated maxima due to considerable cancelation in the overlap region of the separated CD transitions. This is a general phenomenon for overlapping CD transitions of opposite sign (23).

Exciton coupling theory also provides a way to assign the absolute configuration (either A or B, Fig. 5) of the chiral pigment preferentially bound to HSA. The handedness or screw sense that the electronic transition moments of the coupled pyrromethenone chromophores make with each other (Fig. 5) correlates with signed order of the bisignate CD CEs (21). A right-handed screw sense (positive chirality) of the transition moments leads to a (+) longer wavelength CE followed by a (−) shorter wavelength CE. For a left-handed screw sense (negative chirality), the CE signs are inverted. Since the direction of the electric dipole transition moment in the pyrromethenone chromophore has been calculated in theoretical studies (4) to lie along the longitudinal axis of the planar conjugated π-system, the exciton model can predict the CE of the structurally well defined enantiomers, A and B. In these intramolecularly hydrogen-bonded conformations of BR-IX and MBR-XIII, the relative orientations of the two pyrromethene electric dipole moments constitute a left-handed chirality for A and a right-handed chirality for B. Since the induced bisignate CD of both BR-IX and MBR-XIII show (+) long wavelength CEs followed by (−) short wavelength CEs, theory predicts a predominance of enantiomer B bound noncovalently to HSA at pH 7.3.

The enantio-selectivity of albumins for BR-IX and MBR-XIII should not be too surprising, especially since there are many examples of proteins preferring one enantiomer over the other. HSA, for example, has been shown to exhibit a higher affinity for the d-enantiomer ($K_d = 1.3 \times 10^4 M^{-1}$) than for the L-enantiomer ($K_d = 2.3 \times 10^4 M^{-1}$) of the spin labels D- and L-glutamate-5-N-(1-oxyl-2,2,6,6-tetramethyl-4-amino)peridinyl)-2,4-dinitrobenzene (24). The particular preference for one enantiomer over the other is probably dictated by the shape of the protein binding site—a shape which can change with pH or with the albumin species and thereby invert the enantiomeric selectivity (25). For bilirubin, the signed order of the bisignate CEs is reversed on HSA at pH 4 compared with pH 7.3 (5, 6)—an observation consistent with enantiomer A being bound preferentially at pH 4 and enantiomer B at pH 7.3. Similarly, since the signed order of the CEs is also reversed at pH 7.3 when the serum albumin is changed from human to bovine (2, 7), the enantiomeric selectivity changes from B to A, respectively. The enantio-selectivity by albumins for conformation A or B has also been shown for complexation with (chiral) cyclodextrins, which serve as hosts for complexing with BR-IX, MBR-XIII, and MBR-IV but show bisignate CD CEs only for the first two guests (26). Further studies on the circular dichroism of pigment-protein complexes is under investigation in our laboratory.

REFERENCES

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