Effect of the binding of bilirubin to either the first class or the second class of binding sites of the human serum albumin molecule on its photochemical reaction

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The kinetics of the photochemical changes of bilirubin were studied at a constant concentration of bilirubin bound either to the first class or to the second class of binding sites of the human serum albumin molecule. The more the bilirubin binds to the first class of binding sites in the human serum albumin molecule, the more readily geometric photoequilibrium to give (*ZE*)-bilirubin takes place. The more the bilirubin binds to the second class of binding sites or allosterically transformed binding sites induced by added SDS, the more readily structural photoisomerization, i.e. the formation of (*EZ*)-cyclobilirubin, takes place. When the serum bilirubin concentration is at low, safe, values bilirubin binds exclusively to the first class of binding sites and serves as an antioxidant [Onishi, Yamakawa & Ogawa (1971) *Perinatology* 1, 373–379]; at these concentrations human serum albumin protects bilirubin from irreversible photodegradation by only allowing readily reversible geometric photoisomerization. As the serum bilirubin concentration increases to high, and potentially dangerous, values, bilirubin binds to the second class of binding sites, and under these conditions human serum albumin seems to promote the photocyclization of bilirubin. During irradiation human serum albumin seems to act by retaining low, useful, concentrations of bilirubin while facilitating irreversible photoisomerization of excess bilirubin.

INTRODUCTION

The photochemistry of (*ZZ*)-bilirubin and the chemical mechanism of bilirubin removal from the body during phototherapy are very important because of the widespread clinical use of phototherapy for treating neonatal hyperbilirubinaemia. Complexing of bilirubin with the serum albumin molecule has a marked species-dependent influence on bilirubin photoisomerization *in vitro* (McDonagh *et al.*, 1982; Itoh & Onishi, 1985; Onishi *et al.*, 1985) and *in vivo* (Onishi *et al.*, 1980, 1984, 1986). The photochemical reaction of (*ZZ*)-bilirubin bound to human serum albumin is singularly selective, and of the two possible isomers, (*EZ*)- and (*ZE*)-bilirubin, only the (*ZE*)-isomer is observed. A summary of the relative rate constants for the quantitatively important

photochemical reaction [$(\text{EZ})\text{-cyclobilirubin} \xleftarrow{k_5} (\text{EZ})\text{-bilirubin} \xrightleftharpoons[k_3]{k_4} (\text{ZZ})\text{-bilirubin} \xrightleftharpoons[k_2]{k_1} (\text{ZE})\text{-bilirubin}$] while bound

to human or rat serum albumin in aqueous solution is as follows. Except for k_4 and k_3 , the rate constants are considerably larger for human serum albumin (Itoh & Onishi, 1985; Onishi *et al.*, 1985). Since, in the photochemical reaction of the bilirubin–human albumin complex, the rate constant (k_5) of the endovinyl cyclization step and that (k_3) of the reversion from (*EZ*)-bilirubin to (*ZZ*)-bilirubin are much larger (by two orders of magnitude) than that (k_4) of (*EZ*)-bilirubin formation from (*ZZ*)-bilirubin, only (*EZ*)-cyclobilirubin is apparently detected (Itoh & Onishi, 1985). The reason for the different rate constants for human and rat serum

albumins is probably related to differences in bilirubin conformation and salt or acid dissociation at the binding site of the serum albumin molecule. The former is suggested by c.d. spectra (Blauer & King, 1970) and by amino acid sequence studies (Meloun *et al.*, 1975; Behrens *et al.*, 1975; Sargent *et al.*, 1981a,b), and the latter is suggested by acidimetric titration (Jacobsen & Brodersen, 1983; Frandsen & Brodersen, 1986). It has been shown that at least two molecules of bilirubin dianion can be bound reversibly to one of human serum albumin (Jacobsen, 1969). In a model of independent binding, the two binding constants of the first class and the second class of binding sites (Odell, 1973; Jacobsen & Brodersen, 1983) are 5.5×10^7 and $4.4 \times 10^6 \text{ M}^{-1}$ at 37 °C in a 60 mM-sodium phosphate buffer (Brodersen, 1979a). Therefore the kinetics of the photochemical reactions may also provide insight into the molecular conformation of the bilirubin-binding site in serum albumin. In the present paper we describe the differences in the kinetics of the photochemical changes at constant concentration of bilirubin bound either to the first class or to the second class of binding site of human serum albumin (Jacobsen, 1969; Odell, 1973; Jacobsen & Brodersen, 1983) in aqueous solution. A preliminary report of these results has already appeared (Itoh *et al.*, 1987).

MATERIALS AND METHODS

Photochemical experiments

Except where noted otherwise, in all experiments the solutions were deoxygenated with pure (99.99%) N_2 .

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Each 500 μ l portion of the solution in Pyrex tubes was exposed to light. At different periods of exposure to light the tubes were successively transferred to the dark. Each portion was analysed by h.p.l.c. (Itoh *et al.*, 1983). Light-intensity, determined with a Minolta Air-Shields Fluorolite-meter 451 (Minolta Air-Shields), at the surface of the solution was $27.3 \mu\text{W} \cdot \text{cm}^{-2} \cdot \text{nm}^{-1}$.

Except for photochemical experiments, all manipulations were carried out in the dark or under a photographic safe-light and at room temperature.

Preparation for photochemical experiments

Just before each experiment 5 mg of crystalline bilirubin (Tokyo Kasei) was dissolved in 1 ml of 0.05 M-NaOH, and 0.2 ml of the solution was immediately added to each of 10 ml portions of 0.1 M-sodium phosphate buffer, pH 7.4, containing human serum albumin (Sigma Chemical Co.) at ten different concentrations (8.0, 4.0, 3.0, 2.0, 1.5, 1.0, 0.75, 0.50, 0.375 and 0.25 g/100 ml). With the final concentration of bilirubin kept constant at 10 mg/100 ml, solutions of bilirubin-albumin complexes containing ten different bilirubin/albumin molar ratios (1:4, 3:8, 1:2, 3:4, 1:1, 3:2, 2:1, 3:1, 4:1 and 8:1) were prepared.

Bilirubin-human serum albumin complex solution containing various molar proportions of SDS

Bilirubin-human serum albumin complex solution. Solutions of bilirubin-human serum albumin complex were prepared by adding 100 μ l of a bilirubin solution (prepared by dissolving 10 mg of crystalline bilirubin in 1 ml of 0.05 M-NaOH) to 10 ml of human serum albumin solution (2.0 g/100 ml) in 0.1 M-sodium phosphate buffer, pH 7.4. Portions of this solution were mixed with 33 mM-SDS in 0.1 M-sodium phosphate buffer, pH 7.4, to prepare solutions containing six different SDS/albumin molar ratios (0:1, 1:1, 2:1, 3:1, 5:1 and 15:2). The final total volume was adjusted by addition of 0.1 M-sodium phosphate buffer, pH 7.4.

Measurement of light-absorption at 510 nm. Absorption spectra of non-irradiated samples were measured in 1 cm quartz cuvettes in a Shimadzu spectrophotometer. Light-absorption at 510 nm was calculated for each sample.

RESULTS AND DISCUSSION

Effects of bilirubin/albumin molar ratio of the bilirubin-human serum albumin complex on geometric and structural photoisomerization

Geometric photoisomerization (Fig. 1). The samples were irradiated for 15 min with blue-white light and then analysed by h.p.l.c. The geometric photoisomerization reactions are all photoreversible, and the proportion of (ZE)-bilirubin in (ZZ)-bilirubin (representing the geometric photoequilibrium) reached approx. 40% when the bilirubin/albumin molar ratio was below 1:1. However, as the ratios increased from 1:1 to 8:1, the percentage of (ZE)-bilirubin formed in (ZZ)-bilirubin decreased linearly.

Structural photoisomerization. In preliminary experiments the amounts of photochemically formed (EZ)-cyclobilirubin, resulting from endovinyl cyclization, increased more or less during 10 min irradiation irrespective of the various bilirubin/albumin molar

Fig. 1. Relationships between bilirubin/human serum albumin molar ratios and the geometric or structural photoisomerization of bilirubin

○, (EZ)- and (EE)-cyclobilirubin concentrations at 10 min of anaerobic photoirradiation; ●, percentage of (ZE)-bilirubin in (ZZ)-bilirubin at 10 min of anaerobic irradiation. For experimental details see the text.

ratios of the bilirubin-albumin complex (Fig. 2). However, as shown in Fig. 1, the total amount of (EZ)- and (EE)-cyclobilirubin (referred to here as cyclobilirubin) formed during that period was at a maximum at a molar ratio of 2:1 and decreased as the molar ratio was either decreased from 2:1 to 1:4 or increased from 2:1 to 8:1. When the molar ratio was increased above 3:1 some of the (ZZ)-bilirubin aggregated. Such bilirubin

Fig. 2. Effects of bilirubin/human serum albumin molar ratio and of the duration of irradiation on the structural photoisomerization of bilirubin

Bilirubin/human serum albumin molar ratios were as follows: ○, 1:4; ●, 1:2; ■, 1:1; ▲, 2:1; ▽, 4:1; ▼, 8:1. For experimental details see the text.

Fig. 3. Relationships between the light-absorption at 510 nm of bilirubin-human serum albumin complexes containing various bilirubin/albumin molar ratios and the structural photoisomerization of bilirubin at 10 min of anaerobic photoirradiation

Bilirubin/human serum albumin molar ratios were as follows: ○, 1:4; ●, 3:8; □, 1:2; ■, 3:4; △, 1:1; ▲, 3:2; ▽, 2:1; ◇, 4:1; ◆, 8:1. For experimental details see the text.

cannot participate in the photochemical reactions. As shown in Fig. 3, the relative efficiency of cyclization of bilirubin bound to the second class of binding sites within the molar ratio range from 1:4 to 2:1 correlates with increments in light-absorption at 510 nm. Therefore, even though light-absorption at 510 nm increased above a molar ratio of 2:1, the formation of cyclobilirubin decreased. Thus the most effective molar ratio, as evaluated by the amounts of cyclobilirubin formed at 10 min, occurred at a value of 2:1. This strongly suggests that it is predominantly the bilirubin bound to the second class of binding sites of human serum albumin that undergoes cyclization.

Effects of SDS on geometric and structural photoisomerization

The effects of binding of SDS to bilirubin-human serum albumin complexes on the rate of photochemical formation of (ZE)-bilirubin and cyclobilirubin were also investigated. (ZE)-Bilirubin formation decreased as SDS concentration increased (results not shown). How the bilirubin dianion binds to the human serum albumin molecule at the first class of binding sites remains only partially understood. The results of determination of the quantum yield of fluorescence (Lamola *et al.*, 1983) and a resonance-Raman spectroscopic study (Hsieh & Morris, 1988) of the bilirubin-human serum albumin complex show that the bilirubin is completely enclosed within the human serum albumin molecule (Brodersen, 1979b; Jacobsen & Brodersen, 1983). Moreover, the internal hydrogen bonds between propionate groups and the pyrromethene rings of bilirubin are ruptured (Lauffer *et al.*, 1987; Hsieh & Morris, 1988). Therefore the geometric photoisomerization may occur more readily at the first class of binding sites. On the other hand, cyclobilirubin formation increased as SDS con-

Fig. 4. Effects of SDS on light-absorption at 510 nm and structural photoisomerization of bilirubin at 1, 3 and 5 min of anaerobic photoirradiation of bilirubin-human serum albumin complexes

SDS/human serum albumin molar ratios were as follows: ○, 0:1; ●, 1:1; □, 2:1; ■, 3:1; △, 5:1; ▲, 15:2. For experimental details see the text.

centration was increased to give SDS/albumin molar ratios from 1:1 to 5:1 (Fig. 4). We have already shown that the maximum of the action spectrum for photochemical cyclobilirubin formation is at about 510 nm (Itoh & Onishi, 1985). The relative efficiency correlates with the increase in light-absorption at 510 nm of bilirubin-human serum albumin complex solutions induced either by increasing the bilirubin/albumin molar ratio (Fig. 3) or by increasing the SDS/albumin molar ratio (Fig. 4).

Thus the more the bilirubin binds to the first class of binding sites in the human serum albumin molecule the more readily the geometric photoequilibrium takes place. On the other hand, the more the bilirubin binds to the second class of binding sites the more easily endovinyl cyclization takes place. It was concluded that the geometric and the structural photoisomerization reactions of bilirubin takes place on the binding sites of the first class and of the second class respectively. The relative efficiency of cyclobilirubin formation is presumed to correlate with the amount of light-absorption at 510 nm. This absorption can be augmented by SDS. While this paper was in preparation, Malhotra *et al.* (1987) reported the effect of various fatty acids on the photochemistry of albumin-bound bilirubin, and fatty acids of carbon chain length of 10 or more were found to increase by as much as 3-fold the quantum yield for formation of cyclobilirubin. However, they interpreted this effect as being due to an increase in the dihedral angle between the two dipyrrole chromophores of bilirubin.

Bilirubin plays an important physiological role as an antioxidant, as already described by us (Onishi *et al.*, 1971). When bilirubin was placed on unsaturated fatty acid (linoleic acid), breakdown of bilirubin was accelerated, but the decomposition of linoleic acid was prevented. When bilirubin was placed in a tube with saturated fatty acid, no acceleration of bilirubin breakdown was noted. These phenomena suggested that

bilirubin in subcutaneous fat protected polyunsaturated fatty acids from decomposition and at the same time breakdown of bilirubin was accelerated by the existence of polyunsaturated fatty acids in the subcutaneous adipose tissue (Onishi *et al.*, 1971). This activity has attracted special interest recently (Stocker *et al.*, 1987*a,b*).

When the serum bilirubin concentration is sufficiently low and safe that bilirubin binds exclusively the first class of binding sites, it is useful as an antioxidant. Under these conditions human serum albumin seems to protect bilirubin from photocyclization and allows only readily reversible geometric photoisomerization. In the bile the (ZE)-isomer reverts rapidly to (ZZ)-bilirubin (Onishi *et al.*, 1981; Stoll *et al.*, 1981), which can readily be absorbed by the intestinal mucosa (Lester *et al.*, 1961; Gilbertsen *et al.*, 1962; Corchs & Rodriguez Garay, 1970). However, as the ratio of bilirubin to human serum albumin molecules increases, bilirubin begins binding to the second class of binding sites, and human serum albumin seems to promote the photocyclization of bilirubin. Thus during irradiation human serum albumin acts as a balance to retain low, useful, concentrations of bilirubin while facilitating the removal of a toxic excess of bilirubin.

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