Albumin binding of photobilirubin II

Peter MEISEL, Karl-E. BIEBLER, Andre GENS and Klaus JAEHRIG

Department of Paediatrics, Ernst-Moritz-Arndt University, DDR-2200 Greifswald, German Democratic Republic

(Received 17 November 1982/Accepted 23 March 1983)

Photobilirubin II, a stereoisomer of bilirubin, binds to human serum albumin at a single binding site ($K = 2.2 \times 10^6 \text{ M}^{-1}$), presumably the high-affinity bilirubin-binding site. Binding in the secondary (class II) binding sites is of minor importance. The results are discussed with respect to photometabolism of bilirubin and as a possible source of error in the determination of bilirubin unbound to albumin.

In blood plasma bilirubin is present, almost exclusively bound to one site of the albumin molecule, with smaller amounts located at one or two secondary (class II) binding sites (Brodersen, 1979). Owing to its high binding affinity, this process is thought to determine the disposition of bilirubin within the organism, the danger of developing bilirubin encephalopathies in newborn infants as well as the efficacy of treatment procedures in neonatal hyperbilirubinaemia, such as exchange transfusion and phototherapy. With regard to phototherapy, it is now assumed that bilirubin is mainly converted in a light-driven process into its geometric isomers (photobilirubins) followed by movement from the extravascular tissues (mainly skin) to the bloodstream. Probably at this stage, or only within the intravascular space, photobilirubin is bound to albumin, allowing it to be transported to the hepatocyte, where excretion proceeds. In this sense photobilirubin binding to albumin may be one of the rate-limiting steps in phototherapy (McDonagh *et al.*, 1980).

Therefore we have determined some binding parameters of one of the bilirubin photoisomers to human serum albumin. The photobilirubin studied is the 'pigment 430' (Meisel *et al.*, 1981), probably identical with photobilirubin II (Stoll *et al.*, 1979) and the 'unknown pigment' (Onishi *et al.*, 1980b), recently identified as cyclo-(4*E*,15*Z*)-bilirubin (Stoll *et al.*, 1982). Structures of the compounds in question are shown in Fig. 1.

Experimental

Materials and methods

Crystalline human serum albumin was purchased from Institut für Impfstoffe (Dessau, German Democratic Republic); its non-esterified fatty acid content was estimated to be 0.46 mol/mol of protein.

Bilirubin was obtained from Kallies KG (Sebnitz, German Democratic Republic), and horseradish peroxidase from VEB Arzneimittelwerke (Dresden, German Democratic Republic). 'Pigment 430' (photobilirubin) was prepared by irradiating bilirubin/albumin solutions with phototherapy light as described elsewhere (Meisel *et al.*, 1981). Pigment was extracted by acetone precipitation of albumin and purified by t.l.c. on silica-gel plates with butan-1-ol/ethanol/water (6:2:3, by vol.) as solvent. All binding experiments were performed in 0.1M-sodium Veronal/acetate buffer, pH 8.3, at 25°C. In this buffer the risk of colloid formation is

Bilirubin (4*Z*,15*Z*)

Photobilirubin II (4*E*,15*Z*)

(Stoll *et al.*, 1982)

Fig. 1. Structures of bilirubin and photobilirubin II

minimized even at pigment/albumin ratios well above 1:1. Ranges of total concentrations of albumin and pigments used are specified in the legends to Figs. 2 and 3.

Detection of binding to the high-affinity site

The binding parameters of the pigments to the high-affinity site of albumin were determined by the peroxidase technique. Bilirubin (Jacobsen & Wennberg, 1974) as well as photobilirubin (Meisel *et al.*, 1981) are oxidized by H_2O_2 , the reaction being catalysed by peroxidase and suppressed by albumin binding. The concentration of unbound pigment determined at different pigment/albumin molar ratios was used to construct a plot of $1/r$ versus $1/[pigment]_{free}$, where r is the number of occupied

$$Y = y_I + \frac{1}{2} y_{II} \left[(x-1) + n_{II} + \frac{1}{c_{HSA} \cdot K_{II}} - \sqrt{\left((x-1) + n_{II} + \frac{1}{c_{HSA} \cdot K_{II}} \right)^2 - 4n_{II}(x-1)} \right] \quad (6)$$

sites. This function obeys the equation:

$$\frac{1}{r} = \frac{1}{n_I} + \frac{1}{n_I K_I [pigment]_{free}} \quad (1)$$

Subscript I refers to the primary binding site.

Detection of binding to the class II binding sites

The method employed was that of Brodersen & Funding (1977), with modification. Solutions containing different pigment/albumin molar ratios were prepared and difference in absorbancies of albumin-bound (sample cuvette) versus free pigment (reference cuvette) were measured at 480 and 465 nm for bilirubin and photobilirubin respectively (Unicam SP.700-C spectrophotometer). Variations in the pigment/albumin molar ratio were adjusted by changing the albumin concentration but keeping the pigment concentration constant. Possible dimerizations were neglected, and therefore absorbance changes of the difference spectrum are expressed as:

$$Y = \frac{A_{sample} - A_{reference}}{d \cdot c_{HSA}} \quad (2)$$

where A is the absorbance at the wavelength mentioned, d the optical depth of the solution in centimetres, and c_{HSA} the total albumin concentration. In accordance with Brodersen & Funding (1977), binding parameters were calculated from observed Y values on the basis of a model with one high-affinity site and n_{II} equal and independent sites in a secondary class. It is assumed that

$$Y = y_I + \bar{v}_{II} y_{II} \quad (3)$$

where y_I is the contribution to Y made by saturating the high-affinity site with pigment and y_{II} is the contribution made by saturating one site in the second class. Therefore $\bar{v}_{II} y_{II}$ accounts for the increase in Y by binding of $\bar{v}_{II}$ mol of pigment per mol of albumin in equilibrium:

$$\frac{\bar{v}_{II}}{n_{II} - \bar{v}_{II}} = K_{II} \cdot [pigment]_{free} \quad (4)$$

where K_{II} is the binding constant of a secondary (class II) binding site. Total pigment concentration may be expressed by:

$$c_{pigment} = x \cdot c_{HSA} = c_{HSA} + \bar{v}_{II} \cdot c_{HSA} + [pigment]_{free} \quad (5)$$

where x is the pigment/albumin molar ratio.

Elimination of $[pigment]_{free}$ and $\bar{v}_{II}$ from eqns. (3)–(5) gives:

The values of Y observed at pigment/albumin molar ratios above 1 were used for calculating the parameters sought: y_{II} , n_{II} and K_{II} . By using a computer program 'Zero adjustment by nesting of intervals' fitted to this problem, the set of calculated values was compared with the set of observed values Y . Finally, the sum of the squared deviations was calculated in order to isolate the binding parameters giving the best fit. This method is superior to graphical methods for determination of binding parameters, since it gives equal weight to all experimental points.

Results

Fig. 2 shows the double-reciprocal plots of binding parameters of bilirubin and photobilirubin on the high-affinity site of human serum albumin. These plots give straight lines with the intercepts $-K_I$ and n^{-1} on the abscissa and ordinate respectively. One high-affinity site is observed for binding of both bilirubin and photobilirubin, bilirubin having the tighter binding to albumin. The values calculated from such plots are collected in Table 1.

In Fig. 3, the values of Y are plotted as a function of the pigment/albumin molar ratio in order to obtain the binding parameters of the secondary class II sites. With bilirubin, binding at the secondary class II sites contributes considerably to the values of Y measured. In contrast, binding of photobilirubin at the secondary class II sites results only in a minor increase in Y above the value contributed by binding at the primary class of high-affinity sites. Moreover, dilution of the test solution results in a

Fig. 2. Binding at the high-affinity site of human serum albumin

The Figure shows double-reciprocal plots for estimation of the binding parameters for the binding of bilirubin (a) and photobilirubin (b) to albumin; r is the number of occupied sites; the peroxidase method was used. The range of total albumin concentrations was 95–475 μM , and the ranges of pigment/albumin molar ratios were 0.175–0.595 and 0.044–0.172 for bilirubin and pigment 430 respectively.

Fig. 3. Dependence of increase in absorbance on the pigment/albumin molar ratio

(a) Bilirubin concentrations were 22.2 μM (○) and 4.0 μM (●), the cell path lengths being 1 and 5 cm respectively. (b) Photobilirubin concentrations were 32.4 μM (○) and 5.8 μM (●), the cell path lengths being 1 and 5 cm respectively. The broken line indicates the value of Y calculated as contribution of saturating the high-affinity binding site.

Table 1. Binding parameters for the binding of bilirubin and photobilirubin to albumin

Results shown are means $\pm$ S.D. for three determinations.

Parameter	Bilirubin	Photobilirubin
n_1	0.96 ± 0.04	0.92 ± 0.09
$K_1 \times 10^{-6} \text{ (M}^{-1}\text{)}$	62.5 ± 3.4	2.2 ± 0.2
$y_1 \times 10^{-3} \text{ (M}^{-1} \cdot \text{cm}^{-1}\text{)}$	17.3 ± 1.1	6.6 ± 1.2
n_{II}	1.43 ± 0.07	—*
$K_{II} \times 10^{-4} \text{ (M}^{-1}\text{)}$	31.0 ± 2.8	—†
$y_{II} \times 10^{-3} \text{ (M}^{-1} \cdot \text{cm}^{-1}\text{)}$	24.3 ± 1.2	1.3 ± 0.9

* No solution of eqn. (6) in the range $n = 0-5$.

† No solution of eqn. (6) in the range $K = 10^7-10^4 \text{ M}^{-1}$.

loss of absorbance in the difference spectrum of photobilirubin as the pigment/albumin molar ratio increases. As indicated in Table 1, there is no plausible solution of the computer-derived equations (eqn. 6), i.e. binding at the secondary class II sites is of minor importance. Hence, to calculate the value of Y for full saturation of the high-affinity site y_1 , we took twice the value observed at pigment/albumin molar ratio of 0.5 (see Brodersen & Funding, 1977). Such an approach is valid, as virtually all the pigment is bound to the primary binding site at this ratio.

Discussion

Photobilirubin is formed under the influence of blue light by isomerization of native bilirubin *in vitro* or *in vivo*. With regard to undesirable side effects in phototherapy, the possibility of bilirubin displacement by photobilirubin may be of practical significance. Substances able to displace bilirubin from its binding sites on albumin are suspected to increase the risk of bilirubin encephalopathies in jaundiced newborn infants. The presence of an unknown ligand is obvious in infants' serum after the subjects have received phototherapy (Ebbesen, 1981). Studies of bilirubin binding by sera from newborn infants undergoing phototherapy revealed the presence of competitors of bilirubin for albumin binding (Ahlfors & Wennberg, 1977). The findings reported in the present paper suggest that photobilirubin could be a candidate for such a displacing compound revealed *in vivo*. The critical point in question is the issue whether the binding site for photobilirubin is identical with the high-affinity binding site for bilirubin or with a secondary one. According to Hsia (1978), the albumin binding sites for bilirubin each can bind either one bilirubin dianion or two

monoanions. With regard to the structures of bilirubin and photobilirubin (Fig. 1), both binding sites are to be taken into consideration. If photobilirubin is bound to a site different from the high-affinity site for bilirubin, only an allosteric effect could possibly influence the high-affinity site. Even if binding of both pigments were primarily to the same site, effective competition would require high steady-state concentrations of photobilirubin (see Table 1). Such a situation seems unlikely *in vivo* unless liver function is disturbed. In newborn infants with impaired liver excretion undergoing phototherapy high concentrations of photobilirubin bound to albumin may circulate and eventually displace bilirubin from its binding sites (Onishi *et al.*, 1982; Meisel *et al.*, 1982).

Whereas the question of the identity of the photobilirubin-binding site cannot be answered at this experimental stage unambiguously, a comparison of bilirubin displacement by biliverdin may be illustrative. Biliverdin is structurally related to photobilirubin, is likewise bound at one high-affinity site with minor contributions of secondary sites of albumin, and exhibits a binding affinity comparable with that of photobilirubin. At a bilirubin/albumin molar ratio of 0.56 and a biliverdin/albumin molar ratio of 0.41 the apparent free bilirubin concentration rises from 20 to 68 nM on the addition of biliverdin; in serum a less pronounced increase was measured (Ahlfors, 1981). A free bilirubin concentration of 20 nM (50 nM at 37°C) is the limit of developing bilirubin encephalopathies, provisionally suggested by Wennberg *et al.* (1976). No doubt, however, a drastic decrease of the apparent concentration of unbound bilirubin takes place during phototherapy in newborn infants with liver excretion operating well (Ebbesen, 1981; Ebbesen & Jacobsen, 1980).

Being more loosely bound to albumin as compared with bilirubin (Table 1), photobilirubin may disturb the measurements of free bilirubin used to determine the risk of bilirubin encephalopathies in neonatal hyperbilirubinaemia. This was verified with regard to the peroxidase method (Pedersen *et al.*, 1977; Meisel *et al.*, 1981) as well as to the Sephadex-gel-filtration technique (Onishi *et al.*, 1980a). Therefore photobilirubin present in jaundiced serum from infants treated by phototherapy may give misleading results in evaluating the kernicterus risk.

Finally, it should be noted that the calculations made are based on a model with one high-affinity site and two equal sites in the secondary binding category. At least with respect to bilirubin, a model with three different sites may be more compatible with experience (Brodersen & Funding, 1977). In the case of photobilirubin such an extension may be neglected.

References

- Ahlfors, C. E. (1981) *Anal. Biochem.* **110**, 295–307
- Ahlfors, C. E. & Wennberg, R. P. (1977) *Clin. Res.* **25**, 187A
- Brodersen, R. (1979) *CRC Crit. Rev. Clin. Lab. Sci.* **11**, 305–399
- Brodersen, R. & Funding, L. (1977) *Scand. J. Clin. Lab. Invest.* **37**, 257–266
- Ebbesen, F. (1981) *Acta Paediatr. Scand.* **70**, 223–228
- Ebbesen, F. & Jacobsen, J. (1980) *Eur. J. Pediatr.* **134**, 261–263
- Hsia, J. C. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 1424 (Abstr. no. 852)
- Jacobsen, J. & Wennberg, R. P. (1974) *Clin. Chem.* **20**, 783–789
- McDonagh, A. F., Palma, L. A. & Lightner, D. A. (1980) *Science* **208**, 145–151
- Meisel, P., Jährig, D., Jährig, K. & Meisel, M. (1981) *Photobiochem. Photobiophys.* **3**, 83–91
- Meisel, P., Jährig, D., Theel, L., Auff'm Ordt, H. & Jährig, K. (1982) *Photobiochem. Photobiophys.* **3**, 345–352
- Onishi, S., Kawade, N., Itoh, S., Isobe, K. & Sugiyama, S. (1980a) *Biochem. J.* **190**, 527–532
- Onishi, S., Isobe, K., Itoh, S., Kawade, K. & Sugiyama, S. (1980b) *Biochem. J.* **190**, 533–536
- Onishi, S., Itoh, S., Isobe, K., Togari, H., Kitoh, H. & Nishimura, Y. (1982) *Pediatrics* **69**, 273–276
- Pedersen, A. O., Schønheyder, F. & Brodersen, R. (1977) *Eur. J. Biochem.* **72**, 213–221
- Stoll, M. S., Zenone, E. A., Ostrow, J. D. & Zarembo, J. E. (1979) *Biochem. J.* **183**, 139–146
- Stoll, M. S., Vickers, N., Gray, C. H. & Bonnett, R. (1982) *Biochem. J.* **201**, 179–188
- Wennberg, R. P., Lau, M. & Rasmussen, L. F. (1976) *Pediatr. Res.* **10**, 434–439