Interactions of Rat α -Foetoprotein with Bilirubin

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The binding of bilirubin to rat α -foetoprotein has been measured by changes in the absorption spectrum and by the appearance of two intense circular-dichroism bands. Furthermore binding of this bile pigment has been demonstrated by a decrease of its enzymic oxidation rate and by competition experiments with bovine serum albumin-agarose. The binding parameters have been determined as follows: $n = 1.0$ mol bound/mol of protein and $K_a = 2.9 \times 10^6 \text{ M}^{-1}$. Competition of oestradiol for the bilirubin-binding site has not been established.

α -Foetoprotein is a glycoprotein present in relatively high concentrations in foetal serum and amniotic fluid of many species. The biological role of rat α -foetoprotein is not yet clearly understood, though activities such as a strong oestrogen binding (Vallette *et al.*, 1977; Aussel *et al.*, 1978; Versée & Barel, 1978a) and an immunosuppression (Murgita & Tomasi, 1975) have been reported.

Similarities between α -foetoprotein and serum albumin are indicated by sequence homologies (Ruoslahti & Engvall, 1976; Peters *et al.*, 1978), by immunological cross-reactions between the reduced and alkylated forms (Ruoslahti & Terry, 1976), and by a close analogy in physico-chemical properties (Grigorova *et al.*, 1977; Versée & Barel, 1978b). It is generally assumed that α -foetoprotein may serve as a foetal form of albumin in the transport of biological molecules, such as steroids, fatty acids and dyes. Thus we investigated the possible interactions between bilirubin and α -foetoprotein in comparison with the well-known strong binding of albumin for this bile pigment. A preliminary report of this work has already been presented (Versée & Barel, 1978c).

Materials and Methods

The purification procedure for α -foetoprotein was essentially the same as that previously described (Kerckaert *et al.*, 1975; Versée & Barel, 1978a). Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. and purified through a Sephadex G-100 column to obtain the monomeric form.

Bilirubin (Sigma) was used without further purification. The procedure of Jacobsen & Wennberg (1974) was used for bilirubin solutions and for the oxidation of bilirubin by H_2O_2 in the presence of horseradish peroxidase (Sigma). All spectral studies were carried out at room temperature (25°C). Absorption spectra were recorded on a Cary 118 spectrophotometer. Circular-dichroism spectra were recorded on a Cary 61 dichrograph in 10 mm path-length silica cells.

Fluorescence measurements were performed on an Aminco-Bowman SPF spectrofluorimeter.

Bovine serum albumin immobilized on agarose (0.45 mg of protein/ml of suspended gel) was used in competitive-binding studies as a means of measuring the binding of bilirubin to α -foetoprotein as described by Reed *et al.* (1975). Equilibration studies were carried out at 25°C for 30 min in 2 ml vials. All products were dissolved or suspended in a phosphate-buffered saline solution (3.75 mM- NaH_2PO_4 , 16.2 mM- Na_2HPO_4 and 0.1 M- NaCl) at pH 7.4. Each vial contained a constant volume (1 ml) of suspended bovine serum albumin-agarose and various amounts of α -foetoprotein, bilirubin and buffer were combined to give a final volume of 2 ml. After equilibration, filtration on a glass-fibre filter was carried out. The absorbance of the supernatant was recorded between 350 and 550 nm for bilirubin analysis. The amount of bilirubin bound to albumin-agarose was calculated by subtracting the amount of bilirubin in the supernatant from the total amount of bilirubin initially present. The affinity of albumin-agarose for bilirubin was separately determined by means of competition with soluble serum albumin ($K_a = 1.6 \times 10^7 \text{ M}^{-1}$). If $\bar{v}$ represents mol of bound bilirubin/mol of protein and c the molar concentration of free bilirubin, the association contrast was obtained by the equation of Scatchard (1949): $K_a = \bar{v}/[(1-\bar{v})c]$. The equilibrium relationship for two proteins competing for the same ligand is given by the following equation:

$$\left(\frac{\bar{v}}{1-\bar{v}}\right)_y = \frac{K_y}{K_x} \left(\frac{\bar{v}}{1-\bar{v}}\right)_x$$

where the subscripts x and y refer to albumin-agarose and α -foetoprotein respectively. The ratio of the association constants, K_y/K_x , may be considered as an index of competition. Since K_x , the binding constant of bilirubin to albumin-agarose, is known, the value of K_y can be readily obtained from the slope of the linear plot (see Fig. 3).

Displacement experiments of radioactively labelled oestradiol bound to α -foetoprotein with bilirubin

were carried out at pH 8.5 by using the equilibrium-dialysis technique on Sephadex G-25 described by Pearlman & Crépy (1967) and Versée & Barel (1978b). The concentrations of α -foetoprotein and the radioactively labelled oestradiol were kept constant (respectively 100 nM and 7 nM), whereas various concentrations of bilirubin were used (50–5000 nM).

Results and Discussion

Binding between rat α -foetoprotein and bilirubin was suggested by the following data. The absorption spectrum of bilirubin was modified in the presence of a 2 molar excess of α -foetoprotein. As shown in Fig. 1(a) the absorption maximum shifted from 438 nm for free bilirubin in aqueous solution to 424 nm in the presence of α -foetoprotein; in addition, the appearance of a typical shoulder centred around 470 nm was observed. The spectrum of bilirubin in the presence of serum albumin was also included for comparison. Similar absorption spectra of bilirubin complexes with bovine and human serum albumin were reported by Woolley & Hunter (1970) in the presence of fatty acids. The existence of fatty acid-binding sites on human α -foetoprotein has been noted by Parmelee *et al.* (1978). The stoichiometry of bilirubin binding to α -foetoprotein is illustrated by spectrophotometric titration (Fig. 1b). When the change in A_{424} was plotted as a function of the α -foetoprotein/bilirubin ratio, a distinct break at a molar ratio of 1 was observed, suggesting the existence of one binding site.

Although free bilirubin is essentially non-dichroic, a circular-dichroism spectrum from 300 to 550 nm was obtained for bilirubin in the presence of α -foetoprotein at pH 8.5 (Fig. 2). As shown similarly for bilirubin-serum albumin complexes by Blauer *et al.* (1972) and Beaven *et al.* (1973), this spectrum is pH-

dependent. No circular dichroism could be observed at pH 5.0.

The fluorescence of α -foetoprotein obtained on excitation at 290 nm was gradually quenched by the addition of increasing amounts of bilirubin. In the presence of an 8 molar excess of bilirubin over α -foetoprotein, the tryptophan fluorescence of this protein was quenched up to 80%, suggesting an energy transfer between the unique tryptophan residue of α -foetoprotein and the ligand. The intrinsic fluorescence of bilirubin was not significantly enhanced on binding to α -foetoprotein, as shown by Beaven *et al.* (1973) for human serum albumin.

Bilirubin binding to α -foetoprotein was also shown by the peroxidase method of Jacobsen & Wennberg (1974). In this method it is assumed that the rate of disappearance of bilirubin by oxidation is proportional to the concentration of unbound bilirubin and

Fig. 2. Circular-dichroism spectrum of bilirubin in the presence of rat α -foetoprotein. Bilirubin (7 μ M) and α -foetoprotein (25 μ M) at pH 8.5 in 0.01 M-potassium phosphate buffer. The optical path length was 10 mm.

Fig. 1. Effect of α -foetoprotein on the spectrum of bilirubin. (a) shows the absorption spectrum of 18 μ M-bilirubin alone (---), in the presence of 33 μ M- α -foetoprotein (—) and in the presence of 39 μ M-bovine serum albumin (-.-.-); (b) shows the spectral titration of bilirubin with α -foetoprotein (from 0.5 to 12 μ M). All solutions contained 0.1 M-potassium phosphate buffer, pH 7.4.

Fig. 3. Bilirubin distribution between albumin coupled to agarose and α -foetoprotein in solution

Results are calculated from the equation displayed in the Materials and Methods section. $\bar{v}$ represents mol of bound bilirubin/mol of protein. The subscripts x and y refer to albumin-agarose and α -foetoprotein respectively. The slope, K_y/K_x , may be regarded as an index of competition, in the absence of oestradiol ($\circ$) and in the presence of a saturating concentration of oestradiol ($\bullet$).

that the oxidation of bound bilirubin occurs at a negligible rate. The rate of disappearance of bilirubin was undoubtedly extensively slowed down in the presence of α -foetoprotein. However, we were not able to measure the binding equilibrium constant, since the bilirubin bound to α -foetoprotein was not sufficiently protected from oxidation, as shown for bilirubin bound to albumin.

Binding between α -foetoprotein and bilirubin could be demonstrated by competition with bovine serum albumin-agarose (Fig. 3). From the slope of this linear plot a value of $2.9 \times 10^6 \text{ M}^{-1}$ for the binding constant was obtained at 25°C . It appears that α -foetoprotein displays a somewhat lower affinity for this ligand, compared with various albumins [K_a is in the range $(2-7) \times 10^7 \text{ M}^{-1}$; Chen, 1972].

Displacement experiments of oestradiol bound to α -foetoprotein with bilirubin were carried out to determine whether this bile pigment could compete for the steroid-binding site. Even at a dye/protein molar ratio of 50, bilirubin was unable to displace bound oestradiol. Does oestradiol compete for the bilirubin-binding site? Addition of a saturating concentration of oestradiol has no effect on the absorption spectrum of the bilirubin- α -foetoprotein complex, and on the binding of bilirubin to α -foetoprotein as shown by competition experiments with serum albumin-agarose (Fig. 3).

In addition to the well-described oestrogen-binding properties of α -foetoprotein, we propose the existence

of a supplementary bilirubin-binding site. In recent reports Aoyagi *et al.* (1978) and Ruoslahti (1978) have produced evidence that human α -foetoprotein displays a bilirubin-binding capacity.

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