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Dansylation of Human Serum Albumin in the Study of the Primary Binding Sites of Bilirubin and L-Tryptophan

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Binding of bilirubin and of L-tryptophan to dansylated albumins was investigated. Dansylation of less than one lysine residue per molecule of albumin did not affect the bilirubin binding, but decreased the L-tryptophan binding, indicating that dansylation had taken place in or near the L-tryptophan-binding site. Native albumin and albumin–bilirubin 1:1 complex showed the same affinity for L-tryptophan. The results indicate that, although L-tryptophan and bilirubin are bound in the same region, perhaps in a common cavity of the albumin molecule, such a cavity is sufficiently large to contain both ligands.

Attempts have been made to assign binding sites of physiological anions to the various regions of the human serum albumin molecule by referring to the albumin model by Brown (1975) and the sequence found by Meloun *et al.* (1975). One primary binding site of fatty acids has been demonstrated to lie in the *C*-terminal part of albumin (loops 4–9 region) (Peters *et al.*, 1976). The primary indole-binding site has been assigned to the region of loops 3–4 (Sjöholm & Ljungstedt, 1973; Gambhir *et al.*, 1975) and evidence has been presented that the high-affinity bilirubin-binding site is located in the same region of the molecule (Gitzelmann-Cumarasamy *et al.*, 1976; Jacobsen, 1978). In the present work the location of the bilirubin-binding site and that of indole binding has been studied by means of albumin derivatives having specifically modified amino acid residues.

Materials and Methods

Materials

Human serum albumin was obtained from Ab Kabi, Stockholm, Sweden, and was defatted as described by Chen (1967). Horseradish peroxidase and bilirubin were from Sigma Chemical Co., St. Louis, MO, U.S.A. L-[5-^{3}H]Tryptophan (20 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Binding studies

The binding of bilirubin to albumin derivatives was investigated by measuring the free equilibrium concentration of bilirubin by using the peroxidase method, as described in detail previously (Jacobsen, 1975).

The binding of L-tryptophan to albumin derivatives was conducted by an ultrafiltration technique (Kragh-Hansen *et al.*, 1972). The albumin–tryptophan and the tryptophan reference solutions in the same concentration, 4ml, were enclosed in Visking tubing bags (Union Carbide Corp., Chicago, IL, U.S.A.; size 20/32, that had previously been boiled in 3% (w/v) $NaHCO_3$ for 30min, followed by rinsing in demineralized water. The bags were centrifuged in special tubes equipped with porous Teflon filters at 320*g* and 22°C. The ultrafiltrate obtained after 20min was discarded (approx. 75μl), and centrifugation was continued for an additional 1h. The free equilibrium concentration of tryptophan was calculated from

$$[\mathrm{Trp}] = (1-\alpha)\, C_{\mathrm{Trp}}$$

where C_{Trp} is the total tryptophan concentration, and α is given by the equation:

$$\alpha = \frac{\text{c.p.m.}_{\text{ref.}} - \text{c.p.m.}_{\text{sample}}}{\text{c.p.m.}_{\text{ref.}}}$$

where c.p.m.$_{\text{ref.}}$ and c.p.m.$_{\text{sample}}$ are the radioactivities of a certain amount (50μl) of ultrafiltrates of the reference and the sample, respectively.

Dansylation of human serum albumin

Dansyl chloride [10mM in ethanol/acetone (1:1, v/v)] was in 1–4-fold molar excess added to a continuously stirred solution of albumin (0.75mM) in 0.1M-sodium phosphate buffer, pH7.4. The reaction was carried out for 1h at 20°C in the dark. The same procedure was applied to modify albumin, previously complexed with bilirubin in the molar ratio 1:1. The dansylated albumins were then treated with

Abbreviation used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

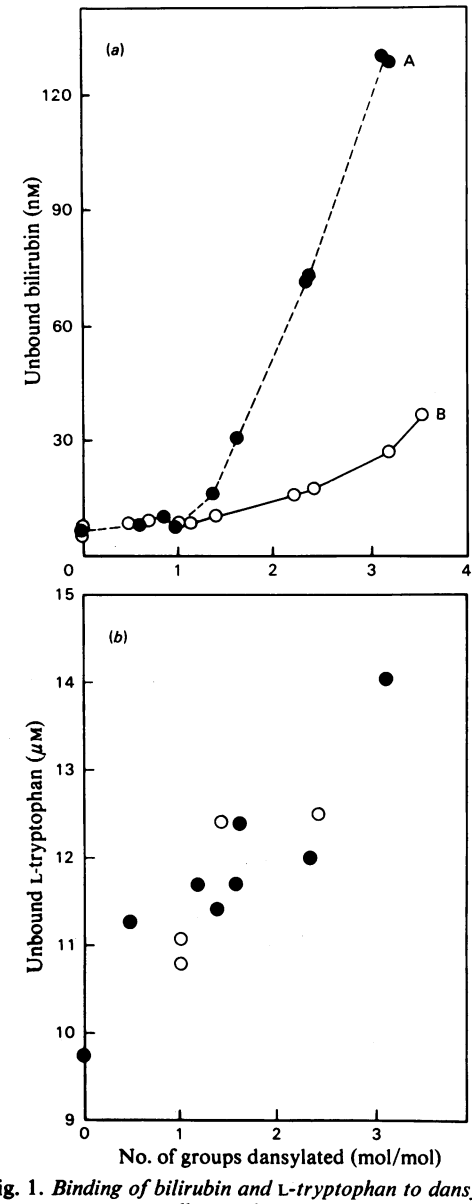

Fig. 1. *Binding of bilirubin and* L-*tryptophan to dansylated albumin derivatives*

The binding affinities of the various albumin derivatives are illustrated by the equilibrium concentrations of free bilirubin (*a*) and L-tryptophan (*b*) respectively. The albumin concentrations were in all cases 30 μM, and bilirubin and L-tryptophan concentrations were 15 μM. The binding experiments were conducted at pH 7.4 and 22°C. The equilibrium concentrations of bilirubin were calculated by using the peroxidase method (J. Jacobsen, 1969; C. Jacobsen, 1975) and the L-tryptophan concentrations were determined by using an ultrafiltration technique (Kragh-Hansen *et al.*, 1972). ●, Human serum albumin dansylated in the absence of bilirubin; ○, human serum albumin dansylated in the presence of bilirubin, followed by removal of bilirubin by charcoal/salicylate (Jacobsen, 1975).

active charcoal (albumin/charcoal, 1:1, w/w) in the presence of sodium salicylate (0.1 M) to remove bilirubin and unchanged dansyl chloride (Jacobsen, 1975). The pH in the solution was kept at 7.4 and the stirring was continued for 40 min. Finally the charcoal was removed by filtration through a Whatman GF filter, followed by removal of sodium salicylate by filtration through a Sephadex G-25 column (2 cm × 50 cm) with water as eluent. The derivatives were freeze-dried and stored at −15°C.

The number of dansyl groups attached covalently to albumin was estimated from ε 3400 $M^{-1} \cdot cm^{-1}$ at 335 nm (Chen, 1968).

Results and Discussion

Affinity determinations

Fig. 1(*a*) shows the correlation between the number of amino groups dansylated in the various albumin derivatives and the equilibrium concentrations of free bilirubin. The concentrations of free bilirubin were calculated as described previously by a kinetic technique based on the observation that free bilirubin, in contrast with bound bilirubin, is oxidized by H_2O_2 in the presence of horseradish peroxidase (J. Jacobsen, 1969; C. Jacobsen, 1972, 1975). To ascertain that all bilirubin was bound to the high-affinity site, an albumin concentration of 30 μM and a bilirubin concentration of 15 μM were selected. The same concentrations were applied in the determinations of the free equilibrium concentration of L-tryptophan, i.e. 30 μM-albumin derivative and 15 μM-L-tryptophan. The results are shown in Fig. 1(*b*). A rise in free ligand concentrations reflects in both cases a decrease in binding affinity.

Dansylation of one lysine residue

It appears from Fig. 1(*a*) that no appreciable change in binding affinity for bilirubin was observed when one lysine residue or less was dansylated per molecule of albumin. This applies to derivatives obtained by dansylation with as well as without bilirubin. Gambhir *et al.* (1975) have published that dansylation of albumin in low reagent/albumin ratios led to dansylation of lysine-195 in the first stage, resulting in blockage of the indole-binding site. They applied essentially the same experimental conditions as in the present work. (Here and below the residue numbering is given in accordance with the sequence of Meloun *et al.*, 1975). As shown in

Fig. 1(*b*), the present results confirm that dansylation of the first group (lysine-195?) affects the L-tryptophan binding.

Dansylation of more than one lysine residue

Fig. 1 also shows that dansylation of additional lysine residues gives derivatives with less affinity for bilirubin or for L-tryptophan. This indicates that dansylation has taken place at residues located in the region of the binding sites. It should be noticed that Gambhir *et al.* (1975) in their experiments found dansylation of lysine-136, lysine-162 and lysine-212 when modification was conducted in the absence of tryptophan. All these residues are located in the loops 3 and 4. Fig. 1(*a*), curve B, shows that bilirubin protects specific lysine groups involved in the bilirubin binding from being dansylated. This observation confirms that lysine residues situated in the bilirubin-binding site actually have been modified in the experiments without bilirubin (i.e. curve A, Fig. 1*a*), and that at least one may be involved in the bilirubin binding, in agreement with earlier findings (Jacobsen, 1978).

Binding of L-tryptophan to albumin–bilirubin complexes

The binding of L-tryptophan to albumin–bilirubin complexes is shown in Fig. 2, which clearly demonstrates an unchanged affinity for albumin–bilirubin complexes containing less than one molecule of bilirubin per molecule of albumin. This finding agrees with Fig. 1, stressing a non-interference between the first molecule of bilirubin and the first tryptophan molecule bound to albumin.

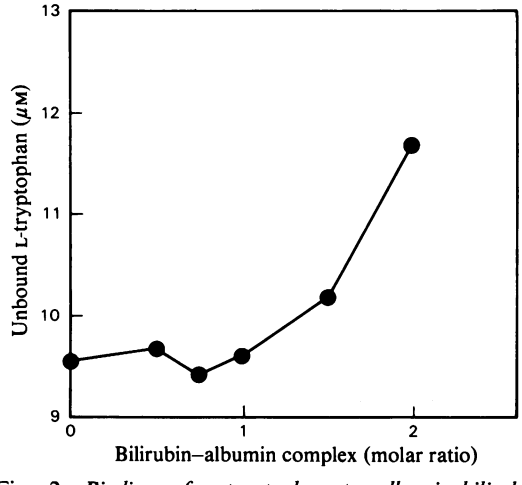

Fig. 2. *Binding of L-tryptophan to albumin–bilirubin complexes*
The concentrations of albumin–bilirubin complexes were 30 μM and that of L-tryptophan was 15 μM.

The primary bilirubin-binding site

It has been demonstrated previously (Jacobsen, 1972) that neither cysteine-34 nor tryptophan-214 is involved in the bilirubin binding, as modification of these residues with 2-nitrobenzenesulphenyl chloride had no influence on the binding affinity. It has been shown also by acetylation with acetylsalicylic acid (which acetylates lysine-199; Walker, 1976) that this lysine residue is not essential for the bilirubin binding (Jacobsen, 1972). It has, on the other hand, more recently been established that lysine-240 is involved in the binding, probably by forming a salt linkage to one of the carboxylate groups on bilirubin (Jacobsen, 1978).

Altogether, the results presented here suggest that between loops 3 and 4 a binding cavity exists that is sufficiently large to contain both an indole compound and a bilirubin molecule. The size of the cavity is restricted in the sense that introduction of more than one dansyl group inhibits the binding of both bilirubin and L-tryptophan.

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