Mouse α₁-Fetoprotein and Albumin

A COMPARISON OF THEIR BINDING PROPERTIES WITH ESTROGEN AND FATTY ACID LIGANDS*

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The binding of estradiol-17β (E₂), diethylstilbestrol (DES), and polyene fatty acids, in particular arachidonate (C₂₀:₄), to α₁-fetoprotein (α-FP) and albumin purified from mouse embryo sera was studied using equilibrium dialysis and electrophoretic techniques.

E₂, arachidonate, and DES bind to α-FP, but with decreasing strength. E₂ is a high affinity, low capacity ligand (Kₐ = 0.8 × 10⁶ M⁻¹ and n₂ = 0.3 sites/mol of α-FP at 25 °C); arachidonate is a weaker ligand disposing of more sites (Kₐ = 0.3 × 10⁶ M⁻¹ and n₂ = 0.5 sites/mol of α-FP); the binding of DES is of comparatively low affinity and capacity (Kₐ = 0.2 × 10⁶ M⁻¹ and n₂ = 0.7/mol of α-FP). In spite of different structures and equilibrium parameters, E₂, DES, and arachidonate are able to compete with each other for binding to the fetoprotein. The C₂₂:₄ and C₂₂:₆ fatty acids are also efficient concentration-dependent inhibitors of E₂ or DES binding.

Albumin binds the fatty acids and DES, but equilibrium parameters are different from those of α-FP. In particular, arachidonate is a better ligand for albumin, where it interacts with at least two classes of apparent sites (Kₐ = 0.3 × 10⁶ M⁻¹ and n₁ = 1; Kₐ = 0.2 × 10⁶ M⁻¹ and n₂ = 30). In contrast to α-FP, albumin virtually does not bind E₂. Also, no competition could be demonstrated between DES and fatty acid ligands for binding to albumin. None of the studied interactions, with either albumin or α-FP, was modified even by high doses of bilirubin. The possible functions of the various binding activities present in fetal sera in the process of growth are discussed.

Growing evidence points to the serum binding proteins as performers of complex functions: extracellular carriers, but also modulators of tissue uptake (1–3), maternal-fetal transfer (4, 5), and degree of biological impact for important metabolites and drugs (3, 6, 7). Studies on ligand-protein interactions in embryo and tumor development are comparatively recent. In fact, at present, the interest problem began to receive considerable attention when the steroids were discovered to associate strongly with rat α₁-fetoprotein (8, 9), a fetal antigen which reappears in some forms of adult cancers. Later such studies enlarged to include novel non-steroid ligands of rat α₁-FP, in particular a number of essential polyunsaturated fatty acids (10, 11), bilirubin (12, 13), and a commonly used estrogen analogue, diethylstilbestrol (7, 14).

Along with the rat, the mouse is the only species known to date which displays higher steroid-protein interactions in the fetal than in the maternal compartment (15, 16); the estrophily of mouse α₁-FP (17, 18) and the elevated corticosterone binding activities of its embryo sera (19) have been demonstrated and explored in detail. The present study was aimed at gaining further insight into the binding properties of the quantitatively major mouse fetal serum proteins α₁-FP and albumin.

A comparison of these two proteins with respect to their binding behavior seemed of particular interest since many authors consider mammalian albums as closely related to the homologous α-FPs in ontogenic and structural, as well as functional, aspects (12, 20, 21). We have sought to determine to what extent the similarities between α₁-FP and albumin persist when binding characteristics are considered.

We report on the application of equilibrium dialysis and electrophoretic techniques to measure the interactions of natural and synthetic ligands—in particular estradiol-17β, diethylstilbestrol, and arachidonate—with mouse α₁-fetoprotein and albumin purified to high electrophoretic and immunological purity.

MATERIALS AND METHODS

Animals and sera—Two-month-old pregnant mice (Charles River, Inbred Strain CD) were killed at the 18th day of gestation. The embryos were removed under ether anesthesia of the mothers and bled by decapitation. Maternal blood was collected through a catheter in the abdominal aorta. Pooled fetal and pooled adult sera were prepared by centrifugation at 4000 rpm and 4 °C. Each fetal protein preparation was obtained from about 5 ml of serum, i.e., collected from groups of at least 120 mixed male and female embryos. A 50 ± 3% sex distribution was assessed in the different groups.

Ligands—We have used the following radioactive compounds (Amersham) of 98–99% regularly checked purity: [2,4,6,7-³H]estradiol-17β, 93 Ci/mmol; [monothethyl-³H]diethylstilbestrol, 106 Ci/mmol; [5,6,8,9,11,12,14,15-³H]arachidonic acid, 147 Ci/mmol. Unlabeled polyunsaturated fatty acids (C₂₀:₄, C₂₂:₄ and C₂₂:₆) were purchased from Nu Chek Prep, unlabeled estradiol, diethylstilbestrol, and bilirubin were Sigma products.

Proteins: Purification and Purity Tests—The α₁-fetoprotein was purified from fetal sera by preparative electrophoresis in 10% polyacrylamide gels essentially as described (18). The purification of the fetal and maternal albumins was achieved in a two-step procedure involving preparative electrophoresis and subsequent chromatography on blue Sepharose/0.05 M Tris-HCl (pH 7) columns (14).

The α₁-FP and albumin preparations were pure according to electrophoretic and immunological criteria. Each showed a single Amido black- or Coomassie blue-stained band on 12% polyacrylamide gel electrophorograms; each showed a single precipitation arc after immunoelectrophoresis with rabbit antisera directed against whole mouse embryo sera (α₁-FP and embryo albumin) or against whole mouse adult sera (maternal albumin). Identical single arcs were obtained with monospecific anti-α₁-FP and anti-albumin rabbit antisera.
Additionally, binding tests applied to α-FP demonstrated the absence of corticosteroid fixation, i.e. of transcortin contamination.

Equilibrium Dialysis Binding Studies—The last step of our purification procedures consisted of a 48-h dialysis against renewed 0.005 M phosphorus, pH 7.4 at 4°C. This left, in either α-FP or albumin preparations, trace amounts of endogenous steroids, undetectable by gas chromatographic analysis but evidenced by radioimmunoassay techniques. Thus the dialyzed α-FPs still retained about 1×10⁻⁷ mol of estrogens (estrone + estradiol)/mol of protein (i.e. a maximum occupancy of 0.00001 sites/mol of protein). To eliminate these traces, the proteins were further treated by adding 1 mg of charcoal/mg of protein, by shaking the mixtures 30 min at room temperature, and by subsequent centrifugation and filtration through glass wool.

Binding Properties of Mouse α₁-Fetoprotein and Albumin

TABLE I

<table>
<thead>
<tr>
<th>Ligand</th>
<th>C valuea</th>
<th>Ksb</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol-17β</td>
<td>600 ± 50</td>
<td>8 ± 0.4</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>150 ± 20</td>
<td>0.3 ± 0.03</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>12 ± 3</td>
<td>0.2 ± 0.05</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

a Means of 8-10 determinations on four αFP preparations from different pools of sera.

b Means of four Scatchard analyses on the 4 αFP preparations.

Accurate evaluation of binding capacities or competitor activities was achieved either by vigorous blending on a Vortex mixer of aqueous buffered mixtures of these compounds, as shown for DES (14), or by directly drying their organic solvent solutions in the assay tubes. Similar results were obtained with either procedure.

The experiments involving bilirubin were carried out under minimal lighting conditions. The required amounts of bilirubin dissolved in chloroform were dried in the assay tubes and ready solubilization was obtained by subsequent addition of the α-FP or albumin solutions.

Electrophoretic Studies—Analytical electrophoresis in 10% polyacrylamide gels was performed on whole sera previously incubated with the labeled ligands in order to locate the bound radioactivity at the different protein fractions. Detailed descriptions of this technique have been given elsewhere (17, 27).

RESULTS

Interactions of α-FP with Arachidonic Acid and Diethylstilbestrol: Comparison to the Binding of Estradiol-17β—C values, association constants (Kb), and number of binding sites (n) were measured at equilibrium for the interactions of purified a-FP with the natural estrogen, the polynene fatty acid, and the estrogen analogue. The results (Table I; Fig. 1) show that mouse α-FP binds not only the phenolic steroid, as already demonstrated (17), but also arachidionate and, to a lesser yet significant extent, diethylstilbestrol.

Compared to the Ez interaction, which is best described as a high affinity, low capacity process (Kb of about 10⁻⁶ M⁻¹ and less than one binding site/mol of protein), the binding of the fatty acid is weaker, by approximately 1.5 orders of magnitude, but displays higher, if limited, capacity (4 to 5 sites/mol α-FP). The binding of DES, by comparison to that of Ez, shows less affinity (Kb ~ 0.2 × 10⁻⁶ M⁻¹) and similar scarcity of binding sites.

Interactions of Fetal Serum Albumin with Ez, Arachidonic Acid, and DES—The three ligands were analyzed for their interactions with the albumin from the same source, as shown in Table II and Fig. 2. It is clear that the binding properties of the albumin are different from those of α-FP.

First, the high Ez binding activity of mouse α-FP contrasts

FIG. 1. Scatchard plots for the interactions of estradiol-17β (inset), diethylstilbestrol (Curve 1), and arachidonic acid (Curve 2) with mouse α-FP. The reaction mixtures consisted of 200 mg of Sephadex G-25 (fine), 5.5, 45, or 15 μg of protein (for Ez, DES, and arachidonate, respectively), 0.5-500 ng of Ez, and 1-3200 ng of DES or arachidonate in 2 ml of 0.15 M phosphorus pH 7.4. Dialysis was for 1 h at 25°C under agitation. Each point is the mean value of a duplicate assay. For Ez: Kb = 0.8 × 10⁻⁶ M⁻¹; n = 0.3. For DES: Kb = 0.17 × 10⁻⁶ M⁻¹; n = 0.68. For arachidonate: Kb = 0.3 × 10⁻⁶ M⁻¹; n = 4.2.
with the inertness of albumin in this respect. In striking opposition to the C values of 600–700 obtained for the E2-a-FP interaction, the same indices measured with E2 and albumin scarcely reached values of about 2, rendering difficult the evaluation of the corresponding affinity and capacity equilibrium.

### Table II

<table>
<thead>
<tr>
<th>Ligand</th>
<th>C value *</th>
<th>( K_a ) ( \times 10^3 ) M (^{-1} )</th>
<th>n ( \times 10^3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol-17β</td>
<td>2 ± 0.2</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>730 ± 385</td>
<td>3.5 ± 0.6</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>15 ± 1</td>
<td>0.2 ± 0.1</td>
<td>33 ± 4.9</td>
</tr>
</tbody>
</table>

* Means of 12-14 determinations on six albumin preparations from different pools of serum.

**Means of four Scatchard analyses on four albumin preparations.

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**For DES:**

\[ \frac{K_a}{n} = \frac{\text{liters/g}}{\text{liter/mol}} \]

**For aromatic acids:**

\[ \frac{K_a}{n} = \frac{\text{liters/g}}{\text{liter/mol}} \]

**For STA:**

\[ \frac{K_a}{n} = \frac{\text{liters/g}}{\text{liter/mol}} \]

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**FIG. 2.** Scatchard plots for the interactions of diethylstilbestrol (Curve 1) and arachidonic acid (Curve 2) with embryonic serum albumin. Experimental conditions were as in Fig. 1. DES assays contained 100 μg of protein and 1-8000 ng of ligand; arachidonate assays contained 5 μg of protein and 1-3200 ng of ligand. The two-sloped curve obtained with arachidonate was interpreted as resulting from two classes of binding sites (n1 and n2) and graphic Rosenthal correction (not shown) was used to calculate the higher affinity sites (n2). For DES, \( K_a = 0.01 \times 10^3 \) M \(^{-1} \); \( n = 3.8 \). For arachidonate: \( K_a = 0.35 \times 10^5 \) M \(^{-1} \); \( n_1 = 1.5 \); \( K_a = 0.11 \times 10^5 \) M \(^{-1} \); \( n_2 = 40 \).**

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**FIG. 3.** Competition experiments with α-FP. Results are plotted as percent bound (i.e., bound radioactive ligand in absence of competitor) versus competitor concentration. A, displacement of \(^{3}\text{H}\)arachidonic acid (~1 × 10^6 cpm; ~0.2 nM) by radioinertilinuribumin, DES, or fatty acid (FA). The displacement curves for the three fatty acids tested as competitors, i.e., the C20:4, C22:4, and C22:6 compounds, were coincident. Incubation mixtures contained 70 nM α-FP (DES and fatty acid experiments) and 90 nM α-FP (bilirubin experiments). B, displacement of \(^{3}\text{H}\)arachidonic acid (~1 × 10^6 cpm; ~0.5 nM) by nonradioactive bilirubin, DES, and E2. Bilirubin and DES experiments, 280 nM α-FP/assay. E2 experiments, 140 nM α-FP/assay. C, displacement of \(^{3}\text{H}\)arachidonic acid (~1 × 10^6 cpm; ~0.7 nM) nonradioactive bilirubin, E2, and fatty acid; 420 nM α-FP/assay. Experimental conditions were as described in the legend to Fig. 1.

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**For the more active albumins,** the two-sloped Scatchard curves indicated at least two classes of apparent binding sites, one of high affinity (\( K_a = 0.4 \times 10^3 \) M \(^{-1} \) and the other, much weaker, of very high capacity (\( n = 30 \) sites/mol of albumin). Similar elevated "\( n \)" values have been reported for secondary sites of human or rat albumin interacting with estrone sulfate (29, 30) or rat albumin with DES (14). In spite of their high capacity, low affinity character, such sites probably contribute significantly to the arachidonate binding potency of purified mouse albumin. DES binding appears comparable with either α-FP or albumin, yet it involves weaker association and more sites in the case of albumin.
the [\textsuperscript{3}H]arachidonic acid-\(\alpha\)-FP interactions. Taken together, these data suggest a competitive character for the different inhibitory reactions and the least partial overlap (or identity) of the sites involved in the binding of \(E_2\), DES, or arachidonate on \(\alpha\)-FP. None of the studied binding reactions was influenced by bilirubin, as in the results of Berde et al. for human \(\alpha\)-FP (32) and unlike the properties of bovine \(\alpha\)-FP as described by Hsia et al. (33).

**Competition Experiments with Albumin**—Mutual displacement experiments, similar to those performed on \(\alpha\)-FP, were carried out on purified embryo or maternal mouse albumins. Fig. 4 illustrates measurements with embryo albumin, arachidonate as the tritiated ligand, and \(E_2\), DES, bilirubin, or the C22:6 acid as the inhibitors. The results showed a very different situation from that found with \(\alpha\)-FP. Indeed, no interference could be demonstrated among the albumin interactions with the natural or synthetic estrogen, arachidonic acid, or bilirubin. The only observed concentration-dependent inhibition was that of [\textsuperscript{3}H]arachidonate by the closely related C22:6 acid. Particularly noteworthy is the lack of inhibitory effect when the highly active albumin ligand arachidonate was tested against the weakly bound [\textsuperscript{3}H]DES. Also bilirubin, known as a high affinity ligand of mammalian albumins (34), failed to displace any of the tritiated compounds. Thus it appears that \(\alpha\)-FP and albumin, even when sharing a ligand, display not only dissimilar affinity and capacity binding characteristics, but also strikingly different sensitivities towards competitors, possibly because of unlike localization or structure of their binding sites.

**Electrophoretic Experiments with Whole Sera**—Since \(\alpha\)-FP and albumin co-exist in vivo as the quantitatively major serum proteins of the mouse embryo, it seemed important to assess their actual relative contributions to the binding of ligands, as they might occur in the nearer to native conditions of whole sera. Consequently we measured the labeling of the two proteins after polyacrylamide gel electrophoresis of fetal sera, preincubated with the different tritiated ligands. The results, shown in Fig. 5, corroborate the indications of the equilibrium dialysis experiments on the isolated proteins. With [\textsuperscript{3}H]E\(_2\), the single radioactive peak is located on \(\alpha\)-FP; the radioactivity of [\textsuperscript{3}H]DES is distributed almost equally between \(\alpha\)-FP and albumin; in the case of the fatty acid, two unequal peaks are seen, the more important at the albumin level. The pregnancy sera of the homologous mothers were likewise analyzed (results not shown). As expected, the liganded \(\alpha\)-FP having disappeared, only albumin shows important labeling, with either [\textsuperscript{3}H]DES or [\textsuperscript{3}H]arachidonic acid, and no \(E_2\) peak is observed. Parallel control runs performed with the different [\textsuperscript{3}H]-ligands, and no protein, failed to display any interferring trailing of radioactivity.

**DISCUSSION**

Developing previous studies from this laboratory, the present results show that \(\alpha\)-FP and albumin, the quantitatively major proteins of mouse embryo serum, both interact efficiently, yet not identically, with important molecules of very different structures: a synthetic nonsteroid estrogen, diethylstilbestrol widely used for experimental, breeding, and clinical purposes; nonesterified fatty acids, particularly polyunsaturated compounds, and among these arachidonic acid, the main precursor of the prostaglandins; also \(\alpha\)-FP, although not albumin, strongly interacts with the phenolic steroids estradiol-17\(\beta\) and estrone. The analogies and differences between the binding properties of \(\alpha\)-FP and albumin, including equilibrium affinity and capacity parameters, competition among ligands for common sites, and labeling behavior of the proteins during electrophoresis of whole sera, were discussed under “Results.” We shall here dwell on some of the more significant aspects.

The contrast between the high estrophily of mouse \(\alpha\)-FP and the inertness of its albumin towards the estrogens, with the other tested interactions shared by the two proteins, suggests the specific participation of the embryonic macromolecule in endocrine events associated with fetal or tumor growth. A number of recent results seem to favor this hypothesis by evidencing circumstances when estrophilic \(\alpha\)-FPs co-exist with likely developmental actions of the feminine hormones. Thus \(\alpha\)-FP was demonstrated inside mouse brain cells, along with a clear effect of estrogens on the innervation patterns of these cells (35). It was also shown that rat \(\alpha\)-FP, the only known estrogen-binding \(\alpha\)-FP besides that of the mouse, provokes a characteristic inhibition of estrogen-dependent tumors (36). However, a direct correlation between estrogen action and estrophily of \(\alpha\)-FP has yet to be proved.

Another noteworthy difference between \(\alpha\)-FP and albumin is seen in the competition studies. With \(\alpha\)-FP, experimental
conditions are readily found where ligands, whether cyclic or noncyclic, natural or synthetic, loose or tight, effectively prevent each other from binding to the macromolecule. Thus the weakly bound analogue DER, when present in concentrations that are high, but commonly used for in vivo treatments (37), can displace natural Ei. The linear arachidonate readily displaces Ei or DES. And the reverse is also true. On the contrary, no such mutual inhibitions occur among the ligand interacting with albumin, except with closely related polyene fatty acids.

These results are of structural and physiological importance. They indicate a different nature for sites and forces involved in the binding abilities of the two proteins, even when the same ligand is concerned. Although refined physical techniques are necessary to clarify this point, our data would agree with a limitation of sites for the binding to α-FP. Moreover, the observations suggest a greater responsiveness of α-FP, in vivo, to changes in medium. For instance, a metabolic or nutritional rise of fatty acids might drive the liberation of α-FP-linked estrogens, thus modifying the physiological potency of the hormones. It does not mean, however, that the more "inert" albumin would be excluded from the factors controlling a situation of this type: since it effectively competes with α-FP for common fatty acid ligands, the albumin might well trap part of the arachidonate-like compounds and so modulate their interactions with α-FP. In short, if the free or protein-bound state of small molecules regulates their biological activities, as now commonly accepted, it is important to know their complex relations to generally more than one binding protein, as well as to metabolic or ingested competitors.

Our results on the mouse cannot be simply extrapolated to other species. Comparison with available data concerning rat (7-11, 14, 31) and human (7, 15, 33, 38) fetal proteins shows important analogies, although no identity with the rat, and great differences, yet no absolute contrast with the human. Comparison with the human is of special interest. In contrast to the murine α-FPs, the fetoprotein of man binds neither natural estrogens nor DES. But, like the two rodent fetoproteins, it displays privileged binding relations to high molecular weight, natural or synthetic, loose or tight, effectively precluding the involvement of a-FP-linked estrogens, thus modifying the physiological potency of the hormones. It does not mean, however, that the more "inert" albumin would be excluded from the factors controlling a situation of this type: since it effectively competes with α-FP for common fatty acid ligands, the albumin might well trap part of the arachidonate-like compounds and so modulate their interactions with α-FP. In short, if the free or protein-bound state of small molecules regulates their biological activities, as now commonly accepted, it is important to know their complex relations to generally more than one binding protein, as well as to metabolic or ingested competitors.

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REFERENCES