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ESTROGEN-BINDING PROPERTIES OF RAT SERUM α_1-FETOPROTEIN AND ITS ISOFORMS. INVESTIGATION OF THE APPARENT NON-INTEGRALITY OF SITES ON THE UNFRACTIONATED PROTEIN

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Summary—Rat fetal serum α_1-fetoprotein (AFP), a heterogeneous glycoprotein, binds estrogens with high affinity but at a fractional number of sites even after treatment with charcoal ($n = 0.6$), which may mean 60% of the protein has 1 site and the remainder none. To investigate the origin of this fractional number of sites the "native" protein (purified by negative affinity chromatography) was further purified (step 1) and fractionated (step 2) into its two main charge variants (electrophoretically "slow" and "fast") by a two-step fast-protein liquid chromatography method. The binding parameters for estrone and estradiol-17β of the "native" and "repurified" proteins and of each charge variant were determined by equilibrium microdialysis. The molar extinction coefficient at 278 nm of each sample was also determined.

(1) The "repurified" AFP and each charge variant had a number of binding sites for estrogens close to unity. This increase in the number of sites could neither be explained by the loss of a non-binding isoform (corresponding to 40% of the protein) during chromatography, nor by the existence of complex negative modulatory interactions between isoforms.
(2) The affinities for estrogens of the "repurified" protein and the two charge variants were slightly decreased compared to that of "native" AFP, except that the "fast" form had the "native" protein's high affinity for estrone—but not for estradiol-17β.
(3) The molar extinction coefficients at 278 nm of the "repurified" AFP and the isoforms were much lower than that of the "native" protein.

These results suggest that the presence of (an) inhibitor(s) of estrogen binding on the "native" protein which is/are removed by the ion-exchange fast protein liquid chromatography (FPLC) column. A ligand absorbing at 278 nm, which may or may not be the inhibitor, is also removed. The isoform heterogeneity with respect to estrone binding is discussed.

INTRODUCTION

Rat α_1-fetoprotein (AFP), a fetospecific protein which binds estrogens with relatively high affinity ($1–5 \times 10^7\ M^{-1}$) [1, 2], is important for the binding of circulating estrogens in the fetal rat. Modeling of the binding distribution indicates that over 99% of estrone and estradiol-17β is bound by AFP in the 19-day fetal rat circulation [3].

This protein is a mixture of heterogeneous isoforms for which there is conflicting evidence concerning estrogen binding. On the one hand the number of estradiol-17β binding sites of rat AFP isolated from fetal serum has been found to be less than one [1, 2, 4], and the two major isoforms separated electrophoretically—the "slow" ($\simeq$60% of the total protein) and "fast" ($\simeq$40% of the total protein) fractions—have been reported to bind estradiol very differently [5]. On the other hand, rat AFP purified from amniotic fluid has an integral number of sites [6] and elutes as a single peak from an estradiol affinity column [7].

In our laboratory rat fetal serum AFP purified by negative immunosorption, using antibodies against whole normal adult serum [8], has regularly been found to bind estrogens at a fractional number of relatively high-affinity sites ($n \simeq 0.5$) [2, 4]. Among other possibilities, this may mean that only about half the AFP molecules have one binding site and the remainder none.

Given the availability of an FPLC method for the rapid and quantitative fractionation of the fetal

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Abbreviations: AFP, α_1-fetoprotein; FPLC, fast-protein liquid chromatography.

protein into its two major isoforms [9, 10], it has become possible to investigate the reason for this apparent fractionality directly.

EXPERIEMNTAL

Binding proteins

AFP was prepared from the serum of 19 ± 2-day-old rat embryos (Charles Rivers CD strain) by a negative immunoabsorption method previously described [8]. The method permits the recovery of at least 95% pure AFP ("native" AFP) which was then submitted to chromatography on a Pharmacia FPLC system. First the protein was purified further by FPLC on a Pharmacia Mono-Q SI column ("repurified" AFP) [9]. Then, on the same column at a different pH [10], the AFP isoforms were separated. The collected fractions were dialyzed against distilled water and lyophilized. Prior to binding measurements and spectroscopic determinations all the samples were treated with activated charcoal as previously described [11].

Elecrophoresis was performed in (80 × 60 mm) 10–14% (w/v) polyacrylamide gel gradients in 5 mM Tris–0.04 M glycine buffer, pH 8.3, using 6.25% (w/v) polyacrylamide as the stacking gel. A bovine serum albumin preparation known to contain dimers (batch No. 33, Miles, Elkhart, Ind., U.S.A.) was used as a reference. Electrophoreses were also carried out with estradiol-17β (rather than estrone, because of its greater solubility in aqueous medium). Rat AFP samples (10 μM) were preincubated for 1 h at 37°C in the presence of estradiol-17β at two different estrogen/protein molar ratios (1/5 and 1/1). In each case the same estradiol concentration was present in the buffer, the gel and the stacking gel. Gels were fixed with trichloracetic acid, stained with Coomassie blue and bands were quantified by scanning with an LKB Ultroscan XL densitometer.

Ligands

Estrone and estradiol-17β came from Roussel-Uclaf (Courbevoie, France) and were dissolved in small volumes of ethanol (grade A; Merck, Darmstadt, Germany) and diluted with buffer. [2,4,6,7-^{3}H]Estrone (3.37 TBq/mmol, 99% pure) and [2,4,6,7-^{3}H]estradiol-17β (2.96 TBq/mmol, 99% pure) were obtained from the Radiochemical Centre (Amersham, England). All compounds were used without further purification.

Buffers

All reagents were prepared in 5 mM sodium phospahte buffer, pH 7.4. Because of the poor estrogen solubility in aqueous medium, the same buffer containing 10% (v/v) ethanol was used for the binding measurements with these ligands. Ethanol, at concentrations not exceeding 10%, does not denature AFP nor significantly disturb its binding of estrogens [2].

Equilibrium dialysis

Binding experiments were performed by micro-equilibrium dialysis at 4°C for 24 h as previously described [11]. The different rat AFP samples were 0.5 μM for all the binding experiments and the ligands estrone and estradiol-17β were 0.04–4 μM, with a constant amount of radioactivity per well (1.35×10^3 Bq $\equiv$ 0.005 μM for estrone and 1.18×10^3 Bq $\equiv$ 0.005 μM for estradiol respectively).

Evaluation of the binding data

Calculations of the binding parameters (number of sites and apparent intrinsic affinity constant) were carried out using a slightly modified version of the Basic translation of the "LIGAND" programme [12]. "LIGAND" does a weighted non-linear least squares fit to the binding data. The main modification concerned weighting: the experimentally determined variances at each point were used for weighting rather than a pre-defined variance function.

Protein determinations

Protein concentrations were determined by the method of Lowry *et al.*[13], assuming a molecular weight of 70,000 for rat AFP, with bovine serum albumin (grade A, Calbiochem-Boehring Corp., La Jolla, Calif., U.S.A.) as standard. The validity of the Lowry method was checked by dry weight [14] determinations with "native" and "repurified" AFP. Molar absorption coefficients of rat AFP samples were determined at 278 nm using a Cary Model 118 spectrophotometer.

RESULTS

Fractionation of "native" rat AFP by FPLC

"Native" rat AFP obtained by negative immunosorption eluted from FPLC step 1 as a single peak ("repurified" AFP) with a yield of over 95%: this concords with the degree of purity of the starting material. Step 2 FPLC fractionation of the eluted peak led to its separation into 2 main fractions (Table 1): two peaks (Peaks 1 and 2) found by peak integration to be approximately 48 and 27% of the "repurified" AFP respectively. The fractions eluted between Peaks 1 and 2 (the gap) and after Peak 2 (the tail) (Table 1) contained small amounts of isoforms and were also collected, dialyzed and lyophilized. As a control, the AFP Peaks 1 and 2 were remixed with the gap and tail in their original proportions in order to yield a "reconstituted" AFP having exactly the same relative isoform concentrations as those in the "repurified" AFP.

Electrophoresis of the isolated peaks and of the "native", "repurified" and "reconstituted" AFP on 10–14% polyacrylamide gel gradients is shown in Fig. 1. "Native" rat AFP shows the typical "slow" and "fast" variants, as do the "repurified" and the "reconstituted" forms. Peak 1 was essentially pure

Table 1. Properties of the rat serum AFP fractions prior to and after FPLC

AFP samples	Elution volume (ml)	Percentage protein in each fraction*	Percentage "Slow" form	Percentage "Fast" form	ϵ 278 nm ($M^{-1} \cdot cm^{-1}$)
"Native"	—	—	69	31	50,000
"Repurified"	—	—	71	29	36,700
Peak 1	50–90	48	100	0	28,000
Peak 2	100–130	27	25	75	26,600
"Reconstituted"	—	—	72	28	29,000
Gap	91–99	15	—	—	—
Tail	131+	10	—	—	—

*Determined from peak area of absorption recorded at 280 nm.

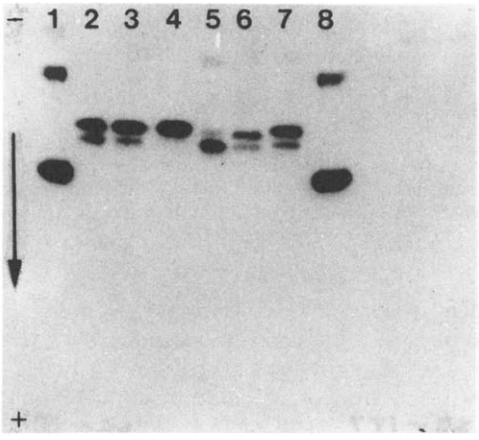

Fig. 1. Electrophoresis of rat AFP at different steps of its (re)purification and fractionation. The experiments were performed in (80 × 60 mm) 10–14% (mass/vol) polyacrylamide gel gradients. The samples were as follows: 4 μg of bovine serum albumin containing a high proportion of dimers (tracks 1 and 8), respectively 4 and 2 μg of "native" AFP (tracks 2 and 6), 4 μg of "reconstituted" AFP (track 3), 4 μg of Peak 1 (track 4), 4 μg of Peak 2 (track 5) and 4 μg of "repurified" AFP (track 7).

and co-migrated with the "slow" AFP variant, but Peak 2 was heterogeneous, though highly enriched in a species co-migrating with the "fast" rat AFP variant. Peak 2 and "repurified" AFP contained a faint band which may correspond to a small proportion of AFP polymers (less than 10% of the protein), having a similar relative mobility to that of albumin polymers relative to albumin. The proportions of "slow" and "fast" variants in each AFP sample, determined by gel scanning, are shown in Table 1 and are in accordance with those previously reported [9, 10]. No difference was observed, either in the mobility or in the proportion of protein in each band, after electrophoresis of the rat AFP samples in the presence of estradiol-17β (not shown).

Binding parameters of rat AFP and the fractionated peaks for estrone and estradiol-17β

The binding parameters (number of sites and apparent intrinsic affinity constant) for estrone and estradiol of the various AFP fractions were calculated for the model of one class of equivalent sites plus non-specific binding. The results are shown in Table 2 and two typical Scatchard plots, for the binding of estradiol-17β by "native" AFP and Peak 1, are shown in Fig. 2. The non-specific binding in all samples was 0.05–0.10. Calculations using a model for two classes of sites plus non-specific binding indicated a very small fraction ($n < 0.1$) of higher affinity sites, but an F-test showed the curve fit was not statistically better than with the model for one site class.

The binding results confirmed that, under our experimental conditions, unfractionated "native" rat AFP strongly binds estradiol and estrone with an affinity of 2–3 × 10^7 M^{-1}, but at a fractional number of sites (mean number of estrogen sites = 0.61), possibly indicating functional heterogeneity among AFP's isoforms [2, 11]. In contrast, the "repurified" AFP, from the first anion-exchange FPLC chromatographic step, showed a significant increase in estrogen-binding sites which approached unity (mean number = 0.95). Retention on the FPLC column of

Table 2. Binding parameters for estrone and estradiol-17β of charcoal-extracted rat AFP at different steps of its (re)purification and fractionation

AFP samples	n	$k \times 10^7$ (M^{-1})	NSB	P
		Estrone		
"Native"	0.60 ± 0.02	2.74 ± 0.11	0.10 ± 0.03	<0.05
"Repurified"	0.87 ± 0.01	1.75 ± 0.05	0.06 ± 0.01	<0.05
Peak 1	0.98 ± 0.02	1.76 ± 0.07	0.07 ± 0.02	<0.10
Peak 2	0.92 ± 0.02	2.62 ± 0.07	0.07 ± 0.02	<0.05
"Reconstituted"	0.85 ± 0.02	2.01 ± 0.06	0.03 ± 0.01	<0.05
		Estradiol		
"Native"	0.61 ± 0.01	1.83 ± 0.06	0.07 ± 0.01	<0.10
"Repurified"	1.12 ± 0.03	0.90 ± 0.03	0.07 ± 0.02	<0.05
Peak 1	0.81 ± 0.03	1.10 ± 0.05	0.05 ± 0.02	<0.05
Peak 2	0.84 ± 0.03	1.09 ± 0.05	0.07 ± 0.02	<0.10

n and k represent respectively the number of binding sites and apparent intrinsic association constant of the high-affinity binding sites. NSB is the non-specific binding and P gives the significance level of the fit.

some isoforms of "native" AFP, not binding estrogens, is unlikely since the protein recovery from the FPLC purification was 95%, while over 30% of the protein would have to be removed in order to explain this increase.

After fractionation of the "repurified" AFP in FPLC step 2, the two main isoforms, Peaks 1 and 2, both present the same number of high-affinity sites for estrogens as the "repurified" protein: mean numbers = 0.89 (Peak 1) and 0.88 (Peak 2). Therefore the two main isoforms (i.e. the "slow" and the enriched "fast" variants) appear homogeneous with respect to their estrogen binding site numbers.

The apparent association constants of the "repurified" AFP and of the two isoforms were slightly modified compared to those of the "native" AFP. The alterations in site numbers and affinities for the two estrogens can be summarized in terms of changes in binding capacity ($n \cdot k$) and these are shown in Fig. 3.

The numbers of sites of the "repurified" and "reconstituted" AFP (Table 2) also exclude the possibility that "native" AFP's fractional site number represents negative modulatory interactions between the isoforms. The results obtained for the "reconstituted" AFP are those to be expected from the binding parameters of the individual components, weighted by their proportions in the mixture.

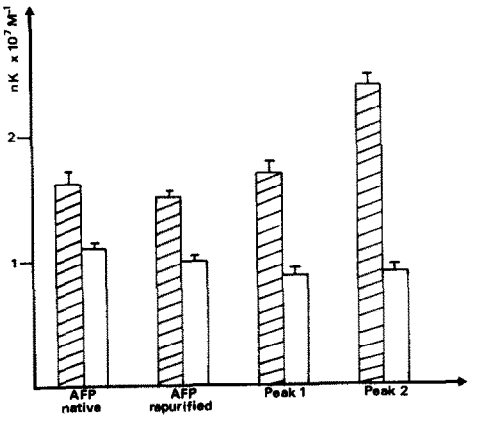

Fig. 3. The binding capacity ($n \times k$ l/mol) of "native" and "repurified" AFP, and its charge variants, for estrone (hatched bars) and estradiol-17β (open bars). Standard deviations were calculated from those of the two binding parameters (n, k) using the standard equation. The binding capacity of Peak 2 AFP for estrone is significantly higher than that of the other preparations ($P < 0.001$).

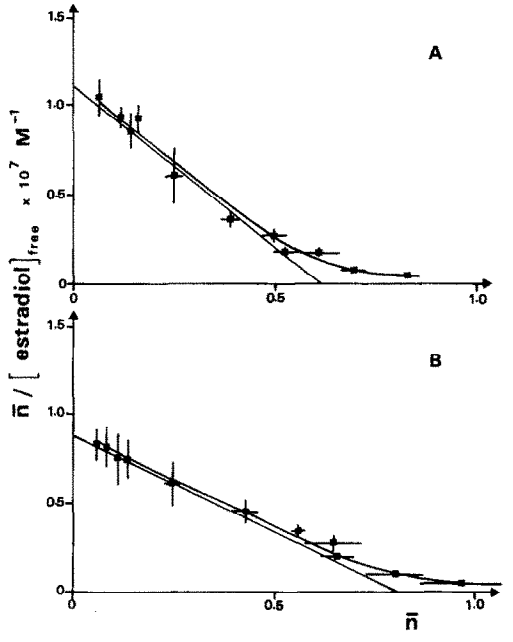

Fig. 2. Scatchard plots of the binding of estradiol-17β by "native" rat serum AFP and Peak 1. The binding of estradiol-17β (0.04–4 μM) by 0.5 μM charcoal-extracted "native" rat AFP (A) and AFP Peak 1 (B) are shown. Equilibrium dialyses between two 80 μl volumes were carried out at 4°C in 5 mM-sodium phosphate buffer, pH 7.4, containing 10% (by vol) ethanol for at least 24 h. The calculated binding curve fits are shown as are the theoretical straight lines for binding after subtraction of non-specific binding. The binding parameters calculated from the curves are presented in Table 2. n = estradiol-17β_{bound}/[protein] is the relative binding saturation and estradiol$_{free}$ is the concentration of the free hormone. Each point is the mean result of four triplicate experiments.

The electrophoresis of "native" AFP and the fractionated peaks in the presence of estradiol (not shown) showed no changes in protein mobility (no formation of a high fraction of dimers...) and this was further evidence against such modulatory interactions involving estrogens and the AFP isoforms.

Spectrophotometric measurements of AFP preparations

The molar absorption coefficients (ϵ $M^{-1} \cdot cm^{-1}$) at 278 nm of all the AFP preparations are shown in Table 1. Repurification of "native" AFP by FPLC leads to a large (30%) decrease of its molar absorption coefficient. The fractionation of "repurified" AFP leads to a further, smaller decrease for the isoforms. The overall value of decrease is shown by the molar absorption coefficient of the "reconstituted" AFP. The molar absorption coefficients of the "native" and "repurified" preparations found by dry weight determination [14] agree with those obtained by the Lowry method [13] to within 3%.

DISCUSSION

The results indicate that the increase in the number of estrogen binding sites originates in the first ion-exchange chromatographic step since "repurified" AFP, unlike the "native" protein, has almost one high-affinity site for the estrogens. Overall, these binding results show that there is no heterogeneity in the mean number of estrogen binding sites of rat serum AFP isoforms corresponding to their structural heterogeneity: the two major "slow" and "fast" variants both have almost one high-affinity site for the estrogens.

Negative modulatory interactions between isoforms were not the source of estrogen site fractionality in the "native" protein, as shown by comparison of the binding parameters of the "reconstituted" preparation with those of the "repurified" AFP and isoforms, as well as the results from electrophoresis of the preparations in the presence of estradiol.

The increase in the number of sites following FPLC purification could mean the FPLC column removes (an) endogenous inhibitor(s) of estrogen binding not previously removed by the charcoal treatment of "native" AFP. Furthermore, the relatively large decrease (30%) in the molar absorption coefficient at 278 nm following the first FPLC step is also consistent with the removal of some substance(s), which absorb in the u.v., from the "native" AFP preparation by the chromatography. This/these chromophore(s) may or may not be the inhibitor. The further smaller decrease of the molar absorption coefficient, following the second FPLC step, would represent the removal of residual endogenous chromophore(s) from the protein by the highly positively-charged column.

These results are not necessarily in contradiction with the finding by Aussel[6] and Aussel and Massayeff [7] that rat amniotic fluid AFP composed of the same isoforms as the serum protein [15], has one high-affinity site for estrogens, since any inhibitor(s) of estrogen binding may be absent from amniotic fluid. Further, Benassayag *et al.*[5] reported that after AFP separation by preparative electrophoresis, the "slow" variant of rat serum AFP bound estrogens at approximately one site while the "fast" variant had almost no such sites. This suggests that the inhibitor(s)—not removed during that electrophoresis and not affecting the protein's mobility—is/are only bound to the "fast" isoform.

It is known that during ion-exchange chromatography, interactions between the protein and the gel generally induce small conformational changes of the protein. We have shown that rat serum AFP is very flexible [16] and Zizkovsky *et al.*[17] have shown that different conformations coexist for AFP. Consequently the removal of endogenous ligand(s) from "native" AFP could be facilitated by such conformational changes during FPLC.

The increase in the number of sites is generally accompanied by a slight decrease in the estrogen affinity of the "repurified" AFP and isoforms. While an alteration in AFP's conformation would explain the slight decreased affinities after removal of inhibitor(s), this is not consistent with the increased affinity of Peak 2 for estrone. As it can be seen from Fig. 3, the presence or absence of the inhibitor on AFP has little effect on its binding capacity for estradiol, but its removal leads to an increased binding capacity of Peak 2 ("fast" variant) for estrone. In a previous work we have shown that the estrogen D-ring, which differentiates estrone from estradiol, is important for binding by rat AFP [2]. Physiologically the existence of an isoform for which an inhibitor affects the binding capacity for estrone, but not estradiol, may be important given that circulating estrone concentrations in the rat fetus are some 10 times those of estradiol-17β [18].

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