BINDING SPECIFICITY OF ESTROGENS AND NORANDROGENS TO RAT α-FETOPROTEIN (AFP)

Vera VERSÉE and André O. BAREL

Algemene en Biologische Scheikunde, Vrije Universiteit Brussel, 65 Paardenstraat, B-1640 Sint Genesius Rode, Belgium

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1. Introduction

Most of the recent estrogen binding experiments with rat α-fetoprotein (AFP) were considered and realized in relation with the well-known microheterogeneity of this protein. Some differences were found [1] in binding capacity of the various forms, while essentially identical binding properties for the iso α-fetoproteins were reported [2–4]. There are only scant reports concerning the binding of this fetal protein with non-steroid ligands. The presence of fatty acids on human AFP has been shown [5], a bilirubin binding site on AFP revealed [6] and interaction between aflatoxin B_1 and AFP reported [7].

Binding specificity studies of AFP with various steroids are necessary since they provide a better insight in the biological role of this carcino-fetal globulin [8,9] particularly in comparison with the rat uterus estradiol receptor [10]. Up to now very few quantitative data have been collected for non-estrogen steroids [2,11,12]. A large number of steroids has been investigated [13] by studying the diminution of the fluorescence of a dye bound to the protein (1-anilino-naphthalene-8-sulfonate). However, this last method implies that competition exists between the dye and the steroid for the same site.

We describe here the displacement experiments of bound radiolabelled estradiol with 18 steroids (estrogens and non-estrogens). The displacement data were examined using a simple linear analysis in order to obtain binding constants between AFP and the considered steroids. The obtained results provide substantial evidence that the steroid does not necessarily have to contain the estratriene skeleton in order to obtain binding with AFP. A strong binding (binding constant 10^7–10^8 M^{-1}) of some 19-norandrogens to AFP was also observed.

2. Materials and methods

2.1. *Chemicals*

[2,4,6,7-^{3}H]Estradiol was purchased from the Radiochemical Center, Amersham. Unlabelled steroids were periodically tested for purity by thin-layer chromatography. Steroids number 1,4,5,9,11,12,14,15 and 17 (see table 1 for a complete list of steroids) were obtained from Sigma Chemical Co., St Louis. Steroids number 3,6,7,8,16 and 18 were kindly provided by Dr Zeelen, Organon, Oss. Steroids number 2 and 10 were kindly provided by Dr Pons, University of Montpellier. Finally steroid number 13 was a gift of Dr Leclercq, University of Brussels. Blue Sepharose was purchased from Pharmacia, Uppsala. All the other reagents were reagent grade.

2.2. *Purification*

Rat AFP was isolated from the amniotic fluid and the fetal serum of Wistar rats by preparative slab gel electrophoresis on polyacrylamide as in [2]. Before electrophoresis, the crude AFP solution was passed through a Blue Sepharose C1-6B column in order to selectively remove most of the albumin fraction.

2.3. *Binding Experiments*

The association constant for the estradiol-AFP interaction was obtained by equilibrium dialysis with Sephadex G-25, at pH 8.5 and 25°C, in a batchwise

fashion as in [2,14] using radiolabelled estradiol. In order to determine the interactions of unlabelled steroids with AFP, displacement experiments of bound radiolabelled estradiol were carried out. A constant amount of [^{3}H]estradiol (10 or 50 pmol) and AFP (150 pmol) was added to each tube containing a constant weight of Sephadex G-25 in buffer, pH 8.5. Various quantities of unlabelled steroid were added (from 50 7000 pmol) in order to displace the bound [^{3}H]estradiol. From the radioactivity of the external volume, the amount of labelled estradiol bound to AFP was calculated as in [15]. The residual binding was plotted against the total amount of added steroid. The quantity of steroid necessary to obtain, for example, 20% displacement was then graphically determined. As a result of the mass law, the equilibrium relationship between estradiol and the steroid for a single binding site is given by the following equation:

$$\left(\frac{F}{B}\right)_A = \frac{K_{ES}}{K_A}\left(\frac{F}{B}\right)_{ES}$$

where B = bound, F = free, K = association constant. The subscripts A and ES refer to steroid and radiolabelled estradiol, respectively. The ratio of the intrinsic association constants, K_A/K_{ES} may be considered as an index of competition. If K_{ES} is known, the value of K_A can be readily calculated from the slope of the linear plot, when $(F/B)_A$ is plotted in function of $(F/B)_{ES}$. The advantage of this equation is to obtain in a very convenient graphical analysis the ratio of the binding constants. As an example of this plot, fig.1 illustrates the displacement of bound [^{3}H]estradiol by 3-acetylestrone and 11α-hydroxyestrone.

3. Results and discussion

3.1. *Binding data with estradiol*

As described [2], rat AFP binds 17β-estradiol maximally at pH 8.5 with an association constant of $5 \pm 1 \times 10^7\ M^{-1}$. The Scatchard plots always gave straight lines suggesting that AFP displays only a single class of binding sites (stoichiometry of binding 0.9 ± 0.1).

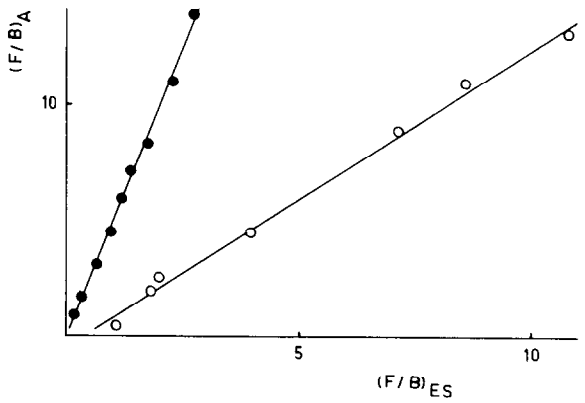

Fig.1. Competition for the estradiol binding site of AFP. Displacement of bound [^{3}H]estradiol with 3-acetylestrone (○—○) and 11α-hydroxyestrone (●—●). Data are presented according to the equation in section 2.3. The free/bound for the steroid $(F/B)_A$ is plotted against the free/bound for estradiol $(F/B)_{ES}$.

3.2. *Binding data with the other steroids*

In order to determine the interactions between AFP and several other ligands, experiments of displacement of tritiated estradiol were carried out. Table 1 summarizes the interference of 18 derivatives with respect to estradiol. For convenience, the ligands were classified as:

(i) Compounds which compete successfully with estradiol;
(ii) Compounds which compete very poorly;
(iii) Those which have no significant displacement capacity.

However, for the last two types of compounds, a 50% inhibition was not reached at saturating concentrations and therefore no competition index could be calculated.

First the identification of the determinant regions of the estratriene skeleton for binding with AFP was considered. As essential factors for estrogen binding we pointed out that addition of a hydroxyl group at position C_{10} (derivatives 11 and 12) or alkylation of the 17β-hydroxyl group (derivative 10) or a change in orientation of the C_{17} hydroxyl group (derivative 14) result in an extensive decrease in affinity for AFP. On the other hand modification at position C_3 (alkylation of the hydroxyl group, derivative 2) or introduction of a hydroxyl group at position C_{11} (comparison between estrone and 11α-hydroxyestrone, derivative 3)

Table 1
Competition between tritiated estradiol and various steroids for binding on AFP

Steroid	Number of derivative	Steroid, pmol added in order to obtain 20% displacement	Competition index K_A/K_{ES}	Binding constant (M^{-1})
(i) Compounds which compete				
17β-Estradiol	ref.	127	1.00	5×10^7
Estrone	1	107	1.85	9×10^7
3-Acetylestrone	2	129	0.95	5×10^7
11α-Hydroxyestrone	3	213	0.33	2×10^7
Equiline	4	606	0.08	4×10^6
17α-Ethinylestradiol	5	940	0.05	3×10^6
19-Norandrost-4-ene-17β-ol	6	92	5.55	3×10^8
19-Norandrost-4-ene-17α-ol	7	775	0.06	3×10^6
19-Norpregn-4-ene-20S-ol	8	125	1.04	5×10^7
19-Nortestosterone	9	1306	0.04	2×10^6
(ii) Compounds which compete poorly				
17β-Acetylestradiol	10	2000	–	–
16α,17β-Estriol	11	3500		–
16β,17β-Epiestriol	12	3500	–	–
17β-Estradiol-6-one(*O*-carboxy-methyl)oxime	13	4360	0.01	6×10^5
17α-Estradiol	14	7000	–	–
(iii) Compounds which do not compete				
17β-Testosterone	15	–	–	–
17α-Testosterone	16	–	–	–
Androstadiene-3,17-dione	17	–	–	–
Androst-4-ene-17β-ol	18	–	–	–

does not modify extensively its binding with this fetal protein. Finally, modification of the estratriene skeleton at position C_6 (addition of a ketoxime group, derivative 13) or introduction of a C_7–C_8 double bond (comparison between estrone and equiline, derivatives 1 and 4) result in an important decrease in affinity. However, these steroids still show some displacement capacity. Therefore, a major factor in the AFP–estradiol interactions resides in the integrity of the β-hydroxyl configuration (change of configuration or alkylation). This hypothesis does not agree with [13] where it was concluded that AFP was very little influenced by modification of the estrogens at C_{17}. The synthesis of appropriate specific affinity labels for AFP should preferentially involve modifications of the estratriene skeleton at positions C_3 or C_{11}. For example, the synthesis of an estrone-3-monohemisuccinate–Sepharose gel would be a more efficient affinity adsorbent for the purification of AFP as the 7β-derivative of estradiol used in [18,19].

According to the well-known estrogenic binding properties of AFP, the importance of an aromatic A ring among the factors intervening in steroid recognition was emphasized [13]. We observed a virtual lack of displacement capacity for all the chosen androgens with a C_{19} methyl group (derivatives number 15–18). On the other hand the nor-derivatives of testosterone and androst-4-ene-17β-ol (derivatives number 6–9) are very effective in displacing bound estradiol. Particularly striking is the comparison between androst-4-ene-17β-ol which has no displacement capacity and its 19-nor derivative which is characterized by an inhibition constant of 3×10^8 M^{-1}, greater than that of estradiol. It may also be noted,

similarly to that observed for the 17α- and β-isomers of estradiol, that the β-enantiomer of 19-norandrost-4-ene-17-ol is much more strongly bound to AFP than the α-enantiomer.

This study clearly shows the high affinity of some 19-norsteroids for the estradiol binding site of AFP. The addition of a C_{19} methyl group prevents the binding of the androgen. In disagreement with the hypothesis defended [13], it is suggested here that AFP interacts strongly with 19-norandrogens where the A ring is no longer aromatic. As a consequence it would be of interest to devise affinity labels starting from the skeleton of 19-norandrogens, since these compounds are generally much more soluble in water than estrogens.

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