BINDING OF ESTROGENS TO MOLECULAR VARIANTS OF RAT ALPHA-FETOPROTEIN

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1. Introduction

In previous reports [1,2], we have demonstrated the occurrence of two molecular variants of rat AFP separable by electrophoresis in acrylamide gels of low porosity. This finding was confirmed by several authors [3–6]. Others [7,8] developed a method of affinity on concanavalin A–Sepharose leading to the separation of two other forms of rat AFP.

We also demonstrated that the binding capacity of rat AFP in one estrogen/AFP molecule. This suggests that the four molecular variants have the same capacity. This finding was confirmed by Soloff et al. [9], while Benassayag et al. [5] concluded that only one variant was able to bind estradiol. This paper reports the results of binding experiments performed with variants isolated both by the electrophoretic and concanavalin A methods.

2. Materials and methods

2.1. *Chemicals*

[2,4,6,7-^{3}H]Estradiol and estrone were purchased from the Radiochemical Center, Amersham, England. Unlabeled steroids were obtained from Roussel-UCLAF, France and periodically tested for purity by thin layer chromatography.

Concanavalin A and Sepharose 6B were obtained from Pharmacia, Upsalla, Sweden.

Amniotic fluid was obtained as previously described [1,2] and treated with charcoal Norit A in order to remove endogenous steroids.

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All binding experiments were made by equilibrium dialysis as described in previous reports [1,2].

2.2. *Preparative electrophoresis in acrylamide gels*

Preparative electrophoresis was performed in acrylamide–agarose gel plates 1 cm thick, 12 cm high, 25 cm wide in multiphor LKB apparatus. Gels contained 11% acrylamide monomer, 1% agarose and 0.2% bisacrylamide in 50 mM Tris–HCl buffer, pH 8.2.

Polymerisation was induced at 56°C by Temed and ammonium persulphate as catalyst. Electrophoresis was run at 10 V/cm for 18 h. After electrophoretic separation, two lateral and one central guide strip were cut and stained with amido black. The areas corresponding to the two AFP variants were cut and homogenized in KH_2PO_4–NaOH buffer, pH 6.0, then filtered through a Sartorius filter (0.2 μm pore size).

2.3. *Affinity chromatography*

A column 2 cm diameter, 6 cm hight, was filled with Con A coupled to Sepharose 6B. Coupling was done by the CNBr method [10]. The column was equilibrated with 0.1 M acetate buffer, pH 6.0, containing 1.0 M NaCl, 10^{-3} M $CaCl_2$, 10^{-3}M $MgCl_2$ and 10^{-3} M $MnCl_2$. Amniotic fluid was applied to this column with or without preincubation in 10^{-9} M [^{3}H]estradiol. Elution was made by adding 0.1 M glucose in the buffer. The eluate was monitored at 280 nm, 5 ml fractions were collected and tested for AFP by double immunodiffusion, using a specific antiserum against rat AFP.

When amniotic fluid was preincubated with [^{3}H]-estradiol, an aliquot of each fraction was counted in a Packard liquid scintillation spectrometer using Packard Instagel as scintillation fluid.

2.4. *Neuraminidase treatment*

Amniotic fluid (1 ml), was dialysed against acetate buffer, pH 5.0 and incubated for 1 h at 37°C with 100 μl of neuraminidase (Koch-Light Laboratories, 500 U/ml).

3. Results

As previously described, 11% acrylamide electrophoresis resulted in the separation of two AFP molecular variants.

Eluates from the gels were diluted to a concentration of 0.25 μg/ml AFP, and studied by equilibrium dialysis, using [^{3}H]estrone and various concentrations of unlabeled steroids. The data were displayed in Scatchard plots. Figure 1 shows that the two AFP electrophoretic variants bind estrone with the same association constant K_a 3.0 × $10^8 M^{-1}$. This value was very similar to the figure obtained with whole pure AFP or amniotic fluid [2]. Since the AFP concentration was 3.5 × 10^{-11} M and the concentration of binding (as determined by the Scatchard plot) was 4.1 × 10^{-11} M, it may be calculated that the number of binding sites by AFP molecule is 1.17, i.e., close to one. This result is in good agreement with values for whole AFP previously reported by ourselves [2] and by Savu et al. [11]. However, the Scatchard curves (fig.1) were not linear, suggesting the presence of a second binding system of lower affinity. This was not previously found with AFP from amniotic fluid. A biphasic curve, but of a different shape, suggesting a second site of higher affinity, was also shown by Vallette et al. [12] using AFP from rat serum. These facts remain unexplained and the possibility of artifact due to the purification procedure is under study.

Affinity chromatography on Con-A–Sepharose allowed the separation of AFP into two variants called AFP Con-A$^+$ and AFP Con-A$^-$, according to their affinity for Con-A. Figure 2 shows a typical separation of these two variants.

A first peak appears in the void volume and a second one is obtained when 0.1 M glucose is infused in the column. AFP assay showed that approx. two-thirds of AFP directly passed through the column, while one-third was adsorbed and eluted with glucose.

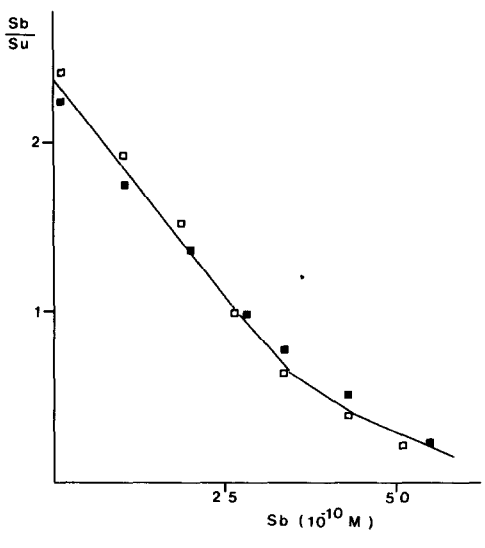

Fig.1. Scatchard plot obtained with the low (□—□) and the fast (■—■) variants of rat AFP separated by electrophoresis on low porosity acrylamide gels. The binding [^{3}H]estrone was measured in the presence of various concentrations of unlabeled estrone. AFP concentration was 0.25 μg/ml. K_a determination leads to a figure of 3.0 × 10^8 M^{-1}.

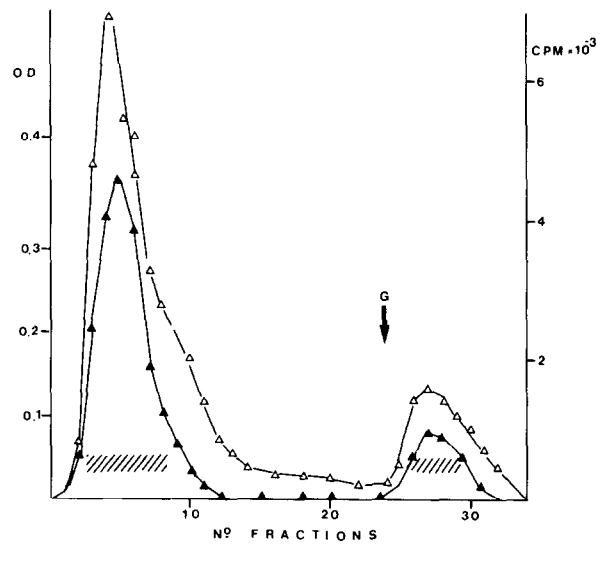

Fig.2. Separation of two variants of rat AFP on Con-A–Sepharose Amniotic fluid was preincubated with [^{3}H]estradiol. Optical density (▲—▲) and radioactivity (△—△) are represented. The presence of AFP in fractions determined by double immunodiffusion is shown as hachted zones. The arrow indicates the addition of glucose in the elution buffer.

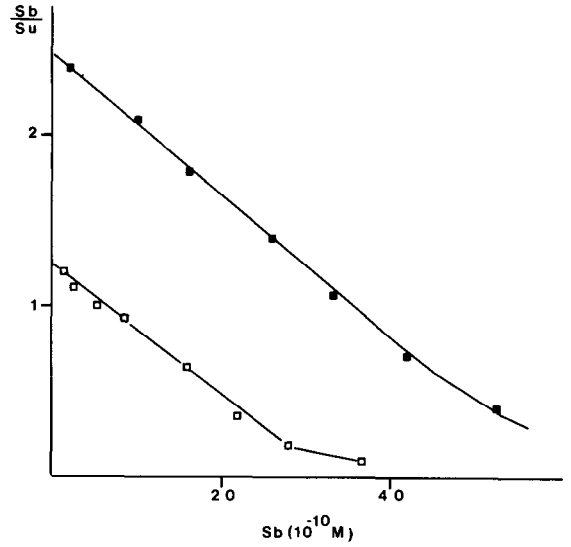

Fig.3. Scatchard plots obtained with AFP–Con-A$^+$ (□—□) and AFP–Con-A$^-$ (■—■) by equilibrium dialysis using [^{3}H]estrone and various concentrations of the unlabeled steroid. The slopes lead to values of K_a of 4.0 and 4.1 × 10^8 M^{-1}, respectively. AFP concentration was 0.20 and 0.45 μg/ml.

When AFP was incubated with [^{3}H]estradiol prior to separation, we obtained two radioactive peaks superimposable to the peaks of optical density at 280 nm and to the presence of AFP as detected by immunodiffusion.

Equilibrium dialysis experiments (fig.3) showed that association constants with estrone were 4.1 × 10^8 M^{-1} for AFP Con-A$^-$, and 4.0 × 10^8 M^{-1} for AFP–Con-A$^+$. Since the concentration of binding sites as determined by Scatchard plots were 3.0 × 10^{-9} M for the Con-A$^+$ variant and 6.0 × 10^{-9} M for the Con-A-one, the concentration ratio of the variants Con-A$^+$/Con-A$^-$ is of 0.5. This ratio obtained by the estradiol binding experiment is identical with the one obtained from Con-A–Sepharose elution data. This strongly suggests that those two variants of AFP have the same binding properties.

Treatment of AFP by neuraminidase does not modify estrogen binding, a result which indicates that carbohydrates are probably not involved in the steroid binding site.

Finally, our results agree with the data of Soloff et al. [9] and do not support the contradictory conclusion [5] that some AFP variants may differ from others in their estrogen binding properties. Nevertheless, it is interesting to note that Benassayag et al. [5] purified AFP variants from sera while Soloff et al. [9] and ourselves, in this work, did it from amniotic fluid. A structural difference in the AFPs obtained from these two different fluids may be advocated to explain their different binding properties; however, there still is no experimental evidence to support this hypothesis.

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