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RAT ALPHA-FETOPROTEIN–ESTROGEN INTERACTION

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SUMMARY

The influence of temperature, pH, ionic strength and sulphydryl reagents on the binding of estrogen to alpha-fetoprotein was studied. Equilibrium dialysis experiments showed that under optimal conditions, the association constant is high ($K_a = 3 \times 10^8 M^{-1}$) and one binding site per alpha-fetoprotein molecule was demonstrated. This binding was shown to be specific for estrone and estradiol. Anti-estrogenic compounds are not bound by alpha-fetoprotein. Rat alpha-fetoprotein appears to differ by several functional differences from uterine estrogen receptors.

INTRODUCTION

Alpha-fetoprotein (AFP), a fetospecific serum protein, is synthetized by the liver and secreted into the plasma during fetal and early post-natal life. Since the original observation of the reappearance of AFP in the plasma of adults bearing primary liver cancer, this protein has been considered a tumor associated antigen [1].

More recently, AFP of rats and mice has been demonstrated to possess a high affinity for estrogens [2, 3, 4]. In the past three years, AFP has been shown to be present in rat uterine cytosols [5, 6] and Uriel *et al.* have suggested [7] that AFP is the major binding protein in rat uterus. A better knowledge of the characteristics of AFP-estrogen and receptor–estrogen interaction would facilitate the understanding of the relative importance of both systems.

On the other hand, Benassayag *et al.* [8] have shown that AFP has about 0.2 binding sites per AFP molecule, suggesting that some AFP molecules do not bind estrogens. The present paper deals with some physical and chemical aspects of the AFP-estrogen interaction, reconfirms that the number of binding sites is about one per molecule and confirms some fundamental differences between AFP and uterine estrogen receptors.

MATERIAL AND METHODS

Chemicals. 1,3,5(10)-estratrien-3,17β diol (estradiol); 1,3,5(10)-estratrien-3-ol-17 one (estrone); 1,3,5(10) estratrien-3, 17β, 16α-triol (estriol) 4, androsten-17β-ol-3-one(testosterone) and 5α-androstan-17β-ol-3-one (androstanolone) were obtained from Roussel-UCLAF (France).

1,3,5(10)-estratrien-3, 17β, 16β-triol (epiestriol); 1,3,-5(10)-estratrien-17-α-ethynyl-3, 17β-diol (ethynyl-estradiol); 1,3,5(10)-estratrien-3-ol-17-one-3-methyl-ester and 1,3,5(10)-estratrien-1-methyl-3-ol-17-one (1-methylestrone) were obtained from Koch Light Laboratories (England); 1,3,5(10)-estratrien-2, 3-diol-17-one (2-hydroxy-estrone) and 1,3,5(10)-estratrien-3-ol were purchased from Steraloids (U.S.A.).

1,3,5(10)-estratrien-3-ol-17-one-6-o-carboxymethyl-oxime was a gift from Dr. Condom, U.E.R. de Médecine (Nice) and 4-estren-17β-ol-3-one (nortestosterone) was a gift from Theramex (Monaco).

U-11100A or nafoxidine, 1-(2-(*p*-(3,4-dihydro-6-methoxy-2-phenylnaphth-1-yl)-phenoxy)-ethyl)pyrrolidine; Mer 25 or ethamoxytriphetol, 1-(p-2-diethyl-aminoethoxyphenyl)-1-phenyl-2-*p*-methoxypenyleth-anol; CI 628, α-(4-pyrrolidinoethoxy) penyl-4-meth-oxy-α'-nitrostilbene monocitrate; were kindly supplied by Upjohn Company, Kalamazoo (U.S.A.), Merrel Laboratories, Cincinatti (U.S.A.) and Parke Davis Company, Detroit (U.S.A.) respectively.

[2,4,6,7 H^3]-estrone, 100 Ci/mmol., was purchased from Amersham (England). Other products and reagents were from Merck (Darmstad, R.F.A.) or Sigma, St Louis (U.S.A.).

Amniotic fluid. Fifteen to twenty day old embryos were carefully removed from inscised uterii of pregnant anesthetized Wistar rats. Amniotic fluid was obtained by puncture of the amniotic sacs. The fluids were pooled, centrifuged to remove cells and stored at $-20°C$.

Isolation of AFP. AFP was isolated from amniotic fluid using an immunoadsorbant anti-AFP and subsequent elution at pH 2.8, according to the method of Nishi and Hirai [9]. Purity was tested by immuno-electrophoresis and acrylamide–agarose gel electrophoresis.

Preparative electrophoresis in acrylamide gels. Preparative electrophoresis was performed in acrylamide-agarose gel plates 1 cm thick, 12 cm high, 25 cm wide in a Multiphor LKB apparatus. Gels contained 11% acrylamide monomer, 1% agarose and 0.2% bisacrylamide in 50 mM barbital–HCl buffer pH 8.5.

Polymerisation was induced at 56°C byTEMED and ammonium persulphate as catalyst. Electro-

phoresis 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 μ pore size).

Equilibrium dialysis. In order to remove most of the endogenous steroids, 1 ml of crude amniotic fluid or 1 ml of an undiluted solution of pure AFP (1 mg/ml) was treated for 10 mn at 37°C with 5 mg of Norit A charcoal and centrifuged. One ml of 1:1000 diluted amniotic fluid or AFP solution was dialyzed (dialysis tubing 8:100′, Union Carbide Corporation) against 15 ml of 10 mM sodium phosphate buffer pH 6.0, containing a mixture of radioactive (0.05 nM) and unlabeled steroids (1.0 to 500 nM) at 4°C for 24 h. Previous experiments have shown that this time was necessary to reach equilibrium.

Each sample was dissolved in Instagel (Packard) and radioactivity was counted in a Tricarb Packard liquid scintillation spectrometer. Standard counting error was inferior to 1, p.100.

AFP Determination. AFP determination was carried out using the method of Mancini *et al.* [10]. A standard curve in the range 0.5 to 500 μg of antigen was set up with pure AFP isolated as described above.

RESULTS AND DISCUSSION

Determination of optimum pH

The binding activity of AFP was studied using estrone and estradiol between pH 2 and 10 with different buffer systems (Fig. 1). The optimum pH was 5.5–6.0, depending on which buffer was used. This value is in good agreement with the results of Raynaud *et al.* [11] showing maximum binding at a pH value close to pH 6.

Influence of ionic strength. AFP was studied by equilibrium dialysis in buffers containing NaCl or KCl in the concentration range 0 to 1 M without a significant difference.

Influence of sulphydryl reagents. Scatchard plots obtained by equilibrium dialysis in the presence of 10^{-3} M parachloro-mercuri-benzoate (PCMB) or 0.1 M Iodoacetamide or with AFP previously treated with 2.5 10^{-3} M PCMB or 1.1 M iodoacetamide at 22°C for 15 h showed no significant difference with the control. Thus, no SH group seems to be involved in the binding of estrogens to AFP.

Specificity towards steroid compounds. Thirteen steroids were studied by competition with [^{3}H]-estradiol. A binding curve was set up by measuring the amount of bound [^{3}H]-estradiol at equilibrium in mixtures containing a fixed amount of [^{3}H]-estradiol and increasing concentrations of unlabeled steroids. The concentration range studied varied from 10^{-9} to 10^{-6}M.

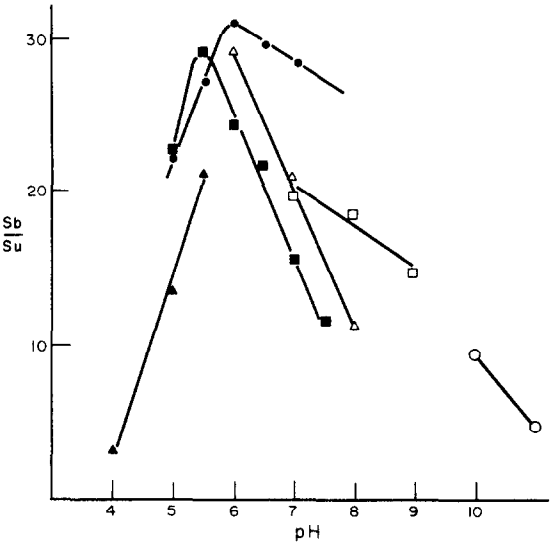

Fig. 1. Variation of the ratio bound/unbound estradiol (Sb/Su) at different pH values. *Sb/Su* was measured by equilibrium dialysis. Each point is an average of three equilibrium dialysis experiments in acetate buffer ▲——▲, potassium phosphate-NaOH buffer ■——■, phosphate buffer △——△, tris-HCl □——□, carbonate NaOH buffer, ○——○ and barbital-sodium acetate–HCl buffer ●——●. Buffer concentration was 50 mM.

Figure 2 shows the curves obtained with several different steroids. In these experiments knowing the concentration corresponding to 50% bound estradiol allows the calculation of the dissociation constant K_D for the steroid studied. The K_a values calculated are reported in Table 1. The inhibition percentage (Table 1) was compared with previous values obtained by Laurent *et al.* [12]. For some compounds, the values are similar, while for others, such as 17-desoxoestrone and nortestosterone, a very large difference was observed. These discrepancies may reflect merely the difference in techniques. In the present work, a direct competition method between estradiol and other steroids was used, while Laurent used a more indirect technique involving 8-anilino-naphthalen as fluorescent marker. AFP binds this compound with a low affinity constant ($K_a = 10^6 M^{-1}$). Consequently, estrogens were shown to compete with a large excess (10^{-6}M) of 8-anilinonaphthalen in fluorescence experiments. On the other hand, Laurent *et al.* used a pH value 8.7 which is very far from the optimum pH. This may explain a loss of specificity due to a different conformation of the protein.

The data reported in Table 1 clearly shows that:

(1) The integrity of the unsaturated ring A is a prerequisite for binding by AFP. All testosterone derivatives showed negligible affinity for AFP. Derivatives obtained by substitution on this ring showed a marked decrease in binding (1 methyl; 2 hydroxy; 3 methyl ether).

(2) Similarly, substitution on ring D also reduces the binding activity, (17-ethynyl-estradiol, estriol, 16-epiestriol, 17-desoxoestrone).

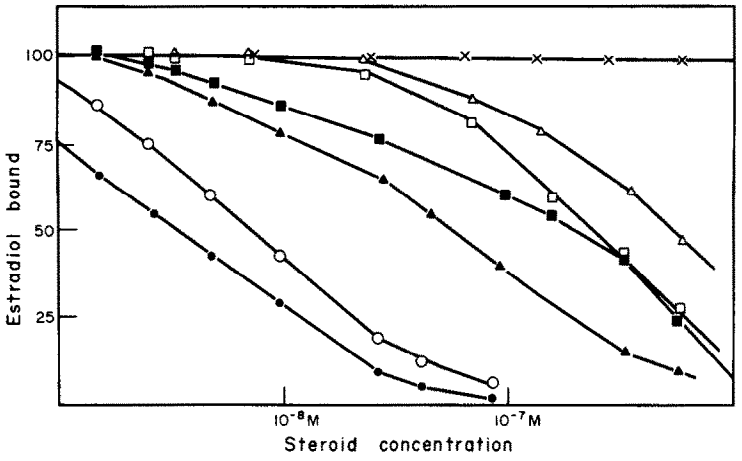

Fig. 2. Inhibition of [H³]-estradiol binding by competition with different unlabeled steroids at various concentrations. Equilibrium dialysis was performed at 4°C for 24 h. Estrone ●——●, estradiol ○——○, 1 methyl-estrone ▲——▲, 17α-ethynyl-estradiol ■——■, estriol □——□, 16-epiestriol △——△ and testosterone ×——×.

(3) 6-carboxy-methyl-estradiol showed negligible binding.

It appears from these results that the integrity of the estradiol molecule is required for its binding to AFP. Any modification of the A cycle or the D cycle results in an important loss of affinity, either by steric hindrance, or by the disappearance of the unsaturated A ring.

Specificity towards antiestrogens

Competition experiments using [³H]-estradiol and anti-estrogenic molecules showed that antiestrogens MER 25, U11-100 A and CI628 were unable to displace estradiol bound to AFP even at a concentration 1000 times greater than that of estradiol.

Estimation of binding capacity

Binding curves were constructed by measuring the amount of bound estrone at equilibrium in mixtures containing a fixed amount of [³H]-estrone and increasing amounts of unlabeled steroid. When the data were displayed in a Scatchard plot, an association constant of $3 \times 10^8\ M^{-1}$ was found. This value is similar to those reported by other workers:

$1 \times 10^8 M^{-1}$ [13, 14] and $2 \times 10^8 M^{-1}$ [11, 4].

The slight difference found may be ascribed to the use of whole serum instead of purified AFP by some authors [12, 13] and to the pH of the reactions which ranged from 7.0 to 7.4 in all the previous works. The pH was 6.0 (i.e. the optimal pH) in the present work.

Assuming a molecular weight of 72000 Daltons for AFP [4], the equilibrium dialysis method gives 0.92 to 1.05 molecules of estrone bound per molecule of AFP. Savu *et al.*[13] found the number of binding sites to be approximately one per molecule, as was the case in the present work. However, in a recent report, Benassayag *et al.* [8] calculated that the number of binding sites for estrogens was close to 0.2. Thus, they suggested that some variants of the AFP molecule may not bind estrogen.

This difference in results might be explained by the occurrence of endogenous estrogens in the sample examined by Benassayag *et al.* [8]. Their paper makes no mention of any attempt to remove the endogenous steroids, while in our experiments all samples were treated with charcoal at 37°C prior to equilibrium dialysis.

On the other hand, the calculation of the number of binding sites was based upon the determination of AFP concentration. In our work, AFP was determined by the immunological method of Mancini *et al.* [10]. Previous works by Benassayag *et al.* [8] make no mention of specific AFP determination. More recent works (unpublished) have shown that the standards used for AFP determination are largely responsible for the discrepancies obtained by different laboratories. In the past, such results with human AFP led to the preparation of an international standard [18].

Table 1. K_a values obtained in competition experiments with 13 different steroids

Compound	K_a	%A	%B
Estrone	2.8×10^8	108	90
Estradiol	1.3×10^8	100	100
17-ethynyl-estradiol	5.5×10^6	40	70
Estriol	4.0×10^6	53	42
16-Epiestriol	1.9×10^6	35	—
1-methyl-estrone	1.4×10^6	59	—
Estrone-3-methyl-ether	1.4×10^6	56	—
17-desoxoestrone	1.4×10^6	57	125
2-hydroxy-estrone	4.0×10^6	32	50
6-carboxymethyl-estradiol	1.8×10^6	5.6	—
Testosterone	—	0	0
5α-Dihydrotestosterone	—	0	—
19-Nortestosterone	1.0×10^6	4	65

%A and %B represent the percentage of inhibition of estradiol binding obtained with 100 ng of each steroid. A: in the present work and B: by Laurent *et al.* [11].

Binding of estrone to molecular variants of AFP

The two molecular variants of AFP previously described [4] were isolated by electrophoresis in low porosity acrylamide–agarose gels. Equilibrium dialysis experiments showed that the two forms are able to bind estrone with the same capacity of 1 estrone bound per AFP molecule and that they have identical association constants $K_a = 3 \times 10^8\ M^{-1}$. These results do not agree with those of Benassayag *et al.* [8] who have claimed that only the slow moving form was able to bind estradiol. They do agree, however, with those of Soloff *et al.* [15] who demonstrated that AFP variants separated on Concanavalin-A Sepharose were both able to bind estrogens.

As Benassayag *et al.* [8] purified their variants from sera while Soloff *et al.* [15] and the present authors purified it from amniotic fluid, it may be advocated that these different fluids are responsible for these discrepancies. Nevertheless, no evidence has actually been found to support this hypothesis.

Thermodynamic considerations

Thermodynamic parameters may be calculated from binding curves obtained at different temperatures (Fig. 3). The association constants determined for AFP–estrone systems are $3.0 \times 10^8\ M^{-1}$ at 5°C, $2.4 \times 10^8\ M^{-1}$ at 23°C and $1.66 \times 10^8\ M^{-1}$ at 37°C. The plot of log K versus $1/T$ gives an approximate straight line and allows the evaluation of the enthalpy. The evaluated thermodynamic constants are:

free energy variation	$\Delta F = -10.78$	kcal/mole
enthalpy variation	$\Delta H = -3.9$	kcal/mole
entropy variation	$\Delta S = +25$	cal/°C/mole

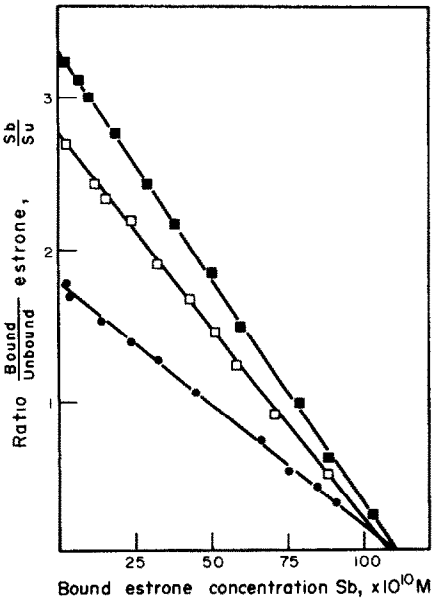

Fig. 3. Scatchard plots of binding data obtained after dialysis of AFP against estrone (see concentration in the text) for 24 h at different temperatures. 5°C ■——■, 23°C □——□ and 37°C ●——●. Each point represents the average of three determinations.

With such an association reaction, one would expect to find a reduction in the degree of freedom, i.e. a decrease in entropy, but the opposite was observed. This is reminiscent of the observations made with the hapten–antibody interaction [16]. Conversely, the binding of estradiol to uterine receptors is associated with a negative entropy change [17].

It has been suggested [16] for antibody–hapten systems that the reaction is accompanied by a release of the water of hydratation from the binding site which results in an increase in the degree of freedom. This increase compensates for any loss in entropy due to the association of the reaction parameters. Such a mechanism may be involved in the binding of estrogens to AFP but further work is necessary to elucidate this problem.

Another difference between uterine receptors and AFP lies in the stability of these proteins. AFP (like antibodies) is a very stable protein in the temperature range extending from 0 to 40°C. This is not true for uterine estrogen receptor which are known to be very labile proteins. For uterine estrogen receptors [16], the K_a decreases twenty fold between 0 and 37°C, while for AFP, the decrease in the same range of temperature is only two fold. This fact may be responsible for the apparently great difference in ΔH and ΔS for those two proteins.

CONCLUSIONS

In conclusion, this work confirms the results of previous studies showing the high affinity of rat AFP towards estrone and estradiol, and that it is in the same range as that of other hormone transporters. However, its affinity constant is inferior to that of rat uterine receptors (K_a being $3 \times 10^8\ M^{-1}$ and 10^9 to $10^{10}\ M^{-1}$ respectively).

Rat AFP differs from uterine receptors by at least three functional features, i.e. its better resistance to temperature variation; its narrow specificity restricted to estrone and estradiol as opposed to that of estrogen receptors which are able to bind several estradiol derivatives and anti-estrogens; its overall large quantitative binding capacity due to the high concentration of AFP in the fetal body fluids.

Finally, our experiments demonstrate that the number of estradiol binding sites per molecule of AFP is very close to 1. This was confirmed by the binding studies on the two AFP variants separated by electrophoresis. Our results do not support the contention (8) that some AFP variants may differ from others in their binding capacities.

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