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EXISTENCE OF MULTIPLE SITES FOR ANS IN AN ALPHA-FETOPROTEIN FRACTION DEMONSTRATION BY FLUORESCENCE POLARIZATION

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INTRODUCTION

Rat alpha-fetoprotein, a specific fetal glycoprotein, has been purified (1-2) and shown to consist of a family of isoproteins (3). A specific function already assigned to this protein is estradiol binding (4) but its close resemblance to rat plasma albumin (5) suggests that alpha-fetoprotein could also be involved in binding and transport of hydrophobic anions. Polyacrylamide electrophoregrams of fetal serum show that only two proteins bands, those of AFP and rat albumin, are stained with ANS giving intense fluorescence. This observation suggested the use of ANS to titrate the binding sites and determine some of their properties in the AFP, just as has already been done with albumin (6). In particular, three points were investigated : stoechiometry of affinity of the ANS-AFP complexes, detection of multiple binding of ANS by the AFP molecules through the depolarization of the fluorescence as the number bound increases and competition for sites between ANS and estradiol.

MATERIAL AND METHODS

The purification of the alpha-fetoprotein has already been described (1). Rat albumin (Sigma), and ANS ammonium salt from Pierce, and estradiol(Roussel Uclaf) were employed.

Absorption spectra were measured with a Perkin Elmer 402 Spectrophotometer, emission spectra and intensities with a Fica MK2 spectrofluorimeter, fluorescence polarization with an SLM polarization photometer.

Abbreviations: AFP, alpha-feto protein; ANS, anilinonaphtalene sulfonate; BSA, bovine serum albumin.

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RESULTS

Binding parameters

The fluorescence intensity of ANS bound to alpha-fetoprotein I_{max}, was determined by addition of increasing quantities (concentrations 0.2μM to 4μM) of AFP to 1.0μM solution of ANS in 0.05M phosphate buffer, pH 7. Fig. 1 shows the fluorescence intensities observed at the increasing ratios AFP/ANS. For comparison an identical titration with bovin serum albumin is shown. The ratio of I_{max}, to the fluorescence of an equal concentration of ANS in absolute ethanol, I_{max}/I_{eth} was 1.45 $\pm$ .05. The curves of Fig. 1 show that four molar equivalents of ANS are bound by BSA but only 0.63 are bound per mole of AFP (M.W. 64.000).

The average dissociation constant of the AFP-ANS complexes was obtained by determination of the fraction of ANS bound, when increasing amounts of this were added to 1.0 M solution of AFP. The results are shown in Fig. 2 by means of a Bjerrum plot (7) in which the logarithm of free ANS concentration is plotted against $\bar{n}$ the number of moles of ANS bound per mole of total AFP. The saturation towards $\bar{n}$ = 0.65 is clearly shown. The figure shows besides the experimental points two theoritical curves centered at log (ANS) = -6.15. The steeper curve-1- of the two corresponds to a cooperative bin-

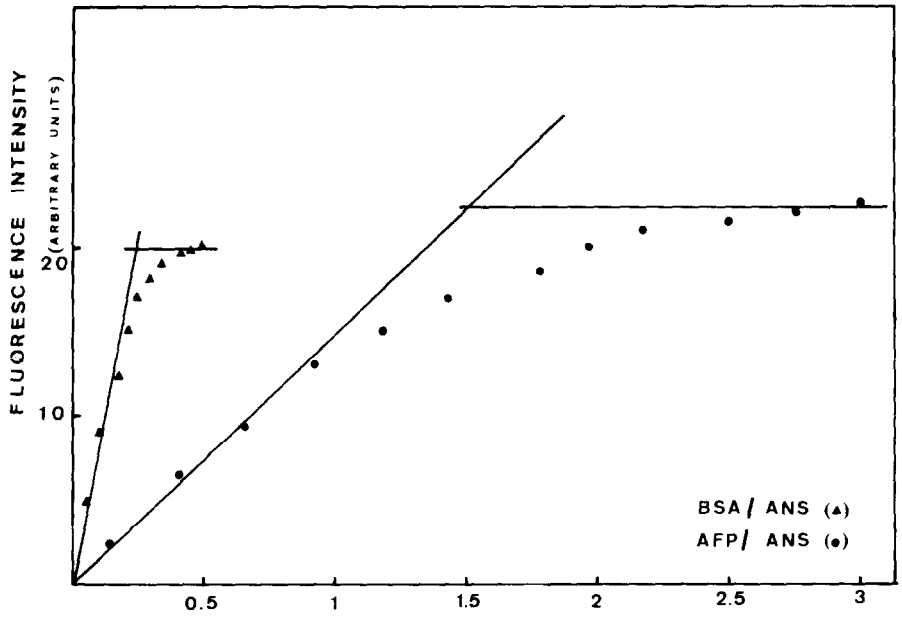

Fig. 1 Saturation curves for ANS (1.0μM) with increasing amounts of AFP (● ●) and bovin serum albumin (▲ ▲).
Phosphate buffer (0.05M) pH7 is used.
Excitation wavelength 370nm, emission wavelength 470nm.

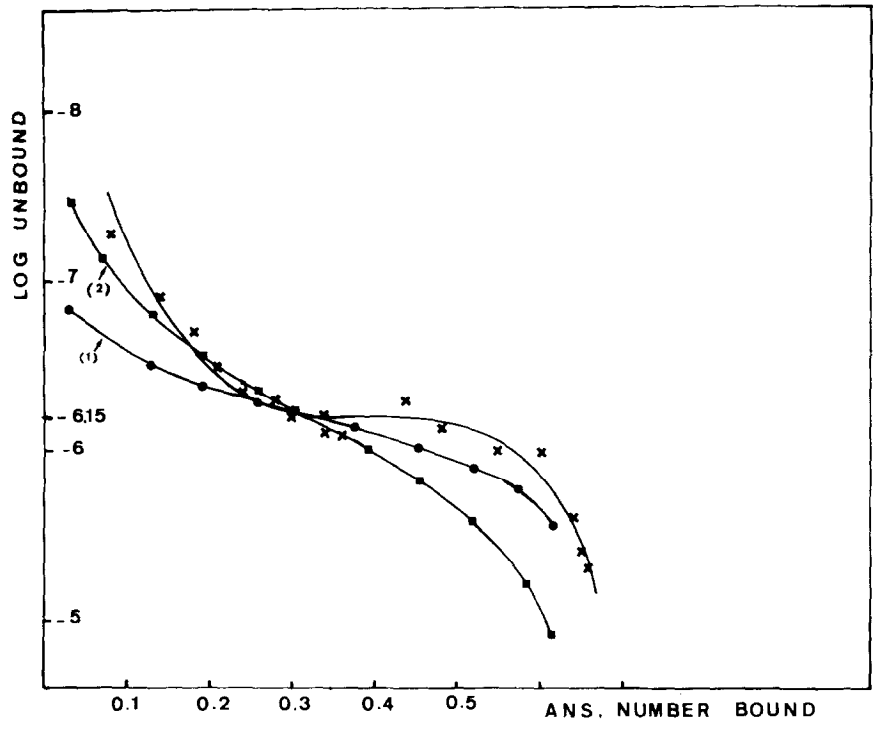

Fig. 2 Titration curve for AFP by ANS (× ×)
Increasing amounts (1μM to 10μM) of ANS are added to a 1.0μM solution of AFP. The bound ANS is given from the ratio $I_{max}/I_{eth.} = 1.45$ where I_{max} and I_{eth} are the intensities of fluorescence of 1μ mole of ANS totally bound to the protein and in ethylic alcohol respectively. The curves 1 and 2 are theoretical curves calculated with Hill coefficients of 2 and 1 respectively.

ding with a Hill coefficient of 2. The less steep curve-2- corresponds to independant binding sites (Hill coefficient = 1). It appears that at low degrees of saturation binding the experimental titration curve is close to that of independant sites but at higher degrees of saturation cooperative interaction among the sites is evident. The causes of this behaviour may be too complex for analysis with the present method but on any plausible model it would require as a minimum three binding sites per molecule of protein. The dissociation constant calculated is $K_D = 7 \pm 0.5 \times 10^{-7}$ M. This figure is quite close to the average dissociation constant of BSA-ANS complexes, 1×10^{-6}M.

Multiple sites for ANS in alpha-fetoprotein

The fractional number of ANS bound by AFP and the complex titration curve are easy to explain if only a fraction of the alpha-fetoprotein molecules is able to fix one or more molecules of ANS. To detect the existence of multiple

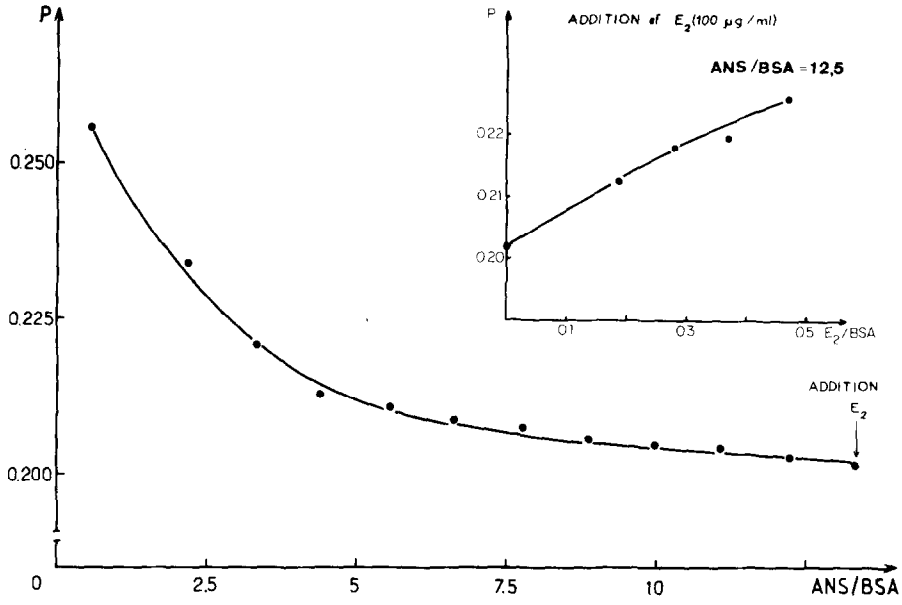

Fig. 3 Polarization curve of saturation of BSA by ANS (BSA concentration : $2.6\ 10^{-5}$M). Showing the decrease in polarization P as the number of bound ANS molecules increases. The insert shows the effect of estradiol additions upon the polarization of the ANS-BSA complex.

binding sites for ANS in the protein we have measured the polarization of the fluorescence at various values of $\bar{n}$. If the AFP population responsible binds a single mole of ANS/mole AFP, we must expect constant fluorescence polarization at all values of $\bar{n}$. If more than one mole is bound, we expect the fluorescence polarization P to decrease monotonically with increase in $\bar{n}$, because transfer or electronic energy among ANS molecules bound to the same protein will result in depolarization of the emitted radiation : as $\bar{n}$ increases, the fraction of the total AFP population with more than one molecule of ANS increases and consequently P decreases monotonically.

Fig. 3 and 4 show the curves for the polarization of fluorescence against increasing concentration of ligand for BSA and AFP respectively. It is quite clear that AFP has more than one binding site for ANS. In fact, the general resemblance to the albumin curve would lead one to suspect the presence of four strong binding sites per molecule for this ligand in agreement with the complex titration curve of Fig.2 . From the value of $\bar{n} = 0.65$ and the reasonable conclusion from the polarization data indicating that the protein with high affinity for ANS binds 3 or 4 moles of it, we deduce that this high-affinity polulation representa 1/5 to 1/6 of the total AFP molecules.

Interaction of estradiol with the ANS-AFP complexes

Addition of estradiol to these complexes results in a significant quenching of the ANS fluorescence. Similar results are obtained with other estrogenic

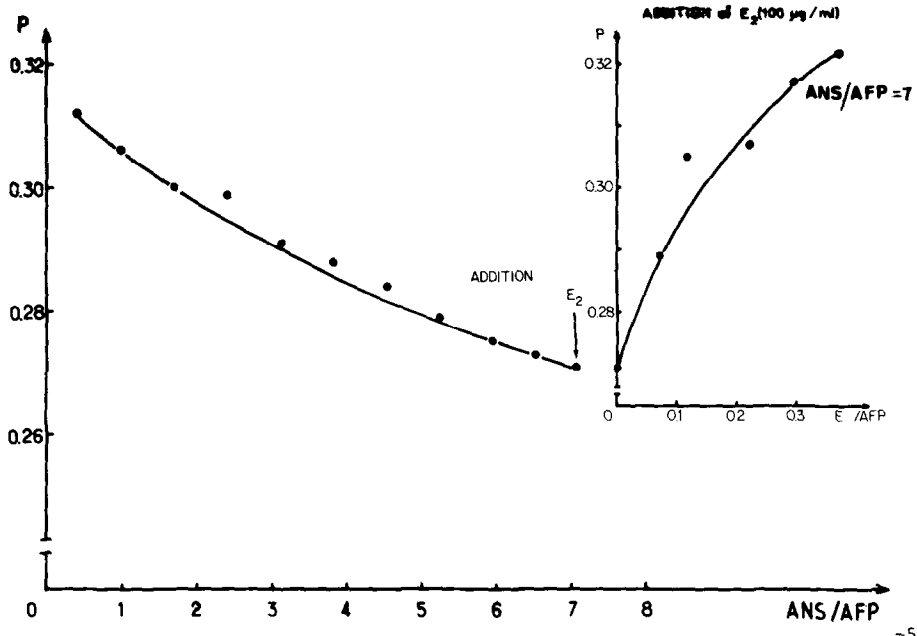

Fig. 4 Polarization curve of saturation of AFP by ANS (AFP concentration 3.3 10^{-5}M) showing the decrease in polarization P as the number of bound ANS molecules increases. The insert shows the effect of estradiol additions on the polarization of the ANS-AFP complex.

compounds : 6-oxo-estradiol, estrone, 6-α-hydroxy-estrone, estratriene, diethylstiboestrol (8). Fig. 5 shows the Scatchard plots obtained with fixed concentrations of estradiol and varying ANS concentrations. The slope of

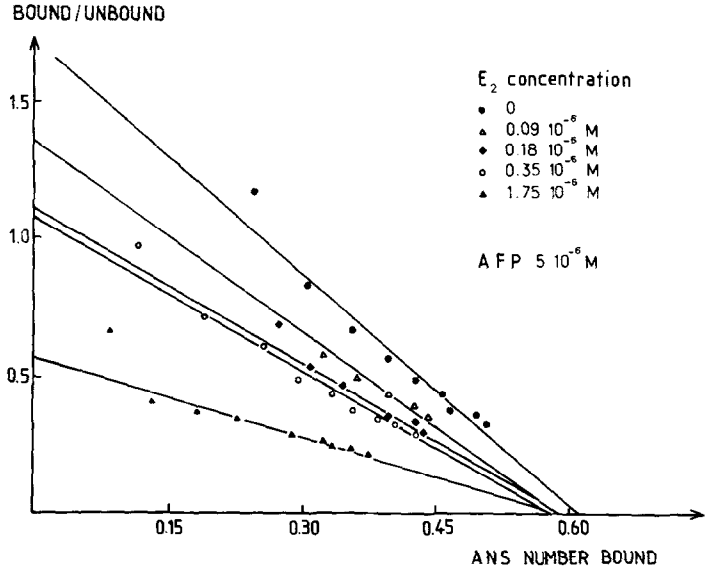

Fig. 5 Scatchard plots for ANS binding to AFP in presence of increasing amounts of estradiol.
Conditions are the same as those indicated in fig. 2.

the lines decreases, indicating a relative increase in ANS dissociation constant when the concentration of estradiol increases, but the total number of ANS sites remains constant indicating competition of ANS and estrogen for the same sites.

Independant evidence of the competition of ANS and estradiol for the binding sites is given by a study of the changes in fluorescence polarization of the ANS-AFP and the ANS-BSA complexes when estradiol is added (fig. 3 and 4). Addition of estradiol results in rapid increase in fluorescence polarization, which for a sufficiently high ratio of estradiol to protein, reaches the value of polarization observed at the lowest ANS/protein ratio and which corresponds to a single molecule of ANS bound to the protein.

CONCLUSIONS

1 - ANS is multiply bound by a small fraction of the total AFP population.

2 - Estradiol competes for all but one of these sites with ANS.

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