IN VITRO INTERACTION OF 1-ANILINO 8-NAPHTALENE SULFONATE WITH EXCITABLE MEMBRANES ISOLATED FROM THE ELECTRIC ORGAN OF ELECTROPHORUS ELECTRICUS

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Summary - In vitro binding of 1-anilino 8-naphtalene sulfonate (ANS) to membrane fragments derived from the innervated, excitable, surface of electroplax cells is accompanied by a strong enhancement of fluorescence intensity and polarization. Effects of temperature on ANS binding indicate that a structural reorganization of the membrane fragments occurs between 30 and 50°C. Fluorescence polarization of bound ANS is insensitive to changes of solvent viscosity upon addition of sucrose. Ca^{++} and d-tubocurarine modifies ANS binding.

I. INTRODUCTION

1-anilino 8-naphtalene sulfonate (ANS) has been commonly used as a fluorescent probe of protein and membrane structure (1-10). In the present communication we describe the in vitro interaction of ANS with membrane fragments derived from the innervated face of electroplax cells (EME). It is shown that the binding of ANS to EME is accompanied by a strong enhancement of fluorescence intensity and polarization, which is interpreted as an interaction of ANS with membrane hydrophobic areas. Study of the effect of temperature on the fluorescence parameters of ANS bound to EME indicates that an irreversible structural reorganization of the isolated membrane fragments occurs, between 30 and 50°C. In contrast with what is generally observed with soluble proteins, the change of solvent viscosity upon addition of sucrose is not accompanied

by any significant alteration of fluorescence polarization of bound ANS. This last effect appears to be a characteristic property of ANS bound to a membrane structure. Several *in vivo* effectors of the excitable membrane of the electroplax, Ca^{++} ions, d-tubocurarine and flaxedil, modify the *in vitro* interaction of ANS to EME.

II. MATERIAL AND METHODS

EME were prepared from homogenates of fresh electric tissue from *Electrophorus electricus* by differential centrifugation in sucrose gradient following the method described by Changeux, Gautheron, Israël and Podleski (11). Except when otherwise indicated, all the experiments were performed in the presence of 0.2 M sucrose and 5×10^{-3} M glycyl-glycine buffered at pH 7.0. Fluorescence intensity and polarization of ANS were measured according to the method of Monnerie and Neel (12) with a fluorescence polarization apparatus.

The wavelength of excitation was 365 mμ and fluorescence was observed at 438 mμ ; in both instances interference filters were used. The polarization p is defined as $p = \frac{2p_n}{p_n + 1}$ where $p_n = \frac{I'_v - I'_h}{I'_v + I'_h}$, I'_v and I'_h being the intensity components measured along the vertical and horizontal directions with unpolarized exciting light.

III. RESULTS AND DISCUSSION

1. Binding of ANS to EME

Exposure of EME to ANS in 0.2 M sucrose is accompanied by a strong enhancement of ANS fluorescence. The fluorescence emission spectrum of ANS bound to EME is similar in shape to the spectrum of ANS bound to bovine serum albumin (BSA), and its maximum occurs at the same wave-length. The change of the absorption spectrum of ANS upon binding is similar to the one observed with BSA : the absorption band at 365 mμ shifts to 371 mμ, and the difference spectrum between bound and free ANS also shows a maximum at 390 mμ.

The amount of ANS bound to EME was estimated by reference to the complex ANS-BSA in the following manner : 1) the variation of fluorescence intensity (I_f) was first measured as a function of increasing concentrations of ANS ; 2) in parallel, but within a smaller range, the difference in optical density at 390 mμ between bound and free ANS was determined ; 3) various ANS-EME mixtures were centrifuged at high speed and the concentration of ANS in the supernatant solution was determined by optical density measurement ($E_{350} = 5 \times 10^3$ per mole). The same number (n) of sites per mass of membrane protein was found with the three methods by extrapolation at infinite ANS concentration assuming that the quantum yield and the extinction coefficient of the complex ANS-EME are the same as for the complex ANS-BSA i.e. 0.75, and $\Delta OD = 2.04 \times 10^3$ per mole at 390 mμ (Fig.1).

This result indicates that the environment of ANS in the membrane is similar to that of ANS bound to BSA, namely a highly hydrophobic environment. In the present experimental conditions we determined : $n = 6 \pm 1$ mole per 10^5 g of protein and $K_D = \frac{1}{K_A} = 4.4 \times 10^{-4}$M. Of interest is the

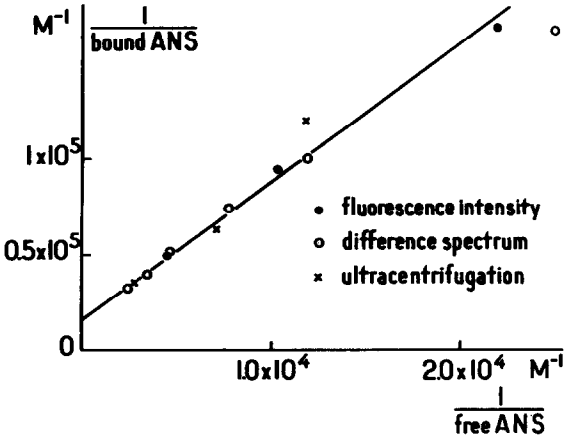

Figure 1. - Quantitative estimate of ANS bound to EME. Three different kinds of experiments were carried simultaneously 1) the fluorescence intensity of bound ANS was first measured, then 2) the difference in optical density at 390 mμ between free and bound ANS ; 3) various mixtures of ANS and ESE were centrifuged at 55.000 RPM in a SW 65 swinging bucket rotor for 90 minutes and the optical density of the supernatant was determined. The concentration of membrane was 1 mg/ml in proteins (using the Folin reagent and BSA as the standard). The temperature was 22°C.

fact that the variation of the reciprocal of the number of ANS bound with the reciprocal of free ANS concentrations follows a straight line within a large range of concentration, which indicates that the ANS binding sites on EME are homogeneous with respect to their affinity for ANS.

The polarization (p) of the light emitted by ANS bound to EME is 0.31 at 20°C a value almost identical to that found with BSA under the same conditions. p does not changes with the number of ANS molecules bound to EME as it does with BSA which suggests that ANS binding sites on EME do not interact as much as they do on BSA.

2. Effect of temperature on ANS binding.

Fig.2 and 3 show the variation as a function of temperature of I_f and p for ANS bound to EME in 0.2 M sucrose. When the temperature (T) is progressively elevated from 0 to 30°C, I_f and p decrease. The variation of 1/p with T/η follows the Perrin law assuming for the viscosity (η) of the solvent that of pure sucrose solution. The rotational relaxation time

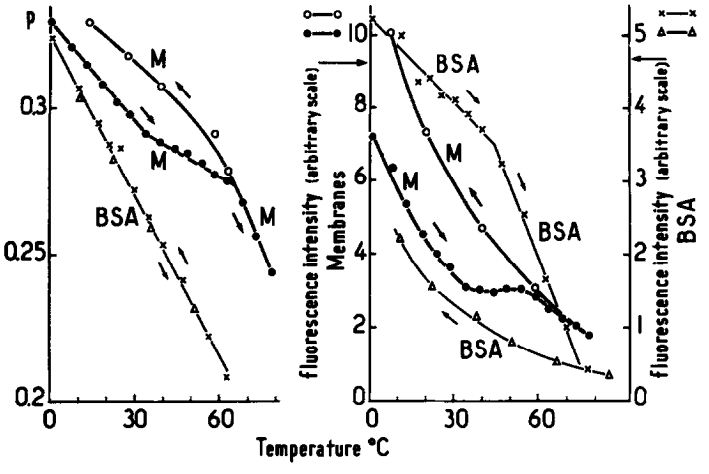

Figure 2. - Effect of temperature on ANS binding to ESE. The concentration of ESE was 0.2 mg of proteins per ml and that of ANS $2x10^{-5}$M. The experiment was carried in the presence of 0.2 M sucrose and $5x10^{-3}$M glycyl-glycine-HCl pH 7.0 at 20°C. Control experiments with BSA were done in the presence of $2x10^{-7}$M ANS and 0.2 mg/ml of BSA in buffer. Readings were made 5 minutes after temperature equilibrium.

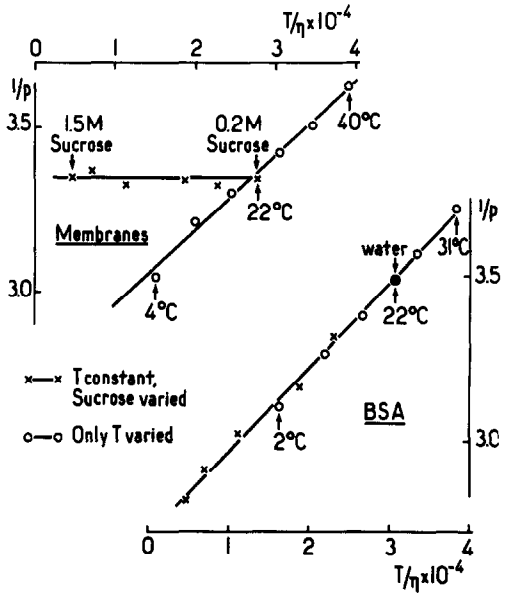

Figure 3. - Compared effects of sucrose on the polarization of fluorescence of ANS bound to ESE and BSA. The concentration of ESE was 0.2 mg/ml in proteins and that of ANS 2.2×10^{-6}M. When the temperature was varied the sucrose concentration was 0.2 M. When sucrose concentration was changed the temperature was maintained constant at 22°C. In the case of BSA when the temperature was varied the solution was made in buffer only. The concentration of ANS was 1.1×10^{-7} M and there was 0.2 mg/ml of BSA. The buffer was 5×10^{-3}M glycyl-glycine pH 7.0.

ρ calculated from these data is ρ = 156 n seconds at 22°C assuming for the life time of the excited state of ANS τ = 15.5 n seconds. Such a value is close to that found with BSA.

A plot of log K_A against 1/T is linear within this range of temperature, the slope corresponds to ΔH° = -5 KCal per mole at 20°C, the ΔF° of binding is -4.9 KCal per mole, yielding an entropy contribution of -0.3 e.u.

Above 30°C a break in the temperature curves occurs, I_f and p decrease more slowly than between 0° and 30°C. Above 50°C, I_f and p start again to decrease rapidly and with a slope close to that measured between 0 and 30°C. If the mixture ANS EME is first heated at 80°C and then progressively but rapidly cooled to 0°C, it is observed that the variation of I_f and p with T now strikingly differs from the variation of the same parameters recorded before heating to 80°C. The curve does not show any discon-

tinuity and the plot of log K agains 1/T is linear from 0° to 80°C. The thermodynamic parameters calculated from this curve are the following : ΔH° = -1.64 KCal per mole, ΔF° at 20°C = -5.29 KCal per mole, ΔS° = -12.5 e.u. ρ is now 380 n sec. assuming τ constant.

Following heat treatment an increase of both the number of ANS binding sites and their affinity for ANS is observed. The simplest interpretation of these results is that an irreversible structural reorganization of EME occurs between 30 and 50°C. By light scattering we followed in parallel the size and shape of membrane particles. No detectable changes of structure were noticed below 55°C although above 55°C an irreversible increase of the average size of the particles was noticed. The structural transition occuring around 30°C thus does not correspond to a gross alteration of membrane structure and is interpreted as a rearrangement of membrane components within the membrane.

3. Effect of solvent and membrane ligands on ANS binding.

It has been shown with the few globular proteins which have been tested in this respect that the polarization of fluorescence of ANS bound to these proteins increases with solvent viscosity. As seen on Fig. 3 when the concentration of sucrose in a suspension of EME is changed from 0.2 M up to 1.5 M, no significant variation of p occurs. Moreover this is true at any point of the heating and cooling curve. Such an independance of ANS fluorescence with respect to solvent viscosity was not observed with the bulk of soluble proteins which were separated from the membrane fragments in the course of EME purification from the total homogenate of electric organ ; neither was it observed with the protein acetylcholinesterase purified from electric organ (kindly supplied by Dr. W. Leuzinger from Columbia University), although in all these last instances a temperature dependance of ANS fluorescence parameters was noticed. On the other hand purified E. coli membranes (kindly supplied by Dr.A. Siccardi from the Institut

Pasteur) show the phenomenon ; the independance of p upon increase of solvent viscosity seems thus to be a characteristic property of ANS bound to a membrane structure.

In addition we noticed that the wavelength of the maximum of fluorescence emission of ANS bound to EME was not changed when the concentration of sucrose was increased from 0.2 M up to 1.5 M ; in contrast the emission spectrum of ANS bound to BSA showed a 2 mμ shift towards shorter wavelengths upon addition of 1.5 M sucrose.

Thus we see that 1) accounting for the high values of p measured, ANS is strongly immobilized by the membrane component to which it binds, 2) since the motion of ANS cannot be perturbed by changes in the solvent viscosity, ANS seems to be sequestred within the membrane phase. It should be emphasized that a definitive interpretation of these results requires the direct measurement of τ for ANS bound to ESE.

Various effectors of the excitable membrane of the eel electroplax were tested on the complex ANS-EME. In the presence of 10^{-3}M Ca^{++} the affinity of ANS to its membrane sites increase by a factor of 5 but the number of sites does not change. The effect of Ca^{++} is reversed by addition of versene. Carbamylcholine and decamethonium, two receptor activators have no significant effect at concentrations up to 10^{-4}M but in the presence of $5x10^{-5}$M d-tubocurarine or flaxedil, two receptor inhibitors, the affinity of ANS to EME increases respectively by a factor of 2 and 1.6 without change of the number of ANS binding sites. We are presently trying to see whether these effects are related or not to the in vivo pharmacological action of Ca^{++} and curare.

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