INTERACTION OF ETHIDIUM WITH THE MITOCHONDRIAL MEMBRANE: COOPERATIVE BINDING AND ENERGY-LINKED CHANGES

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Summary: Binding of ethidium, a cationic dye, to mitochondrial membranes occurs at a site of low polarity, and has the characteristics of a homotropic cooperative interaction. Energy conservation induces an increase in the affinity of the membrane for the dye.

INTRODUCTION

The structure and function of biological membranes has been the object of much interest during the last few years (1-3). Recently, new physico-chemical techniques have yield informations on the protein-lipid interactions and kinetics of membrane transitions (4-8).

The utilization of small organic fluorescent molecules such as anilinonaphtalene sulfonates, as environment sensitive probes, has been of great interest, to elucidate the polarity, viscosity, proximity relations and electric charge in membranes (9-15).

We now report our studies on the binding of a dye, ethidium, to the membrane of intact rat liver mitochondria.

Ethidium has fluorescent properties which are sensitive to environment polarity and has been utilized for investigating energy-linked transitions in mitochondrial membranes (16-18).

METHODS AND MATERIALS

Mitochondria were prepared as previously published (19). Binding of ethidium was measured by the following method: after centrifugation of the membranes (20,000 xg, 5 minutes) the concentration of the dye in the supernatants was evaluated from its absorbance at 480 nm by comparison with standards of known concentration and subtracted from the concentration of added dye.

Absorbance measurements were performed in a Hitachi-Perkin-Elmer spectrophotometer (Mod. 124). Protein was measured by a biuret method (20).

Ethidium was obtained from Calbiochem.

All other chemicals were reagent grade products.

RESULTS AND DISCUSSION

Binding of ethidium to mitochondria does not follow the simple Langmuir isotherm (Fig. 1). A plot of the concentration of free ethidium as a function of the amount of ethidium bound to mitochondria (expressed per mg of protein) yields a sigmoidal curve.

The presence of ATP, at concentration usually employed for driving energy requiring reactions, produces a marked change in the shape of the curve of ethidium binding to the mitochondrial membrane.

The unusual binding curves indicate that the interaction with the membrane of ligand molecules is accompanied by marked effects on the subsequent binding of additional molecules.

An analysis of the substances which can substitute for ATP in inducing the observed change in the ethidium binding curve has revealed that neither ADP or AMP are effective.

On the other hand mitochondrial respiratory substrates, such as succinate (1 mM) and ascorbate (1 mM) plus tetramethyl-

-p-phenylenediamine (0.1 mM) has been found as effective as ATP in inducing the ethidium binding change.

These observations, together with the inhibitory effects of uncouplers (1 μM FCCP) and respiratory poisons

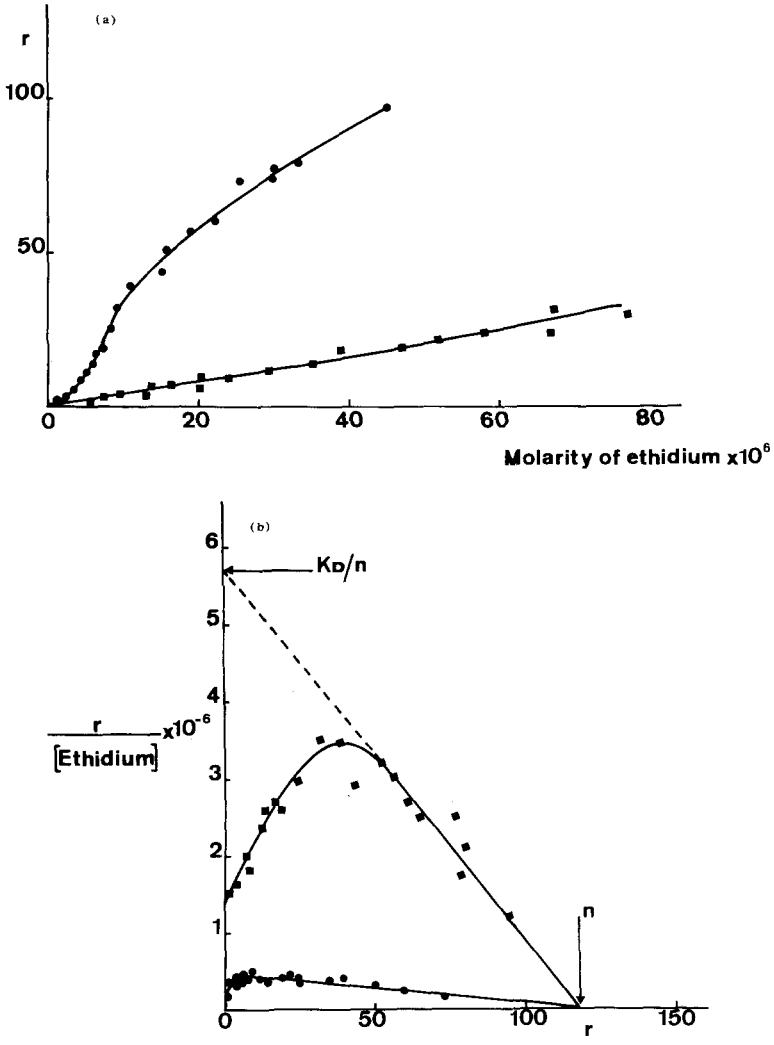

Figure 1 a,b. Binding of ethidium to mitochondria.

Mitochondria (1 mg protein/ml) were suspended in a medium of 0.25 M sucrose, 5 mM Tris-HCl pH 7.5 2 μM rotenone (to inhibit the oxidation of endogeneous substrates). In the upper curves 1 mM ATP was also present. Ethidium concentration was varied according to the figure. r is the amount of ethidium bound per mg protein. The amount of ethidium bound per mg of protein divided by ethidium free has the dimensions of $1 \times g^{-1}$.

(0.5 mM KCN or 2 μM antimycin A), indicate that ATP (or respiratory substrates)-induced ethidium binding changes represent an energy-linked reaction.

An analysis of the data of fig. 1a according to Scatchard (21) is presented in fig. 1b. The complex curve obtained deviate from the straight line, characteristic of ligand binding to identical and independent sites (22).

The intercept with the abscissa of the extrapolated linear part of the Scatchard plot (corresponding to infinite ethidium concentration) yields a value of 1.2×10^{-4} moles/gram of mitochondrial protein, independent of the presence of ATP.

The concentration of ethidium which gives 50% saturation is 2×10^{-5} M in the presence and 3×10^{-4} M in the absence of ATP. The effect of ATP is therefore to increase the affinity of the mitochondrial membrane for ethidium. This can be also concluded from the values of the ordinate intercept of the extrapolation of the linear portion of the Scatchard plot (K_D/ sites).

A plot of the data of fig. 1a, according to Hill (23), gives a curve having a maximum slope of 1.65 which is significantly different from the value of 1.0 expected for non-cooperative ligand binding.

It appears that a cooperative homotropic interaction occurs in the mitochondrial membrane (24). An analysis of the system in terms of known models (24-26) will be the object of a study to be presented elsewhere.

We believe that ethidium binding may arise from a coordinated membrane transition to a conformational in which the dye has greater affinity. Such a transition can be associated either with binding of dye molecules or with energy conservation in the membrane.

Some characteristics of ethidium binding site can be analyzed by taking advantage of the fact that interaction of the dye with the membrane results in a red shift of the dye

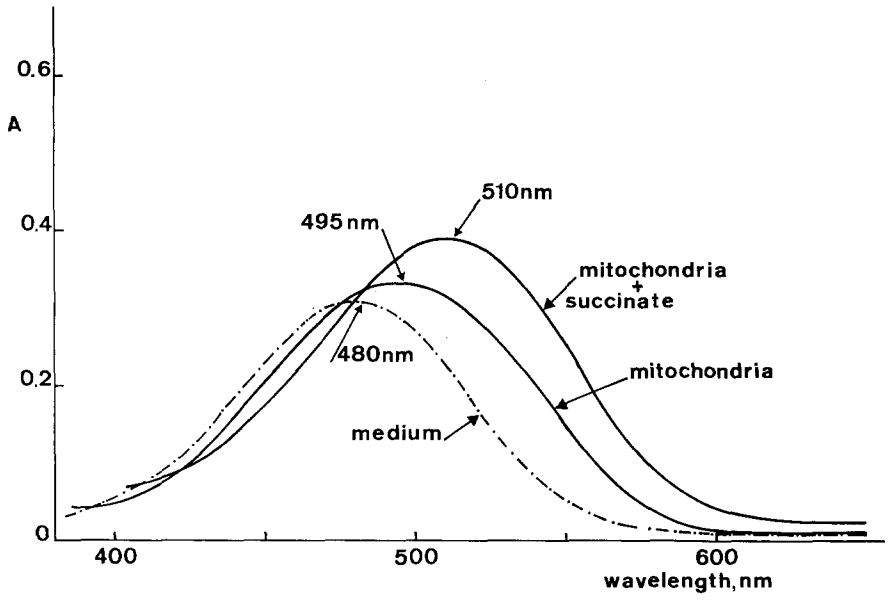

Figure 2. Spectral changes of ethidium in mitochondria. Conditions as in figure 1. Ethidium concentration was 84 μM. Succinate was added at a concentration of 1 mM.

absorbance maximum form 480nm in water to 495 nm (fig. 2). Energy conservation (i.e. addition of succinate) provokes an additional binding of the dye and a further red-shift of the absorbance maximum to 510 nm.

The value of 520 nm, which represents the limiting absorbance of ethidium in the presence of excess protein can be compared with the same wavelength maximum of ethidium in solvents having a dielectric constant of about 20.

A relatively low polarity characterizes the site of ethidium binding to the membrane.

The finding that cooperative homotropic effects mediate the binding of an organic cation, ethidium, to the mitochondrial membrane and that energy conservation modifies its binding suggests the following considerations:

1. The structural properties of the mitochondrial membranes must be such (i.e. subunits) that cooperative interactions might occur.

2. Cooperative effects may be important in energy conservation or energy propagation from one to other portions of the membrane.

3. The appearance of negative regions associated with energy conservation in the membrane of intact mitochondria postulated previously (9,10), is in agreement with ethidium binding data.

4. Cooperative binding of charged molecules by mitochondria and the effect of energy conservation on it, may play a regulatory role in cell metabolism by controlling the concentration of the substrates or coenzymes.

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