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LOCALIZED ENERGIZATION OF THE MITOCHONDRIAL INNER MEMBRANE BY ATP

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Summary

Studies were made to determine whether the energy-dependent binding of ethidium to the mitochondrial inner membrane reflects the membrane potential or the energization of localized regions of the membrane.

The number of binding sites of ethidium in mitochondria energized with ATP was 72 nmol/mg protein and decreased with increase in the amount of the ATPase system ($F_1 \cdot F_o$) inactivated by oligomycin. These findings clearly show that the energy-dependent binding of ethidium to the mitochondrial inner membrane energized with ATP does not reflect the membrane potential, in good accord with the previous conclusion (Higuti, T., Yokota, M., Arakaki, N., Hattori, A. and Tani, I. (1978) Biochim. Biophys. Acta 503, 211—222), but that ethidium binds to localized regions of the energized membrane that are directly affected by ATPase (F_1), reflecting the localized energization of the membrane by ATP.

Recently it has been shown [1—4] that positively charged anisotropic inhibitors (e.g., ethidium, acriflavine and tetraphenylarsonium) inhibit energy transduction in oxidative phosphorylation in mitochondria by binding to, and neutralizing, negative charges created on the C-side, but that these cations have no inhibitory effect on submitochondrial particles, which are inside-out relative to the membranes of intact mitochondria [5—8]. Conversely negatively charged anisotropic inhibitors (e.g., anilinonaphthalene sulfonate and tropaeolin OO) inhibit energy transduction in submitochondrial particles, but have no appreciable inhibitory activity in intact mitochondria.

On the basis of these findings, we proposed the anisotropic charge model for ATP synthesis and H^+-transfer in mitochondria [9, 10]. This model was an extension of the hypothesis by Azzi and coworkers [11, 12]. This model suggests that energization of localized regions of the membrane occurs by respiration or ATP hydrolysis.

In good accordance with this model, it has been found [13, 14] that the energy-linked response of anilinonaphthalene sulfonate on submitochondrial particles is not due to electrophoretic transport down the membrane potential, as in the scheme of Mitchell [15—17].

The present studies were made to find out whether the energy-dependent binding of ethidium to intact mitochondria [12, 18—20] reflects the membrane potential [15—17, 21—24] or the energization of localized regions of the mitochondrial inner membrane; in this study, part of the ATPase system ($F_1 \cdot F_o$) in the membrane was inactivated with oligomycin.

Ethidium bromide was purchased from Nakarai Chemicals Co., Kyoto (Japan). Oligomycin was obtained from Sigma Chemicals Co., St. Louis, MO. Other reagents used were as described previously [1].

Rat liver mitochondria were isolated by the method of Hogeboom [25], as described by Myers and Slater [26], except that 0.25 M sucrose containing 2 mM Tris (pH 7.4) was used for homogenization and two washings [27]. Protein was estimated from the content of cytochromes $a + a_3$ [27, 28].

Bertina et al. [29, 30] found that the mitochondrial ATPase system ($F_1 \cdot F_o$) binds one mol of oligomycin/mol F_o with a low dissociation constant. Their finding means that the amount of the ATPase system in the mitochondrial inner membrane is equal to the amount of the oligomycin-binding site in the membrane and also approximately equal to the minimum amount of oligomycin required for 100% inhibition of state 3-respiration. Therefore, oligomycin binds to the F_o molecule of the ATPase system that it first hits and does not interfere with all F_o molecules in a random fashion.

We examined whether ATP-dependent binding of ethidium to the mitochondrial inner membrane reflects the membrane potential [15—17, 21—24] or energization of localized regions of the membrane by inactivating part of the ATPase system in the membrane with oligomycin.

The energy-dependent binding of ethidium to mitochondria energized with ATP reached a steady state after 3 min, as shown in Fig. 1. On addition of oligomycin (0.055 μg (0.068 nmol) [31]/mg protein), which caused 52% inhibition of state 3-respiration, both the initial velocity and extent of the energy-dependent binding of ethidium decreased to 56% of the control (Fig. 1).

Fig. 2 shows a typical plot of the effect of ethidium concentration on its energy-dependent binding at equilibrium to mitochondria energized with ATP in the absence and presence of oligomycin. The concentration of oligomycin used was 0.057 μg/mg protein, which caused 53% inhibition of state 3-respiration. The findings of Bertina et al. [29] show that this inhibition is due to inactivation of 53% of the ATPase system in each mitochondrion.

Scatchard plots [32] of the data in Fig. 2 are shown in Fig. 3. The energy-dependent binding of dye (n: number of binding sites of ethidium in the energized mitochondria) to the membranes at infinite dye concentration in

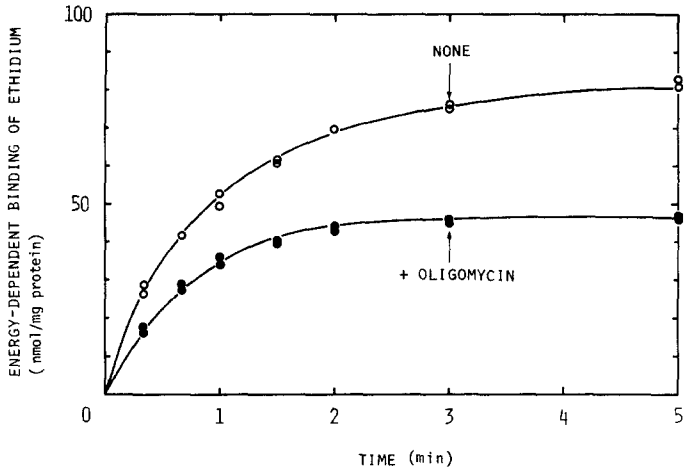


Fig. 1. Time course of energy-dependent binding of ethidium to mitochondria energized with ATP in the absence and presence of oligomycin. Mitochondria (1 mg protein/ml) were preincubated for 5 min at 25° C (± 0.1° C) in the presence of 2 mM ATP/± 0.055 μg of oligomycin per mg protein/1 μg of rotenone/50 mM sucrose/25 mM Tris/15 mM KCl/5 mM $MgCl_2$ /2 mM EDTA, in a final volume of 1.5 ml at pH 7.4 in a test tube. Then 300 nmol of ethidium bromide was added the mixtures were incubated for the indicated times, and rapidly cooled to about 0° C by inserting a smaller test tube filled with ice water into the test tube of reaction mixture and transfering these test tubes to an ice bath. The resulting mixtures were centrifuged at 8000 × *g* for 2 min in an Eppendorf, Model 3200, microcentrifuge, and the remaining dye was estimated by measuring its A_{480} with a Hitachi, Model 556, two-wavelength, double-beam spectrophotometer. The amount of energy-dependent binding of ethidium was calculated by subtracting the amount of energy-independent binding (+ 3 μg of oligomycin/mg protein) from the total binding.

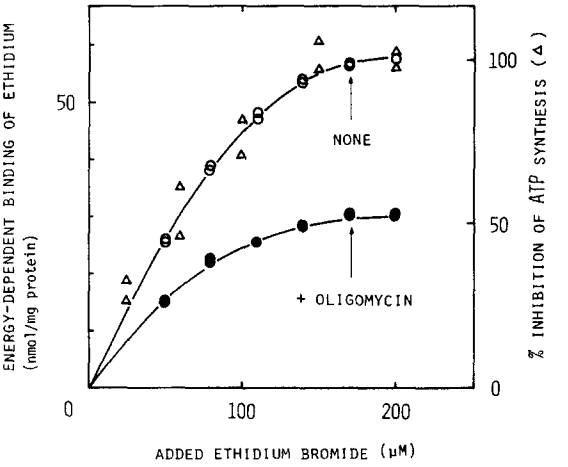
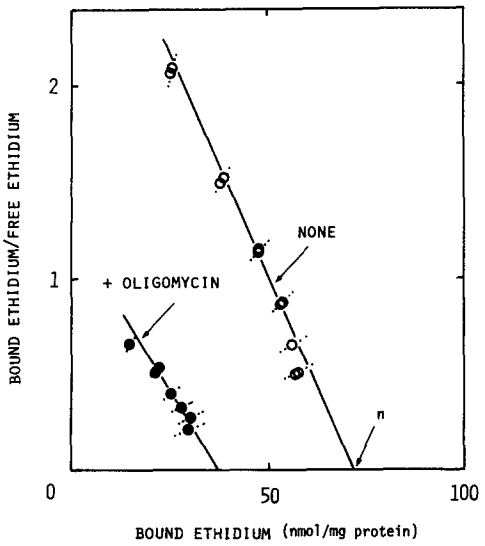


Fig. 2. Effect of ethidium bromide concentration on its energy-dependent binding to mitochondria energized with ATP in the absence and presence of oligomycin. Mitochondria (1 mg protein/ml) were preincubated for 5 min at 25° C (± 0.1° C) in the presence of 2 mM ATP/± 0.057 μg of oligomycin per mg protein/1 μg of rotenone/50 mM sucrose/25 mM Tris/15 mM KCl/5 mM $MgCl_2$ /2 mM EDTA, in a final volume of 1.5 ml at pH 7.4. Then various amounts of ethidium bromide were added and the mixtures were incubated for 5 min. Other procedures were as for Fig. 1. The percentage inhibition of ATP synthesis in mitochondria was obtained from Ref. 1.

the absence and presence of oligomycin, represented as the intercepts of the linear portions of the plots, are 72 and 38 nmol ethidium per mg protein, respectively. The decrease in *n* caused by oligomycin is in good accord with the % inhibition of state 3-respiration by the inhibitor. The dissociation con-

stants (K_D) of the dye-mitochondria in the absence and presence of oligomycin, calculated from the slopes of the lines, are 22 and 29 μM. These values of K_D are not essentially different, assuming that the limits experimental error are ± 2%. These results support the finding of Bertina et al. [29, 30] that oligomycin binds to the ATPase system that it first hits and does not interfere with all ATPase system in a random fashion. Fig. 4 also shows that oligomycin caused approximately equal inhibitions of energy-dependent binding of ethidium to membranes energized with ATP and state 3-respiration. The minimum amount of oligomycin required for complete inhibition of state 3-respiration and the energy-dependent binding of ethidium (Fig. 4) was approximately equal to the amount of the ATPase system in rat liver mitochondria [29]. Thus this result shows that all molecules of added oligomycin bound to the ATPase system in a molar ratio of 1/1, in good accord with the finding of Bertina et al. [29, 30].

If the energy-dependent binding of ethidium to the mitochondria reflects the membrane potential [15—17, 21—24], the values of n in the absence and presence of oligomycin should be equal. However, the present results clearly show that the value of n is dependent on the amount of active ATPase system in the mitochondria. Therefore, the present findings clearly show that the energy-dependent binding of ethidium to the mitochondrial inner membrane energized with ATP does not reflect the membrane potential, in good accord with the previous conclusion [1], but that ethidium binds to localized segments of the membrane that are directly affected by an ATPase (F_1), reflecting the localized energization of the membrane by ATP.



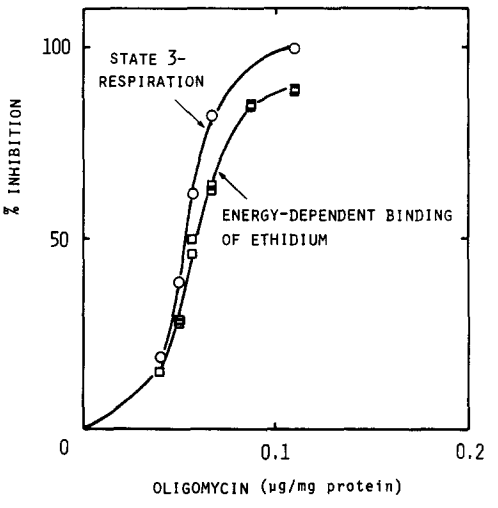


Fig. 3. Scatchard plot of energy-dependent binding of ethidium bromide to mitochondria in the absence and presence of oligomycin. Values were calculated from data in Fig. 2. The dotted lines show the effects of ± 2% error in the concentration of added ethidium bromide.

Fig. 4. Effects of oligomycin concentration on the energy-dependent binding of ethidium to mitochondria energized with ATP and state 3-respiration in the membranes. Conditions were as for Fig. 2 except that 200 μM ethidium bromide was added. The amount of energy-dependent binding of ethidium to mitochondria in the absence of oligomycin was 58.2 nmol ethidium/mg protein. The percentage inhibition of state 3-respiration was calculated after correction for state 4-respiration.

The present conclusion is in good accord with previous findings by Ferguson et al. [14] using anilinonaphthalene sulfonate as an indicator for energization of submitochondrial particles.

Fig. 2 also shows that the dose-response curve of the energy-dependent binding of ethidium to the membrane in the absence of oligomycin closely coincided with the dose-response curve for its inhibition of ATP synthesis in the membranes [1], indicating that the energy-dependent binding of ethidium to the membranes inhibits energy transduction in oxidative phosphorylation. This is why ethidium acts as an energy-linked probe for intact mitochondria [12, 18—20] and also why the dye inhibits energy transduction in the membranes [1] and is consistent with the anisotropic charge model [4, 10].

Like the oligomycin-effect on the energy-dependent binding of ethidium, the maximum amount of ethidium-induced H^+-ejection from the mitochondria energized with ATP decreased with increase in the amount of ATPase inactivated by oligomycin.

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