REDISTRIBUTION OF THE ELECTRICAL CHARGE OF THE MITOCHONDRIAL MEMBRANE DURING ENERGY CONSERVATION

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SUMMARY

The interaction of the fluorescent dyes 1-anilino-8-naphthalene sulfonic acid (ANS) and tetramethyldiamino-diphenyl-ketoimine hydrochloride (Auramine-O) with the membrane of intact and fragmented mitochondria has been studied. The fluorescence changes accompanying the energization of the membrane can be accounted for by binding changes of the dyes, induced by charge changes of the membrane.

INTRODUCTION

The fluorescent dye 8-anilino-1-naphthalene sulfonic acid (ANS) has been employed to obtain structural information on proteins and membranes (1,2,3). ANS, in fact, changes some of its fluorescence properties as the result of the noncovalent interaction with proteins and membranes (4,2,3).

The high quantum yield and the blue shift that are observed when ANS is dissolved in non polar solvents has led to the conclusion that ANS interacts in proteins and membranes with regions of low dielectric constant, or of low Z value (7,8). ANS has been shown to bind to mitochondrial membrane fragments and also to undergo reversible quantum yield changes when the membrane fragments were energized (2).

In the present study the mechanism of the fluorescence transitions associated with energy conservation in intact and fragmented mitochondria has been investigated. On the basis of the analysis of the dye membrane interaction it is proposed that an intermediate step in the process of energy conservation consists in a redistribution of the electrical charges on the mitochondrial membrane (9).

METHODS AND MATERIALS

Rat liver mitochondria were prepared in 0.22 M mannitol, 0.07 M sucrose and 200 μM EDTA pH 7.2 essentially according to the methods of Schneider (10) and Lardy and Wellman (11). Pigeon heart mitochondria (PHM) were prepared according to the method of Chance and Hagihara (12). Mitochondrial fragments (ETPH) were prepared according to Hansen and Smith (13). Fluorescent changes were measured in a compensated fluorometer as previously described (2). Rapid reactions were measured in a stopped flow apparatus (14). ANS binding was measured after incubation of the sample at room temperature for 5 minutes. Subsequently the samples were centrifuged at zero degrees (mitochondria at 10,000 xg for 10 minutes, fragments at 100,000 xg for 30 minutes). The fluorescence of the supernatants and of the pellets was measured in a Hitachi-Perkin-Elmer spectrofluorometer after treatment with 3% Triton-X-100 which produced maximum enhancement. The excitation and emission wavelength were 366 and 470 nm respectively. ANS (from K and K) was recrystallized twice from hot aqueous solutions of its Mg salt. All other reagents were analytical grade.

RESULTS

Energy linked changes of ANS fluorescence in intact mitochondria. The addition of ATP to a suspension of PHM in an isotonic mannitol-sucrose-tris medium in the presence of 100 μM ANS resulted in a decrease of ANS fluorescence that was complete in about 15 seconds (Fig. 1). The presence of 10 μM rotenone (added 5 minutes before the addition of ATP) prevented changes of pyridine nucleotide, and thus eliminated a possible source of interference with the fluorescence measurements of ANS.

The addition of 5 μg oligomycin induced an increase of ANS fluorescence that was completed after about 100 seconds. In the presence of FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone), no changes of ANS fluorescence were observed on addition of ATP (lower trace of Fig. 1). Similar to ATP, energization of the membrane by succinate induced a fluore-

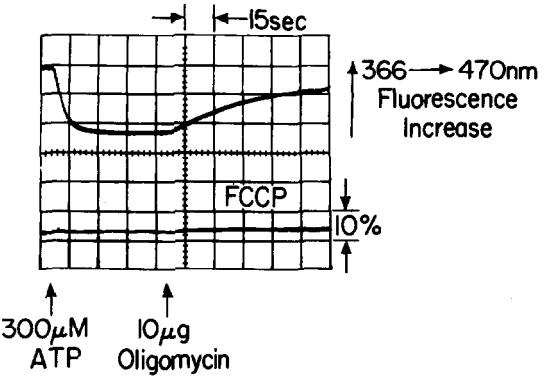

Figure 1. The energy linked fluorescence decrease of ANS in intact mitochondria.
The ANS fluorescence was measured in the following medium: 250 mM mannitol, 50 mM sucrose, 20 mM tris-HCl, pH 7.4, 10 μM rotenone, 100 μM ANS and 2 mg/ml of pigeon heart mitochondria protein.

scence decrease, prevented or reversed by cyanide, antimycin A or FCCP. These experiments suggest that energy conservation in intact mitochondria is accompanied by a decrease of the ANS fluorescence.

Energy linked changes of ANS fluorescence in mitochondrial fragments.

In Fig. 2 the addition of oxygen to an anaerobic suspension of ETPH (state 5), in the presence of ANS, induces a transition to a coupled aerobic state (state 4), which is accompanied by an increase in the ANS fluorescence of

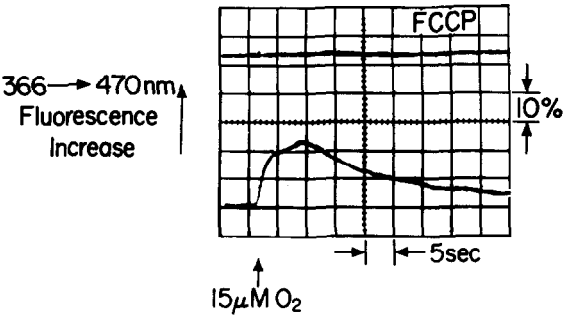

Figure 2. The energy linked fluorescence increase of ANS in mitochondrial fragments.
ANS fluorescence changes were measured in the stopped flow apparatus. The incubation medium contained: 250 mM mannitol, 50 mM sucrose, 20 mM tris-HCl, pH 7.4, 10 mM succinate, 100 μM ANS and 2 mg/ml sonicated submitochondrial particles.

20%. When oxygen has been consumed, the ANS fluorescence decreases and the process can be repeated for several times. In the presence of FCCP (upper trace of Fig. 2) no changes of ANS fluorescence are detectable under these conditions, indicating that in this case also ANS is measuring an energy linked change of the membrane. The addition of ATP or of an oxidizable substrate to ETPH results also in a fluorescence increase, which is reversed (or prevented) by oligomycin or respiratory inhibitors respectively. The comparison between Fig. 1 and 2 indicates that both in mitochondria and in submitochondrial particles ANS fluorescence measures an energy linked function, however, the direction of the changes is opposite in the two systems

Energy linked binding changes of ANS and Auramine-O in mitochondria and mitochondrial fragments. The ANS fluorescence changes described above in intact and fragmented mitochondria could be explained by changes in the environment of bound ANS or by changes of binding of ANS to the membrane in the energized and non-energized state. Binding studies were therefore carried out by measuring the amount of ANS bound to the pellets (and remaining in the supernatants) of intact or fragmented mitochondria in the energized and non-energized state, after centrifugation.

Table I. Changes of the Auramine-O and ANS binding to intact and fragmented mitochondria in the energized state.

The incubation medium contained: 3 ml of 250 mM mannitol, 50 mM sucrose, 20 mM tris-HCl pH 7.4, 10 μM ANS (5 μM ANS in the experiment with mitochondrial fragments) or 100 μM Auramine-O, 5 μM rotenone and 1.2 mg protein/ml. The system was incubated for 10 minutes before adding succinate or ATP for reducing, in the case of mitochondria, the reserves of ATP and of endogenous substrates to zero. The numbers represent percent changes of the binding.

	AURAMINE-O		ANS	
	RLM_W	ETPH	RLM_W	ETPH
7 mM succinate + 1 μM FCCP	0	0	0	0
7 mM succinate	+50	-20	-20	+18

The results are reported in Table I, where the binding of the negatively charged ANS and of the positively charged Auramine-0 (15) was measured. It appears that the energization of the membrane of intact mitochondria induced by succinate is accompanied by a 50% increased fluorescence and binding of Auramine-0 and a 20% decrease of the bound ANS. In fragmented mitochondria (ETPH), energization of the membrane is accompanied by an 18% increased binding of ANS and by a 20% decrease of bound Auramine-0. No fluorescence or binding changes of an uncharged fluorescent derivative of ANS, 8-anilino-1-naphthalene sulfonamide, were measurable.

CONCLUSIONS

The changes of ANS fluorescence in mitochondrial membrane fragments have been reported to be the expression of a new state of the membrane associated with energy conservation (2). The experiments described above suggest that fluorescence transitions in the mitochondrial membrane and membrane fragments can be accounted for by binding changes of the ANS molecules.

The mitochondrial fragments, which are considered to have a morphological polarity opposite to that of intact mitochondria (16,17), have been shown here to exhibit opposite responses to dye binding in the energized vs. non-energized state (18). The comparison between the opposite binding changes of ANS, which is negatively charged, and Auramine-0, which is positively charged, reveals that the interaction between dyes and the energized membrane is electrostatic in nature.

In Fig. 3, the mitochondrial membrane is schematically represented with a charge distribution in the non-energized and the energized state that is consistent with the dye binding studies reported in this communication. In the left portion of the diagram the non-energized membrane is arbitrarily represented without a net electrical charge, in opposition to the energized membrane (right portion of the diagram) that bears instead an external surface that is more negative than the medium and an internal

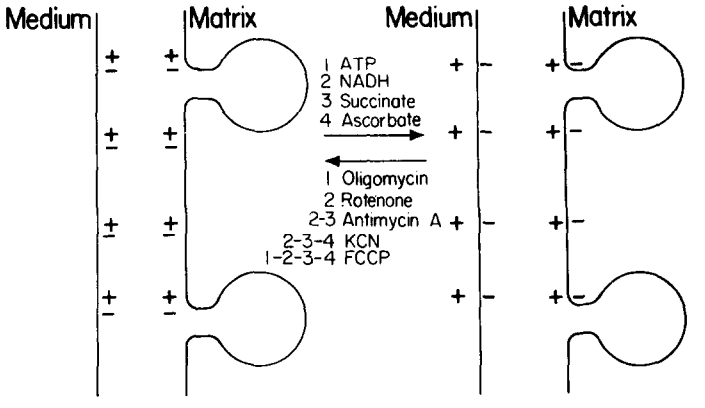

Figure 3. Schematic representation of the changes associated with energy conservation in the mitochondrial membrane. The diagram on the left represents a mitochondrial membrane in the non-energized state. The diagram on the right represents a membrane in the energized state. Arrows indicate the direction of the transitions. Equal numbers indicate the inhibitor of the transition induced by a certain substrate.

surface that is more positive than the matrix. It is probably impossible to conclude from this study only whether the charge changes associated with the energized state of the mitochondrial membrane are the result of a change of membrane potential (19) or of conformational changes of the membrane. It is probably important, however, in the mechanism of ATP synthesis, that an asymmetrical charge distribution is associated with energy conservation in the mitochondrial membrane.

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