ENERGY DEPENDENT INTERACTION OF OLIGOMYCIN AND DICYCLO HEXYLCARBODIIMIDE WITH THE MITOCHONDRIAL MEMBRANE *

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The characteristics of the oligomycin and DCCD inhibitions of energy linked reactions supported by ATP, indicate that the inhibition is increased by the presence of ATP. It is proposed that such an effect can be attributed to an energy linked change in the structure of some component of the ATPase, facilitating oligomycin interaction with the membrane.

1. Introduction

Ion transport [1], reversed electron transport [2], transhydrogenase [3] and the response of fluorescent indicators [4] are energy requiring reactions and may be supported by the coupled hydrolysis of ATP, both in intact and fragmented mitochondria.

Oligomycin and dicyclohexylcarbodiimide (DCCD), are potent and specific inhibitors of the above reactions [5, 6], and also of the ATP synthesis coupled to the flow of electrons in the mitochondrial respiratory chain [6].

In the present study, the characteristics of oligomycin and DCCD inhibitions of two energy requiring reactions, the ATP supported ANS fluorescence changes and the ATP supported reversal of electron transport from succinate to NAD, have been investigated.

Evidence will be proposed, that oligomycin and DCCD inhibitions of ATP requiring reactions, are greatly facilitated by the presence of ATP. This phenomenon can be accounted for by structural changes of the mitochondrial membrane promoted by ATP.

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2. Methods and materials

Rat liver mitochondria were prepared according to the method described earlier [7]. Particles were derived from them by ultrasonic disruption (at 3 A for 1 min in a Branson Sonifier), in a medium containing 1 mM EDTA at pH 8.5. These particles have all the typical reactions of submitochondrial particles such as coupled NADH and succinate oxidations, reversal of electron transport [2] and transhydrogenase [3], ion movements [8] and energy linked fluorochrome changes [4].

ANS was obtained from K and K laboratories, Inc., oligomycin from Sigma, all other reagents were analytical grade. ANS was recrystallized twice from hot solutions of its Mg^{2+} salt.

Fluorescence was measured in a Eppendorf fluorometer.

3. Results

3.1. *Oligomycin inhibition of the ATP-induced ANS response in mitochondria*

ANS responds with fluorescence changes to variations of the energy state of the membrane of mitochondria and submitochondrial particles [9].

In fig. 1, 200 μM ATP was added to a suspension of mitochondria preincubated with ANS and rotenone

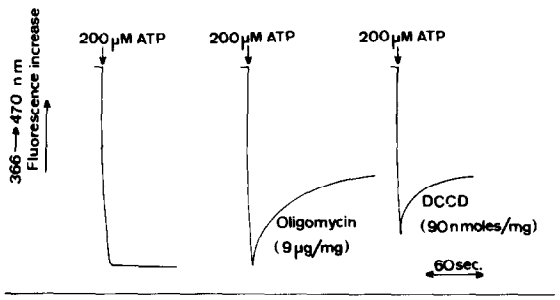

Fig. 1. ATP induced ANS responses in rat liver mitochondria in the presence and absence of oligomycin and DCCD. Mitochondria, 2.5 mg protein/ml, were incubated for 5 min in 250 mM sucrose, 10 mM tris-HCl pH 7.5, 5 mM $MgCl_2$, 10 μM rotenone, and 12 μM ANS. After this time ATP was added, preceded (or not) by oligomycin or DCCD. (Experiment AO44).

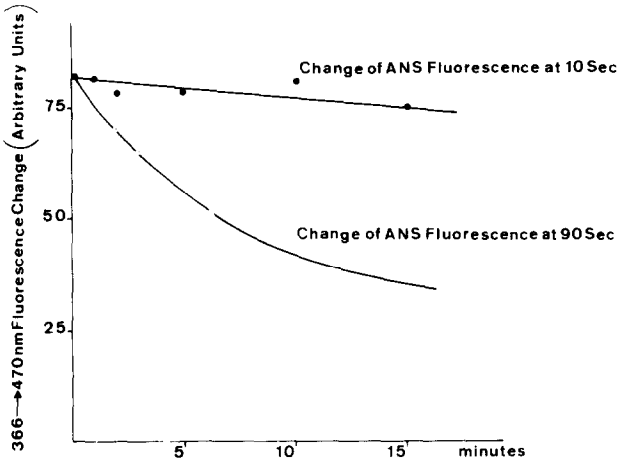

Fig. 2. ATP induced fluorescence changes of ANS in rat liver mitochondria: effect of the time of preincubation with oligomycin. The experimental conditions were as in fig. 1. Oligomycin, 10 μg, was incubated before the addition of ATP for the times indicated. The upper curve represents the initial fluorescence decrease on addition of ATP, the lower curve represents the final fluorescence level attained 90 sec after the addition of ATP. (Experiment AO55).

for 5 min. The fluorescence decrease indicates an energization of the membrane, produced by ATP. In the presence of oligomycin or DCCD the addition of ATP produces also a fluorescence decrease, which, in this case, is followed by a fluorescence increase, which is complete in about 1 min. It should be noted that no fluorescence response would be expected on addition of ATP if the inhibitor had already interacted with the membrane. Vice-versa, the transient decrease of ANS fluorescence on addition of ATP, in the presence of oligomycin or DCCD, suggests that the inhibitor has not fully reacted with the membrane prior to ATP.

This point is further clarified by plotting (fig. 2) the ANS fluorescence change at 10 sec (upper curve) and 90 sec (lower curve) after ATP addition, vs the time for which the mitochondrial suspension was allowed to react with the inhibitor (3.3 μg oligomycin/mg protein, at room temparature). The extent of membrane energization (10 sec after ATP addition) is virtually independent of the presence of oligomycin and of the time (up to 10 min) for which the inhibitor has been in contact with the membrane suspension.

What appears to be strongly influenced by the presence of oligomycin and the time of preincubation is the fluorescence decrease taken at 90 sec after the ATP addition. The same results were obtained by using DCCD instead of oligomycin.

The experiments of fig. 2 suggest, in conclusion, that an energized state of the mitochondrial membrane can be induced by ATP even in the presence of oligomycin. This energization is however transient and followed by an inhibition which is a function of the time of preincubation with the inhibitor. Energization appears therefore to be necessary to obtain a complete oligomycin inhibition of the ATP induced ANS response in the mitochondria.

3.2. *Oligomycin inhibition of the ATP-induced response in submitochondrial particles*

The inhibitory effects of oligomycin on the ATP induced ANS changes were also studied in submitochondrial particles, and the results reported below are very similar to those in intact mitochondria.

The addition of ATP to ANS treated submitochondrial particles (fig. 3) produces an energy dependent fluorescence increase that lasts for several minutes, and is completely reversed by uncouplers and by oligomycin (not shown). If oligomycin is added before ATP, the inhibition of the ATP induced ANS fluorescence increase appears to be only 20% at 10 sec from the addition of ATP, but increases with time and becomes complete about 1 min after the addition of ATP.

The response of submitochondrial particles to ATP after pretreatment with DCCD was not different from that reported above.

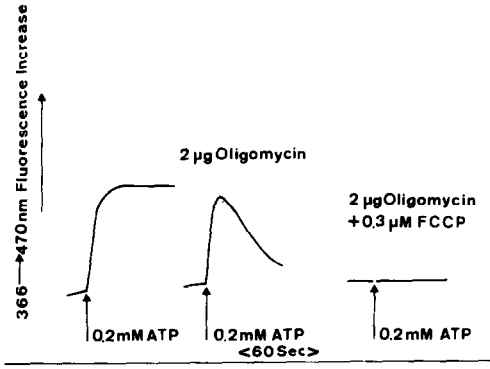

Fig. 3. ATP induced ANS response in rat liver submitochondrial particles: effect of oligomycin and FCCP. Submitochondrial particles, 1.0 mg/ml were incubated in the medium of the experiment of fig. 1. Oligomycin, 1 μg/ml, was added 1 min before the addition of ATP. (Experiment AO51).

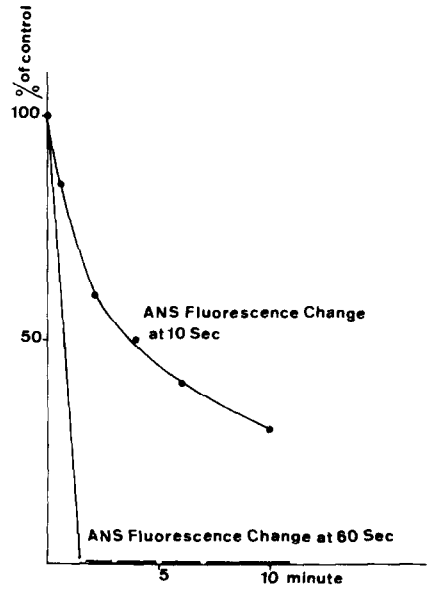

Fig. 4. ATP-induced fluorescence changes of ANS in rat liver submitochondrial particles: effect of the time of preincubation with oligomycin. The experimental conditions were as in fig. 1. 300 μg Submitochondrial particles and 0.25 μg oligomycin/mg protein were also used. The reaction was initiated by 0.2 mM ATP. The upper curve represents the initial fluorescence increase on addition of ATP, the lower curve is the final fluorescence change 60 sec after the ATP addition. (Experiment AO51).

In fig. 4, the oligomycin inhibition of the ATP induced ANS response at 10 sec after the ATP addition was plotted as a function of the time of incubation of the particles with 1 μg oligomycin/mg protein, (upper curve). The lower curve is the inhibition by oligomycin of the ANS fluorescence change, at 60 sec after the ATP addition. Also in this case, as in intact mitochondria, there is evidence of an ATP requirement (together with a time requirement) for the inhibition by oligomycin of the ATP induced ANS response.

3.3. *Oligomycin sensitivity of the ATP induced ANS response and reversal of electron transport in submitochondrial particles*

In the experiments reported in fig. 5, the ANS response to ATP and the reversal of electron transport from succinate to NAD in submitochondrial particles were compared, as a function of the amount of oligomycin present in the incubation medium.

The two upper curves represent the extent of the transient ANS fluorescence increase and the initial rate of NADH formation after the addition of ATP. The lower curve is the final fluorescence change or the final rate of NADH formation (the values were taken 90 sec after ATP addition). Also in the case of the reversal of electron transport on addition of ATP the initial rate of NADH formation is faster than the rate at 90 sec, indicating that, even at high oligomycin concentrations, the inhibition is not complete until ATP is added and allowed to react with the membrane.

4. Conclusions

From the experiments reported above, the following conclusions can be derived:

1) the ATP induced ANS response and reversal of electron transport from succinate to NAD is inhibited by oligomycin in a time-concentration dependent reaction, in agreement with previous observations [6].

2) the oligomycin and DCCD inhibition appears also to be ATP dependent. In fact, at low inhibitor concentrations (0.5 μg/mg protein) or after short preincubation times (30–60 sec), the reaction proceeds for 10–30 sec, before a full inhibition of ANS fluorescence response or reversal of electron transport is established. A preincubation time with oligomycin

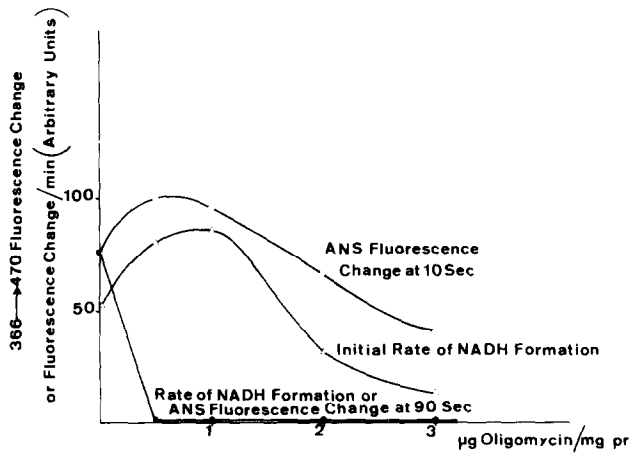

Fig. 5. ATP induced reversal of electron transport and ANS fluorescence changes in submitochondrial particles: effect of oligomycin concentration. The conditions of the ANS experiments were as in fig. 3. Oligomycin was added 1 min before ATP. The reversal of electron transport was studied in the following medium: 0.25 M sucrose, 10 mM tris-HCl, 10 mM $MgCl_2$, 5 mM NAD, 5 mM succinate and 1 mM KCN. (Experiment AO52).

10–30 sec longer does not induce a greater inhibition of the reaction. A preincubation time ten times longer or an inhibitor concentration ten times higher is required to obtain a full inhibition even in the first 10–30 sec after ATP addition.

3) The ATP facilitation of oligomycin-DCCD inhibition does not appear to be linked to a transport and concentration of the inhibitor at the active site, promoted by ATP. DCCD is, in fact an uncharged molecule and mitochondrial membranes are able to concentrate only charged species.

4) The effect of ATP, of facilitating the oligomycin-DCCD inhibitory effects does not appear to be linked to a facilitation of the transit of the inhibitor across the membrane before reaching its binding site. When in fact the ATPase inhibitory site on the ATPase is exposed and easily accesible, as in submitochondrial particles [10], still ATP exerts a definite influence in inducing the oligomycin inhibitory effects.

5) The oligomycin inhibition is maximal only some 10–20 sec after ATP addition and therefore appears to be influenced more by the energization of the membrane induced by ATP during such a period [4], than by the binding of ATP *per se*.

6) In conclusion the study presented here suggests that the energized state, following the addition of ATP to the mitochondrial membrane, is able to produce an alteration of some part of the membrane which leads to an increased sensitivity of the ATP requiring reactions to the inhibitors oligomycin and DCCD. A structural modification of the ATPase system induced by energization may be the physical entity responsible for the higher sensitivity to inhibitors such as DCCD and oligomycin, in the presence of ATP.

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