ATP-INDUCED INHIBITION OF MITOCHONDRIAL ATPase BY OLIGOMYCIN

I. E. DROBINSKAYA, I. A. KOZLOV and V. P. SKULACHEV
Department of Bioenergetics, A. N. Belozersky Laboratory of Bioorganic Chemistry and Molecular Biology, Moscow State University, Moscow 117234, USSR

Received 21 August 1978
Revised version received 2 October 1978

1. Introduction

Oligomycin, introduced into biochemical research by Lardy et al. [1–3], inhibits oxidative phosphorylation and all the ATP-dependent processes occurring on the coupling membrane of mitochondria. In several laboratories it has been shown that oligomycin greatly reduces the proton conductivity of the mitochondrial membrane fragments, which are devoid of coupling factor F_1 (reviewed [4]). This observation suggests the inhibiting effect of oligomycin to be due to the inactivation of the H^+-conducting system operating between factor F_1 and the external surface of the mitochondrial membrane. Such an explanation of the oligomycin effect is in good agreement with the chemiosmotic principle of oxidative phosphorylation [5,6]. On the other hand, there are indications that, besides the above-mentioned action, oligomycin gives rise to some other effect(s) closely related to the energy coupling mechanism. The complex character of the inhibiting effect of oligomycin follows from the fact that uncoupler-protonophores, which greatly increase the proton conductivity of the mitochondrial membrane, do not reverse oligomycin action. Possibly, the binding of oligomycin to the hydrophobic proteins of H^+-ATPase results in conformational changes of the enzyme complex in toto which affect the catalytic properties of factor F_1. Thus, Klein et al [7] showed that oligomycin induces a decrease in the affinity of the membrane-bound factor F_1 to the protein ATPase inhibitor. The occurrence of some relations between the factor F_1 conformation and oligomycin binding to the hydrophobic proteins of ATPase is also indicated by the data of Lee and Ernster [8] who established that detachment of factor F_1 from submitochondrial particles leads to a great increase in the affinity of the particles to oligomycin. Azzi and Santato [9] showed that the oligomycin inhibition of the ATP-dependent processes occurring on the mitochondrial membrane increases if the oligomycin treatment is carried out in the presence of ATP. Bertina et al. [10] noted that the kinetics of inhibition by oligomycin depend on the energisation of the mitochondrial membrane.

In this paper we show that a small portion of ATPase of submitochondrial particles is insensitive to oligomycin. It is rendered oligomycin-sensitive by addition of ATP. The effect of ATP cannot be explained by the generation of $\Delta\bar{\mu}_{H^+}$ coupled with ATP hydrolysis. It has also been shown that the binding of the non-hydrolysable analog of ATP, AMP–PNP, does not increase the H^+-ATPase affinity to oligomycin.

2. Materials and methods

Beef heart mitochondria were isolated by the method in [11]. The submitochondrial particles were obtained by the ultrasonication of beef heart mitochondria by the method in [12]. The ATPase activity of submitochondrial particles was determined by the pH-metric method [13,14]. The values obtained were 0.35–0.70 μmol/min . mg. The oxidative phosphorylation rate was measured in a reaction mixture containing submitochondrial particles (0.4 mg/ml), 0.01 M AMP, 0.005 M K_2HPO_4, 0.01 M $MgSO_4$, 0.001 M

Abbreviation: PCB^-, phenyl dicarba undecaborane anion

NADP, 0.25 M sucrose, 0.001 M glucose, 5×10^{-6} M rotenone, hexokinase (EC 2.7.1.1) and glucose-6-phosphate-dehydrogenase (EC 1.1.1.49). The rate of ATP synthesis was about 0.1 μmol/min . mg. PCB^- was used as a probe for membrane potential (for the method, see [15]). Protein concentration was measured by the biuret method [16].

3. Results and discussion

In fig.1 PCB^- responses of the submitochondrial particles are shown. It can be seen that, when added after ATP, oligomycin completely inhibits ATP-supported membrane potential formation (curve a). Note that the effect of oligomycin added after ATP developed in ~3 min. On the other hand, 3 min incubation of the particles with oligomycin prior to ATP addition fails to abolish the ATP-induced generation of membrane potential as shown by a temporary PCB^- uptake (curve b). A similar picture was observed if the particles (40 mg/ml) were preincubated with oligomycin (from

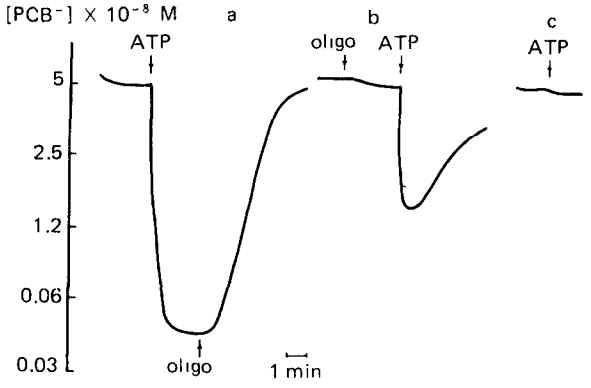

Fig.1. ATP-dependent generation of membrane potential in submitochondrial particles. The incubation mixture in the measuring cell contained 50 mM Tris–H_2SO_4 buffer (pH 7.5), 0.25 M sucrose, 2×10^{-6} M PCB^- and 0.7 mg/ml submitochondrial particles. Additions: 1 mM ATP, 1 mM $MgSO_4$ and oligomycin 1.3 μg/ml. (a,b) control preparations of submitochondrial particles; (c) submitochondrial particles preincubated with oligomycin for 15 min at 20°C. The preincubation medium contained: 10 mM HEPES–H_2SO_4 buffer (pH 7.5), 0.25 M sucrose, 4 mM ATP, 4 mM $MgSO_4$, 4 μg oligomycin and 4 mg particles in total vol. 0.08 ml. Preincubation medium, 0.05 ml, was added to the measuring cell containing 3 ml incubation mixture (see above).

0.5–2 μg/mg protein of the particles) for 15 h at 0°C or for 15–60 min at room temperature. The decrease in the pH of the preincubation medium to 5.0, as well as the addition of 2,4-dinitrophenol were without effect.

On the other hand, if the oligomycin-containing preincubation medium is supplemented with ATP, 15 min preincubation proves sufficient to completely prevent the PCB^- uptake induced by ATP addition to the incubation mixture (fig.1c). As was shown in the control experiment, the difference between fig.1b and 1c cannot be accounted for by the presence of a small amount of ADP formed from ATP during preincubation under conditions of fig.1c. Addition of the corresponding amount of ADP to the incubation mixture does not prevent ATP-induced PCB^- uptake in the samples without oligomycin (not shown).

From the results obtained it follows that the presence of the substrate, Mg-ATP, in the incubation mixture accelerates the development of inhibition of mitochondrial ATPase by oligomycin. Similar results were obtained by Azzi and Santato [9] who studied the effect of oligomycin on the ATP-dependent changes in the anilino-8-naphthalenesulphonate fluorescence. The effect observed may be a result of:

(1) Energisation of the membrane when the particles are preincubated with Mg-ATP;

(2) ATP-dependent changes in the structure of the ATPase complex.

To choose between these two possibilities, we preincubated submitochondrial particles with oligomycin in the presence of succinate (fig.2a), ATP and 2,4-dinitrophenol (fig.2b), or the non-hydrolysable ATP analog, AMP–PNP (fig.2c). It was found that ATP accelerates the interaction of oligomycin with particles in spite of the presence of 2,4-dinitrophenol, whereas succinate and AMP–PNP are ineffective.

The results obtained show that the hydrolysis of ATP and not the energisation of the membrane nor the binding of the ATPase substrate is responsible for the enhancement of the oligomycin inhibition. It is noteworthy that AMP–PNP has been used in several laboratories as an effective inhibitor of mitochondrial ATPase (reviewed [4]). There are grounds for supposing that this substrate analog is bound by the active site of ATPase when the mitochondrial membrane is de-energised [4,17]. The AMP–PNP concentration

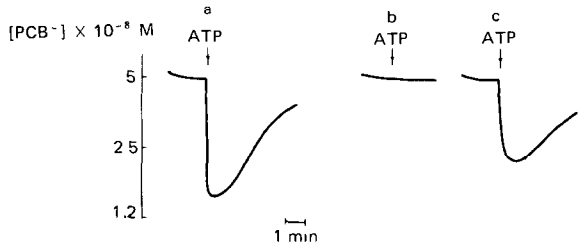

Fig.2. ATP-dependent generation of membrane potential in submitochondrial particles preincubated with oligomycin in the presence of succinate (a), ATP and 2,4-dinitrophenol (b) and AMP–PNP (c). For the contents of the incubation mixture in the measuring cell, see fig.1. Additions: 1 mM ATP and 1 mM $MgSO_4$. Before addition of submitochondrial particles to the measuring cell, they were preincubated for 15 min in a medium containing 10 mM HEPES–H_2SO_4 buffer (pH 7.5); 0.25 M sucrose, 40 mg/ml particles, oligomycin 1 μg/mg protein of the particles, and also 50 mM succinate (a); 4 mM ATP, 4 mM $MgSO_4$ and 200 μM 2,4-dinitrophenol (b); 50 μM AMP-PNP (c), in total vol. 0.08 ml. In the case of (a) the particles were treated with 2.5 mM NaCN before being added to the measuring cell. In all cases, 0.08 ml preincubation medium was added to 3 ml incubation mixture.

used in fig.2c (50 μM) is saturating for mitochondrial ATPase.

In the same series of experiments, the effect of oligomycin on the rate of ATP hydrolysis by submitochondrial particles was studied. The particles were preincubated for 15 min in the following medium: 10 mM HEPES buffer (pH 7.5), 0.25 M sucrose, oligomycin (1 μg/mg protein), with or without 4 mM ATP and 4 mM $MgSO_4$. Small aliquots of the preincubation medium were removed to measure ATPase activity for 1 min. The incubation mixture for ATPase activity measurements contained 3 mM Tris–HCl (pH 8.3), 0.25 M sucrose, 20 μM 2,4-dinitrophenol, 2 mM $MgSO_4$ and 2 mM ATP. A result, which seemed surprising, was obtained. It was found that both in the presence and in the absence of ATP in the preincubation mixture oligomycin strongly inhibits ATPase activity. The degree of inhibition was as high as ~90–95%.

By comparing the last observation with the data obtained in experiments with PCB^- (see above) or anilinonaphthalene sulfonate [9], we can conclude that ATP accelerates the reaction of oligomycin with a very small portion (apparently < 5%) of the H^+-ATPase pool, which is still competent to form a membrane potential under conditions when the main portion of the H^+-ATPase is arrested by oligomycin in an ATP-independent manner.

It should be stressed that ATPase can produce a measurable membrane potential at a low rate of ATP hydrolysis, if the proton-translocating capacity of the H^+-ATPase in samples without oligomycin is greatly in excess of the capacity of the $\Delta\bar{\mu}_{H^+}$-consuming processes, including PCB^- transport. This suggestion was confirmed in the experiment shown in fig.3. Figure 3 demonstrates Mg^{2+} titration of the ATPase rate in the presence of 20 μM 2,4-dinitrophenol and the amplitude of the ATP-dependent PCB^- uptake in submitochondrial particles. One can see that a 3-fold decrease in the ATPase activity due to the lowering of the Mg^{2+} level gives rise to a rather small decrease in the PCB^- response. The semi-maximal inhibition of the latter required a more than 20-fold decrease in the ATP hydrolysis rate.

Thus, it can be concluded that in submitochondrial particles there are two portions of H^+-ATPase, both competent in proton translocation, differing in the mode of reaction with oligomycin. One of them, present in a large amount, does not require ATP hydrolysis to combine with oligomycin. The other, present in small amounts is inhibited by oligomycin only when ATP hydrolysis takes place. The accompanying membrane energisation proves unnecessary for the ATP hydrolysis effect.

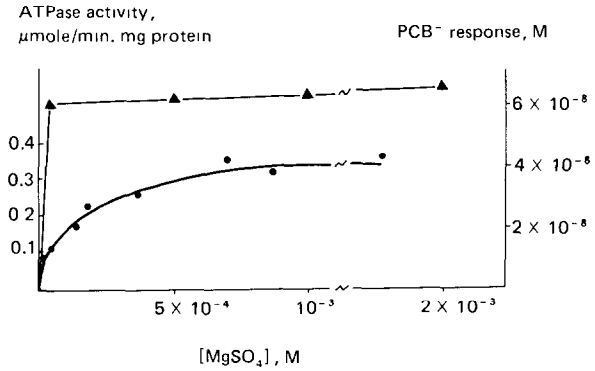

Fig.3. The Mg^{2+} dependence of the ATP-linked PCB^- response and of the rate of the ATPase reaction in submitochondrial particles. For the incubation mixture see fig.1.

References

[1] Lardy, H. A., Johnson, D. and McMurray, W. C. (1958) Arch. Biochem. Biophys. 78, 587–597.
[2] Lardy, H. A., Connelly, J. L. and Johnson, D. (1964) Biochemistry 3, 1961–1968.
[3] Lardy, H. A., Wittonsky, P. and Johnson, D. (1965) Biochemistry 4, 552–554.
[4] Kozlov, I. A. and Skulachev, V. P. (1977) Biochim. Biophys. Acta 463, 29–89.
[5] Mitchell, P. (1961) Nature 191, 144–148.
[6] Mitchell, P. (1966) Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research, Bodmin.
[7] Klein, G., Satre, M. and Vignais, P. (1977) FEBS Lett. 84, 129–134.
[8] Lee, C. P. and Ernster, L. (1966) in: Regulation of Metabolic Processes in Mitochondria (Tager, J. M. et al. eds) vol. 7, pp. 218–234, Elsevier, Amsterdam.
[9] Azzi, A. and Santato, M. (1970) FEBS Lett. 7, 135–138.
[10] Bertina, R. M., Steenstra, I. A. and Slater, E. C. (1974) Biochim. Biophys. Acta 368, 279–297.
[11] Crane, F. L., Gleen, J. L. and Green, D. E. (1956) Biochim. Biophys. Acta 22, 475–487.
[12] Hansen, M. and Smith, A. L. (1964) Biochim. Biophys. Acta 81, 214–222.
[13] Chance, B. and Nishimura, M. (1967) Methods Enzymol. 10, 641–690.
[14] Kozlov, I. A. and Kononenko, V. A. (1975) Bioorgan. Khim. 1, 489–493.
[15] Grinius, L. L., Jasaitis, A. A., Kadziauskas, Yu. P., Liberman, E. A., Skulachev, V. P., Topali, V. P., Tsofina, L. M. and Vladimirova, M. A. (1970) Biochim. Biophys. Acta 216, 1–12.
[16] Gornell, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol. Chem. 177, 751–766.
[17] Melnick, R. L., Tavares De Sousa, J., Maguire, J. and Packer, L. (1975) Arch. Biochem. Biophys. 166, 139–144.