The effect of oligomycin on rat liver mitochondria respiring in state 4

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It has been found that oligomycin inhibits up to at least 50% state-4 mitochondrial respiration. A time dependence of oligomycin inhibition has been shown. A titration curve for state-4 respiration of sigmoidal profile has been presented. The possibility of misreading this oligomycin effect, so far never reported, has been excluded by evaluating the quality of mitochondrial preparations used in respect to their morphological, functional and electrochemical properties. The conclusion has therefore been put forward that the most part of respiration in steady-state-4 is driven by ATP synthesis.

Oligomycin *Respiratory state* *Respiration rate* *Membrane potential* *Rat liver mitochondria*

1. INTRODUCTION

Oligomycin has long been known as a specific inhibitor of that portion of respiration that is tightly coupled to phosphorylation [1,2]. The effects and the mechanism of action of this inhibitor have therefore been investigated essentially only under the conditions of state-3 respiration, and little, if any, attention has been paid to the possible effect of the oligomycin on mitochondria respiring under the conditions of state 4. In fact it is a commonly accepted view that the respiration observed in state 4 is not coupled to phosphorylation, but results from a number of energy-dissipating processes such as cycling of Ca^{2+} [3] and/or H^+ [4,5], passive leakage of protons [6]. The observation that oligomycin reduces the rate of state-3 respiration to values similar to those seen in state 4 [7] strengthened this view.

Recently, evidence has been obtained that a limited but significant synthesis of ATP occurs in rat liver mitochondria respiring under the conditions of state 4 [8,9].

Abbreviations: DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

It thus seems of interest to study the effect of oligomycin on state-4 respiration, also in view of the fact that oligomycin may be proved to provide a convenient tool to evaluate the contribution of different energy-utilizing processes to the respiration measured under the conditions of state 4.

2. MATERIALS AND METHODS

Bovine serum albumin, fatty acid-free, and oligomycin were from Sigma (St Louis MO).

Rat liver mitochondria were isolated in 0.25 M sucrose according to a standard procedure [10].

Electron microscope analysis by the negative staining technique was used to test the homogeneity and the intactness of the mitochondrial preparations [10,11]. Furthermore, the inability of these preparations to oxidize externally-added NADH was taken as evidence of the structural and functional integrity of the inner membrane [12].

The efficiency of the energy-transducing membrane was tested by measuring the transmembrane potential ($\Delta\psi$). $\Delta\psi$ was measured at 25°C, in a final volume of 1.5 ml by monitoring with a tetraphenylphosphonium-selective electrode the movements of tetraphenylphosphonium across the membrane, as in [14]. An inner mitochondrial

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volume of 1.1 μl/mg protein was assumed [15].

The respiratory control index (RCI) and the phosphorylative capacity, as determined from the polarographic traces [13], were the parameters used to test the potential functional ability. Only preparations having RCI higher than 4 were used. The oxygen uptake was assayed with a Clark oxygen electrode at 25°C in a final volume of 3.0 ml.

The incubation medium for assaying both the metabolic and the electrochemical parameters had the following composition: 100 mM NaCl; 10 mM $MgCl_2$; 10 mM Tris–HCl buffer; pH 7.4; 10 mM NaK–phosphate buffer (pH 7.4) and 1.6 mM Na-pyruvate plus 0.4 mM L-malate as the substrate.

The mitochondrial concentration, as determined by the biuret method, ranged between 3–3.5 mg protein/ml.

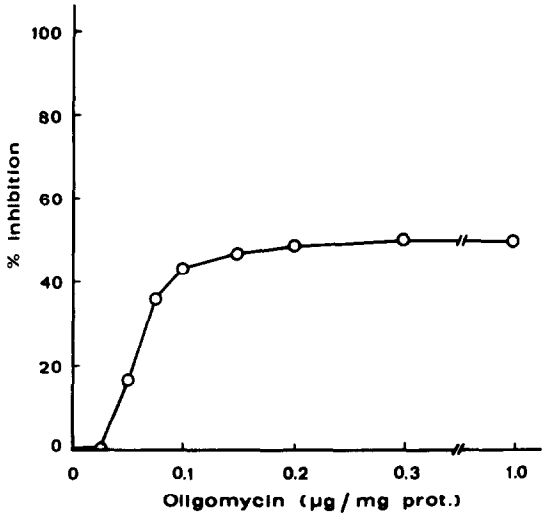

Fig.1. Inhibition by oligomycin of state-4 respiration. Mitochondria were incubated for 1 min under state 4(b) conditions. Oligomycin was then added in a constant volume of 10 μl. All other conditions as in table 1.

3. RESULTS AND DISCUSSION

Table 1 shows that addition of oligomycin (1 μg/mg protein) to rat liver mitochondria in respiratory state 4, reduces the respiratory rate by about 50%. Addition of 25 μM DNP or 1 μM FCCP reverses this inhibition (not shown).

Fig.1 illustrates the relation between concentration of oligomycin and inhibition of state-4 respiration. It appears that the inhibition–concentration curve is sigmoidal and presents its steepest part in the range 0.05–0.1 μg inhibitor/mg protein. The maximal inhibition is attained at 0.3 μg oligomycin/mg protein. Both the kinetics of inhibition by suboptimal concentrations of oligomycin and the concentration for maximal inhibition observed in state 4 are very similar to those found in mitochondria in state 3 [15]. In fact, sigmoidal inhibition–concentration curves were observed when oligomycin was added to mitochondria in respiratory state 3 [15,16], or in the presence of an ADP regenerating system; i.e., hexokinase plus glucose [2,17].

Table 1

Distribution of control of state-4 respiration in isolated rat liver mitochondria

Conditions	Respiratory rate (ngatoms $O.min^{-1}.mg\ protein^{-1}$)	Differential contribution of energy-utilizing processes to state-4 respiration
State 4	12.50	100%, total respiration
+ 1 μg oligomycin/mg	6.25	50%, coupled respiration
+ 1 mM EDTA	11.37	9%, Ca^{2+}-cycling
+ 0.1% albumin	10.62	15%, fatty acids oxidation
		26%, other processes

Mitochondria were incubated as described in section 2, for 1 min in state 4(b); i.e., after state-3 to state-4 transition, before addition of oligomycin. State 3 was obtained by adding 0.33 mM ADP. When present, EDTA and albumin were added to the incubation medium before addition of mitochondrial suspension. The data are for 1 of 5 identical experiments in which the results were within 5% of each other

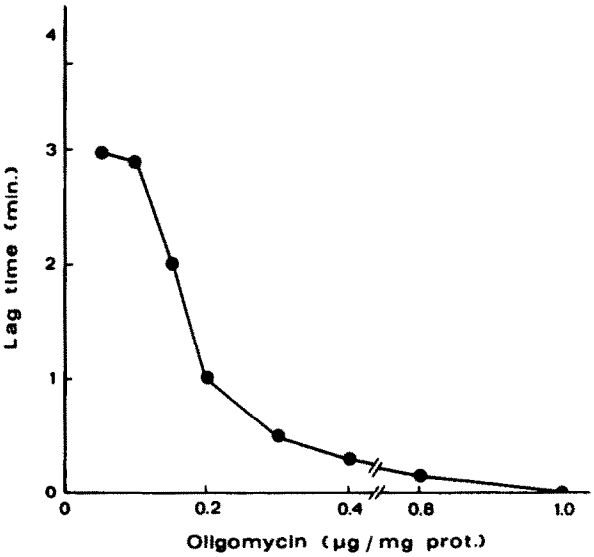

Fig.2. Effect of oligomycin concentration on the onset of maximal inhibition of state-4 respiration. Experimental conditions as in fig.1.

Oligomycin inhibition of state-3 respiration was observed to be a time-dependent reaction [15,16]. A lag phase is similarly seen when oligomycin is added to mitochondria respiring in state 4. Fig.2 shows that there is a sigmoidal relation between lag phase and oligomycin concentration.

In order to investigate a possible contribution of structural damage at the level of the inner membrane to the occurrence of oligomycin inhibition of state-4 respiration, the membrane potential of mitochondria was measured (table 2). It appears that upon addition of 2 mM pyruvate plus malate, rat liver mitochondria develop a membrane potential of about 180 mV (negative inside). This potential is maintained for periods longer than 6 min. An essentially similar value of $\Delta\psi$ is observed after transition from state 3 to state 4(b); i.e., when all added ADP is phosphorylated to ATP. Similar results are obtained when ATP is added to mitochondria in state 4. Under all 3 conditions, the extent of oligomycin inhibition is essentially the same. The similarity of $\Delta\psi$-values and of the degree of inhibition by oligomycin in the absence and in the presence of ATP, both endogenous and exogenous, clearly indicate that the adenine nucleotide translocator is not rate-limiting under these conditions [18]. A similar conclusion has been reached in [16] for oligomycin inhibition of state-3 respiration.

From the results obtained by the use of oligomycin, it appears that about 50% of the respiration observed in state 4 is coupled to phosphorylation. This is in agreement with the recent findings of a respiration-dependent ATP synthesis in mitochondria respiring under state-4 con-

Table 2

Effect of oligomycin on oxygen uptake of rat liver mitochondria in respiratory state 4

Metabolic conditions	$\Delta\psi$ (mV)	Respiratory rate (ngatoms O.min^{-1}.mg protein^{-1})		% Inhibition by oligomycin
		− Oligomycin	+ Oligomycin	
State 4(a)	178	12.1	6.29	48
State 4(b)	181	12.8	6.40	50
State 4(a) + ATP	180	12.6	6.30	50

Mitochondria were incubated as described in section 2, for 1 min in state 4(a), in state 4(a) in the presence of 0.33 mM ATP, and in state 4(b). Later, 10 μl oligomycin solution (1 μg/mg protein) in dimethylformamide were added. Under the conditions used, the mitochondria maintained a linear respiration in state 4 for more than 6 min in the absence of oligomycin. State-4 respiration was unaffected by dimethylformamide. The transmembrane potential ($\Delta\psi$) was measured as described in section 2, in the presence of 20 μM tetraphenylphosphonium chloride

ditions [9]. In table 1 an evaluation of the contribution of different energy-utilizing processes to the whole mitochondria respiration in state 4 is shown. The portion of respiration consequent to Ca^{2+}-cycling was evaluated by the percentage of the respiration reduction in the presence of EDTA, whereas that due to fatty acid oxidation was measured by the use of albumin. It appears that the cycling of Ca^{2+} accounts for about 10% of the respiration observed under the conditions used; i.e., in the presence of 10 mM Mg^{2+}; the oxidation of fatty acids accounts for 15% of the total respiration and the phosphorylative process accounts for about 50% of the oxygen consumption. The remaining part of respiration may be due to passive leakage of protons through the membrane [6] although other energy-utilizing processes cannot be excluded.

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