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AN INVESTIGATION ON THE EFFECT OF OLIGOMYCIN ON STATE-4 RESPIRATION IN ISOLATED RAT-LIVER MITOCHONDRIA

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The inhibitory action of oligomycin on State-4 respiration in rat-liver mitochondria has been investigated in detail with regard to the extent, mode and characteristics of the inhibition. The possibility that this effect may be due either to some damage of the mitochondrial preparation used or to the presence of heavy contaminations by microsomes has been excluded. It has been found that the concentration of specific binding sites is the same in State 4 as in State 3. The extent of the inhibition appears to be related to the ADP concentration, rather than to ATP/ADP ratios. The inhibition of this antibiotic on State-4 respiration does not depend on the experimental conditions used (i.e., choice of substrates or composition of the reaction medium). In agreement with these observations, it has been found that the membrane potential of State 4 is significantly increased when oligomycin is added. All these results provide further evidence to the conclusion that a large portion of State-4 respiration is linked to phosphorylation.

Introduction

One of the long-standing problems of Bioenergetics is why the respiration of isolated mitochondria is not zero in respiratory State 4 according to the operational definition given by Chance and Williams [1], i.e., in the experimental steady state obtained by incubating isolated mitochondria in the presence of substrate, inorganic phosphate and oxygen, but in the absence of added ADP. In fact, in spite of consistent oxygen consumption, no net accumulation of ATP is measured under this condition [2]. The occurrence of a number of energy-utilizing and/or energy-dissipating processes was therefore proposed to account for oxygen uptake in this state [3–8]. These include: energy-dependent Ca^{2+} cycling [4,5]; recycling of H^+ through the inner membrane [6–8]; extramitochondrial ATPase activity [3], etc.

More recently, evidence has been obtained showing that a respiration-dependent ATP synthesis also occurs under the conditions of respiratory State 4 [9–11] and that the net accumulation of ATP is prevented by the concomitant activity of a mitochondrial ATPase [11]. This activity has been proposed to play an important physiological role in the structural and functional transition from respiratory State 3 to respiratory State 4 [11].

In the attempt to define the precise aliquot of State 4 respiration, that is linked to ATP synthesis, oligomycin has been used as a specific inhibitor of coupled respiration [12]. However, little, if any, information exists in the literature as to the effect of oligomycin on the respiration of isolated mitochondria incubated in respiratory State 4. Oligomycin, as a specific inhibitor of tightly coupled respiration [13,14] exhibits its maximal effect

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

on State-3 respiration [15,16]; however, there exist in the literature some scattered indications of effects also on State-4 respiration [17–19]. For example, it has been reported that in rat-liver mitochondria the value of the respiratory control index increases about two-fold if it is measured as the ratio of the rate of oxygen uptake in the presence of ADP (State 3) to the rate of oxygen uptake in the absence of ADP but in the presence of oligomycin (State 4 plus oligomycin) [18,19]. This indicated that oligomycin lowers the rate of State 4 respiration by a 50%. Furthermore, in a study aimed at analyzing the effect of different monovalent cations in the incubation medium on mitochondrial respiration, it has been reported that oligomycin inhibits to some extent the respiration of isolated liver mitochondria incubated under conditions essentially similar to those of State 4. [17].

It seemed therefore of interest to investigate in some detail the extent, mode and characteristics of the effects of oligomycin on State 4 respiration as well as on the membrane potential of isolated mitochondria in respiratory State 4.

Preliminary reports of this research have been presented elsewhere [20,21].

Materials and Methods

Rat-liver mitochondria were isolated in 0.25 M sucrose according to a standard procedure [22]. Microsomes were prepared as described by Sottocasa et al. [23].

The incubation medium for assaying the metabolic and the electrochemical parameters, unless otherwise indicated, had the following composition: 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl buffer (pH 7.4), 10 mM sodium-potassium phosphate buffer (pH 7.4) and 1.6 mM sodium pyruvate plus 0.4 mM L-malate as the substrate. The oxygen uptake was measured with a Clark oxygen electrode at 25 °C in a final volume of 3 ml. The concentration of mitochondrial protein was 3.0–3.5 mg per ml. The respiratory states were those defined by Chance and Williams [1] on the basis of the factors limiting the respiratory rate.

The transmembrane potential ($\Delta\psi$) 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 according to Kamo et al. [24] and to Lötscher et al. [25]. The inner mitochondrial volume was assumed to correspond to 1.1 μl per mg protein [26]. The mitochondrial concentration ranged from 3.0 to 3.5 mg protein per ml.

ATP and ADP concentrations in the mitochondrial suspensions were determined spectrophotometrically according to the procedure described by Jacobus et al. [27].

Glucose-6-phosphatase activity was measured at 37 °C as described by Baginski et al. [28].

The concentration of cytochrome P-450 was calculated from the CO difference spectrum of the reduced sample [29].

Protein concentration was determined by the Lowry method [30].

Oligomycin, ATP, ADP and hexokinase type III were purchased from Sigma (St. Louis, MO, U.S.A.). Other enzymes and nucleotides were obtained from Boehringer (Mannheim, F.R.G.)

Results

Table I shows the effect of oligomycin on State-4 respiration. It appears that addition of 1 μg of oligomycin per mg protein to isolated rat-liver mitochondria in respiratory State 4, inhibits the oxygen uptake by a 50%. Addition of uncouplers, such as dinitrophenol or FCCP, completely reverses the inhibition.

The inhibition by oligomycin on State 4 respiration does not depend on a partial transition to State 3, caused by ADP produced by a contaminating extramitochondrial ATPase or by an activated ATPase in damaged mitochondria. In fact, as shown in Table II, atractyloside, a specific inhibitor of ADP/ATP translocator, has a limited inhibitory effect on State-4 respiration, and does not prevent the further effect by oligomycin. On the basis of the effect of atractyloside it can be deduced that from 10 to 15% of respiration measured in State 4 may be related to the production of limited amount of ADP by an extramitochondrial ATPase and not to the activation of a mitochondrial ATPase due to structural damage. In fact atractyloside completely abolishes

TABLE I

EFFECT OF OLIGOMYCIN ON OXYGEN UPTAKE OF RAT-LIVER MITOCHONDRIA IN RESPIRATORY STATE 4

Mitochondria were incubated as described in Materials and Methods for 1.5 min in State 4(b), i.e., after transition from State 3 to State 4. State 3 was obtained by addition of 0.33 mM ADP. Oligomycin was then added. DNP or FCCP were added after 2 min incubation in the presence of oligomycin. The data are for one of four identical experiments in which the results were within 5% of each other.

Conditions	Addition	Respiratory rate (ngatom 0/min per mg protein)	Inhibition by oligomycin (%)
State 4	none	12.6	–
State 4	1 μg/mg oligomycin	6.3	50
State 4 + oligomycin	25 μM DNP	50.6	0
State 4 + oligomycin	2.5 μM FCCP	51.4	0

the effect of added ADP (Table II) thus showing that the structure of mitochondria is well preserved, a conclusion further supported by the observation that the mitochondrial preparations used do not oxidize external NADH [34], and have a transmembrane potential typical of intact mitochondria, i.e., of about 180 mV under State-4 conditions [24]. Measurements of G-6-Pase activity, as a marker of microsomal contamination in the mitochondrial preparations used, indicates that this contamination is very limited; this conclusion is also supported by measurements of cytochrome P-450 in the same mitochondrial fractions: the concentration of this cytochrome is in fact that structurally present in mitochondria [31,32] (Table III). The zero effect of ouabain, an inhibitor of sodium-activated microsomal ATPase [33], on State-4 respiration further supports this conclusion (Table III).

Fig. 1 shows that the maximum inhibition level of State-4 respiration is attained in the presence of 0.3 μg of oligomycin per mg mitochondrial protein; higher concentrations of this antibiotic, do not enhance the extent of the inhibition. The same figure illustrates the characteristics of the inhibition-concentration curve for State 4 as compared to that for State 3. Both curves are sigmoidal and the maximal inhibition is attained in both conditions at the very same concentration of inhibitor. The chief difference lies in the extent of the maximal inhibition attained: 90% in the case of State-3, and 50% in the case of State-4, respiration.

The inhibitory effect of oligomycin on State-4 respiration does not depend on the composition of the reaction medium used, as far as the ionic species are concerned. In fact, as shown in Table IV, the inhibition of oligomycin on State-4 respiration is the same if Na^+ is substituted for by either

TABLE II

EFFECT OF ATRACTYLOSIDE ON OXYGEN UPTAKE OF RAT-LIVER MITOCHONDRIA IN RESPIRATORY STATE 4

Mitochondria were incubated as described in Materials and Methods for 2 min in State 4. 4 μM atractyloside or 1 μg per mg protein of oligomycin were then added. 0.33 mM ADP or 1 μg per mg of oligomycin were added after 2 min incubation in the presence of atractyloside. All other conditions as in Table I.

Conditions	Addition	Respiratory rate (ngatom 0/min per mg protein)	Inhibition of respiration (%)
State 4	–	12.2	–
State 4 + atractyloside	–	10.8	12
State 4 + atractyloside	ADP	10.8	12
State 4 + atractyloside	oligomycin	6.6	46
State 4 + oligomycin	–	6.5	47

TABLE III

EVALUATION OF THE MICROSOMAL CONTAMINATION IN THE MITOCHONDRIAL FRACTION FROM RAT LIVER

Mitochondria were incubated for 3 min in State 4 in the presence of added 0.33 mM ATP. Then 0.2 mM ouabain was added. Cytochrome P-450 concentration and glucose-6-phosphatase activity were estimated as described in Materials and Methods.

Fraction	Cytochrome P-450 (nmol per mg protein)	Glucose-6-phosphatase (nmol Pi/min per mg protein	State 4 respiration	
			− Ouabain	+ Ouabain
Microsomes	1.098	273.70	–	–
Mitochondria	0.111	9.01	12.9	12.9
Microsomes / Mitochondria	–	30.4	–	–

K^+ or sucrose in the reaction medium. Similarly, the inhibition is found the same by varying the concentration of Mg^{2+} from 10 mM to 2.5 mM.

The inhibitory effect of oligomycin does not depend on the choice of substrate and it is observed in the presence of both NAD − linked substrates and succinate (Table V). This finding is consistent with previous observations on the effect of oligomycin on State-3 respiration [13,14,35,36]. It appears from Table V that the higher the respiratory control, the lower the degree of inhibition by oligomycin, the lowest inhibition being observed in the presence of succinate as the substrate.

The degree of oligomycin inhibition appears to correlate with the absolute concentration of ADP rather than the ATP/ADP ratios (Table VI). This has been found by using an experimental approach similar to that described by Jacobus et al.

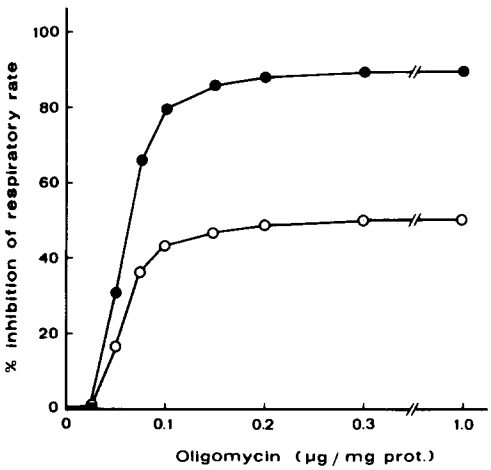

Fig. 1. Inhibition by oligomycin of State-4 and State-3 respiration. Mitochondria were incubated for 1 min under State 3 (●), obtained by the addition of 1.5 mM ADP, and for 1 min under State 4 (○), i.e., following the exhaustion of the added 0.33 mM ADP. Oligomycin was then added. All other conditions as in Table I.

TABLE IV

EFFECT OF COMPOSITION OF THE INCUBATION MEDIUM ON THE INHIBITION BY OLIGOMYCIN OF STATE 4 RESPIRATION

Mitochondria were incubated for 3 min in State 4, before addition of 1 µg/mg protein of oligomycin. Where indicated 0.1 M NaCl in the incubation medium was substituted for by 0.1 M KCl and 0.2 M sucrose, respectively. When different concentrations of Mg^{2+} were used, the osmolarity of standard medium was maintained constant by adding appropriate amounts of NaCl. All other conditions as in Table I

Incubation medium	Concn. of Mg^{2+} (mM)	State 4 respiration	Inhibition by oligomycin (%)
Sodium	10	12.8	48
Sodium	5	13.0	49
Sodium	2.5	13.8	51
K^+	10	12.2	45
Sucrose	10	12.3	44

TABLE V

EFFECT OF VARIOUS SUBSTRATES ON THE MAXIMAL INHIBITION BY OLIGOMYCIN OF STATE-4 RESPIRATION

Mitochondria were incubated in State 4(b) for 1.5 min as described in Table I. Oligomycin (1 μg per mg protein) was then added. The respiratory control index R.C.I. is the ratio of respiratory rate in State 3 to that in State 4. ADP/0 ratios were determined from the polarographic traces. All other conditions as in Table I

Substrate 2 mM	State-4 respiration	R.C.I.	ADP/0	Inhibition of State-4 respiration by oligomycin (%)
Pyruvate +				
L-Malate	12.8	4.34	2.70	50
Citrate	13.2	4.35	2.68	48
α-Chetoglutarate	14.4	5.50	2.64	40
L-Glutamate +				
L-Malate	13.9	6.50	2.80	39
Succinate	20.4	6.90	1.90	38

[27]. In this experimental design, the respiratory rates are measured either by varying the concentration of ATP in the presence of unvaried concentration of ADP maintained by a given amount of hexokinase as the ATP-regenerating system, or alternatively by making variable the concentration of ADP by varying the amount of hexokinase, in the presence of unvaried concentration of ATP. The results are illustrated in Table VI. It appears from the table that the degree of oligomycin inhibition correlates with the rate of respiration and the concentration of ADP, but not with the ATP/ADP ratios.

The effect of oligomycin on State-4 respiration addes further support to the conclusion, reached by a different experimental approach, that a large portion of the respiration measured under the conditions of State 4, is normally coupled to phosphorylation, the accumulation of ATP being prevented by the concomitant action of a mitochondrial ATPase [11]. Consistent with this conclusion are the results illustrated in Fig. 2. The figure shows the variations of the transmembrane potential by varying the metabolic states of isolated mitochondria in the absence and in the presence of oligomycin. When incubated in State-4 conditions with pyruvate as the substrate, the mitochondria develop a transmembrane potential

TABLE VI

EFFECT OF ATP/ADP RATIOS ON THE INHIBITION BY OLIGOMYCIN OF MITOCHONDRIAL RESPIRATION

Mitochondria were incubated for 1.5 min in State 4(a) in the presence of ATP. Hexokinase, at the indicated concentrations, plus 10 mM D(+)-glucose, was then added. After 1.5 additional min, oligomycin (1 μg per mg protein) was added. The control State-3 rate was determined upon the addition of 0.5 mM ADP into the medium containing ATP, but lacking glucose and hexokinase. State 4 was approx. 22% of the ADP state-3 rate. The ATP and ADP concentrations were determined in control samples as described in Materials and Methods. All other conditions as in Table I. IU, enzyme units.

Conditions	State 3 respiration (%)	ATP/ADP	ADP (μM)	Inhibition of respiration (%)
(1) Constant ATP = 1.0 mM				
(a) variable hexokinase = 0.25 IU	28	65	15	56
(b) variable hexokinase = 2.5 IU	88	10	180	80
(2) Constant Hexokinase = 1.5 IU				
(a) variable ATP = 1.0 mM	90	45	98	82
(b) variable ATP = 20 μM	27	8	13	55

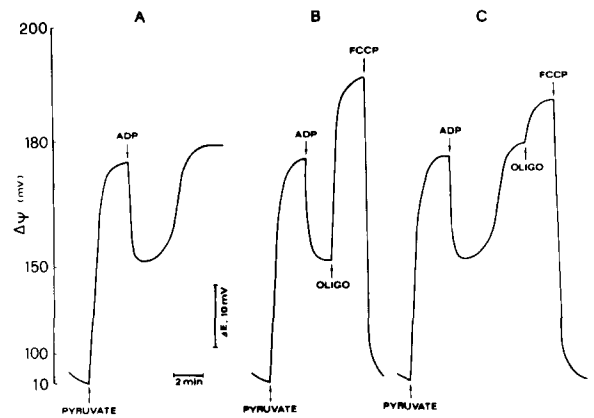

Fig. 2. Effect of oligomycin on mitochondrial $\Delta\psi$ under different metabolic conditions. Mitochondria were incubated as described in Materials and Methods. The transmembrane potential ($\Delta\psi$) was measured as described in Materials and Methods in the presence of 20 μM tetraphenylphosphonium chloride. ΔE, electrode potential. All other conditions as in Table I.

(negative inside) of about 176 mV. In agreement with previous observations [6,24,37], addition of ADP, that causes transition to State 3, induces an immediate fall of $\Delta\psi$ to about 152 mV. When all the ADP added is phosphorylated to ATP, the transmembrane potential returns to nearly its initial value, i.e., 178 mV. The fall in the transmembrane potential clearly correlates with the utilization of the energy during ATP synthesis [24,38]. If oligomycin is added during respiratory State 3, the transmembrane potential increases to values significantly higher than those observed after the transition to State 4, 192 and 178 mV, respectively. A similar increase in the value of the transmembrane potential is observed if oligomycin is added to mitochondria in respiratory State 4: 187 vs. 176 mV.

Discussion

It is well established in the literature that oligomycin inhibits respiration only when tightly coupled to phosphorylation [13,14]. Therefore, the inhibitory effect of oligomycin on the oxygen consumption by mitochondria respiring in State 4, reported in the present results, indicates that a large portion of State-4 respiration is linked to ATP synthesis. This conclusion is further supported by the effect of oligomycin on the transmembrane potential. In fact, it has been found that addition of oligomycin to mitochondria respiring in State 4 results in a significant increase in the membrane potential. This means that the value of membrane potential measured under State 4 conditions is not the maximum that can be achieved. Since the maximum value of the membrane potential may be attained only when net ATP synthesis is blocked [39], the difference between the membrane potential in State 4 in the presence and absence of oligomycin, is evidence that part of the energy made available via respiratory chain is utilized for the phosphorylation process.

ATP, however, does not accumulate in the reaction medium. One possible explanation is that in the conditions where oligomycin is found to have the inhibitory effect on State 4 respiration, there are activated ATP-utilizing reactions occurring. This could depend on either the choice of substrate or the composition of the reaction medium. However, the inhibitory effect of oligomycin on State-4 respiration is found with all the substrates of Krebs cycle, both those NADH-linked and succinate. In particular, it has to be noted that the inhibiting effect of oligomycin is seen with substrates, such as pyruvate or L-glutamate, that do not utilize ATP to be transported [40]. Furthermore, the inhibitory effect does not vary if Na^+ is substituted for by either K^+ or sucrose in the reaction medium, and it is essentially the same within a wide range of variation of Mg^{2+} concentrations that are compatible with the maintenance of normal respiratory controls [41]. It thus appears that the inhibitory effect of oligomycin on State-4 respiration does not depend on the experimental conditions used.

Measurements of microsomal contamination present in the mitochondrial preparations used, indicate that the amount of ADP produced via microsomal ATPase is too low to account for the portion of State-4 respiration inhibited by oligomycin. In fact, by using the calculation procedure suggested by Sottocasa et al. [23] the microsomal contamination present in the mitochondrial preparations used does not exceed about 3% of the total protein. By taking into account the value of the specific activity for microsomal ATPase re-

ported [42], a concentration no higher than 4 μM ADP can be expected to be maintained via microsomal ATPase by the contaminating microsomal fragments present in the preparations used. It appears from a study correlating the respiratory rates with the ADP concentrations [27] that at this ADP concentration there is an increase of State-4 respiration rate of about 10%. This conclusion is in agreement with the observation regarding the degree of inhibition by atractyloside. The microsomal ATPase cannot therefore be the only ATP-utilizing reaction accounting for the portion of State-4 respiration inhibited by oligomycin which is of the order of 50%.

One possible conclusion is that there is also an activated intrinsic mitochondrial ATPase occurring during State 4 [11]. This ATP-utilizing reaction could provide energy for maintaining the intramitochondrial level of Mg^{2+}, which has been shown to be characteristic of this respiratory state [41]. It could also provide energy for maintaining the structural steady state characterizing the respiratory State 4, which also requires energy to be maintained [43,44]. Preliminary observations seem to indicate that, at the ultrastructural level oligomycin causes a conformational change from the expanded configuration, characteristic of State 4, to a more condensed configuration.

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