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PHOSPHORYLATING EFFICIENCY OF ISOLATED RAT LIVER MITOCHONDRIA RESPIRING UNDER THE CONDITIONS OF STEADY-STATE 4

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A limited, but significant net formation of ATP was observed during the very first period of respiratory State 4. The synthesis appeared to depend on respiration, since it was completely inhibited by KCN or by 2,4-dinitrophenol. Accordingly, State 4 respiration was observed to be inhibited to a large extent by oligomycin. After the initial increase, the level of ATP remained unmodified under the conditions of steady-state 4. Also, the maintenance of the equilibrium level of ATP was very sensitive to KCN or 2,4-dinitrophenol. Under the very same conditions of State 4, the mitochondria exhibited a significant ATPase activity, which appeared to be competitively inhibited by ADP. Therefore, it might be concluded that the apparently constant level of ATP observed in State 4 results from a balanced equilibrium between a respiration-dependent synthesis and a continuous hydrolysis. A comparison between the amount of ATP hydrolysed in State 4 and the amount of oxygen consumed under the same conditions indicated that the phosphorylating efficiency of respiring mitochondria in State 4 is as high as in State 3.

Introduction

In recent years, several possibilities have been considered to account for the oxygen consumption exhibited by isolated mitochondria under conditions of controlled respiration, i.e., in State 4 according to Chance and Williams [1]. In fact, under these conditions the oxygen uptake does not appear to be associated with formation of ATP. In 1972, Stucki and Walter [2] suggested that the apparent uncoupled situation was the result of an extramitochondrial ATPase activity, most probably due to microsomal contamination. This explanation appears rather untenable, since State 4 respiration does not depend on the method of mitochondrial preparation nor on the tissue used. Subsequently, Ernster and Nordenbrand [3] showed that the phosphorylating efficiency, i.e., the value of the P/O ratio, is diminished when the phosphorylating system is made rate limiting. They concluded that under these conditions, i.e., when the capacity of the electron-transport system is in excess of that of the phosphorylating system, other energy-dissipating reactions can compete with the synthesis of ATP. One of the possible energy-dissipating reactions was proposed to be a recycling of H^+ through the inner membrane [4,5]. However, the possible contribution of this reaction to total respiration of State 4 has not been quantified because of the lack of specific inhibitors. Another energy-utilizing reaction may involve Ca^{2+} cycling. In this regard, Drahota and co-workers [6] proposed that the respiration occurring in State 4 would provide energy for the maintenance of Ca^{2+} equilibrium within the mitochondrion: a passive efflux of Ca^{2+} would occur in State 4, which

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Abbreviation: FCCP, carbonyl cyanide *p*-trifluromethoxyphenylhydrazone.

would be counterbalanced by a respiration-dependent Ca^{2+} uptake. In agreement with this proposal, Stucki and Ineichen [7] observed an inhibition of State 4 respiration by ruthenium red, an inhibitor of Ca^{2+} transport. It should, however, be noted in this regard that ruthenium red does not inhibit the rate of State 4 respiration at concentrations that cause complete inhibition of Ca^{2+} transport [8].

More recently, Lemaster and Hackenbrock [9], in a study aimed at determining the energy equivalence of State 4, observed a limited, but significant synthesis of ATP during this respiratory state.

In the present research, the kinetic characteristics of ATP synthesis under the condition of State 4 as well as the correlation of this synthesis with the respiratory activity were studied. The results show that there is a significant formation of ATP under the steady-state conditions of State 4 and that ATP synthesis is closely coupled with respiration. It was also found that the accumulation of ATP is prevented by a concomitant ATPase activity.

A preliminary report of this work has been previously presented [10].

Materials and Methods

Rat liver mitochondria were isolated in 0.25 M sucrose according to a standard procedure [11]. Mitochondrial protein concentration was estimated by the biuret method.

The oxygen uptake was measured with a Clark type electrode. The respiratory states were defined as in Refs. 1 and 12. The composition of the metabolic medium was as follows: 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl buffer (pH 7.4), 10 mM sodium/potassium phosphate buffer (pH 7.4), 1.6 mM sodium pyruvate plus 0.4 mM L-malate as the substrate and aliquots of mitochondrial suspensions corresponding to 2–4 mg protein per ml. The final volume was 3 ml and incubation temperature 25°C.

The ATP concentration was determined, under State 4 conditions, as follows: aliquots of 50 μl were collected at the indicated times and immediately added to 1 ml of boiling water. After 2 min they were rapidly cooled and the ATP content was determined by bioluminescence photometry, employing purified firefly luciferase [13]. Luminescence was detected by an LKB 1250 luminometer.

The ATPase activity of respiring mitochondria in State 4 was determined by employing [γ-^{32}P]ATP as the substrate [14]. The rate of ATP hydrolysis was estimated by measuring the amount of $^{32}P_i$ liberated. $^{32}P_i$ liberated was measured according to the method of Lindberg and Ernster [15]. When the hydrolysis of endogenous ATP had to be measured, the activity of the ATPase was assayed by measuring the rate of decay of ATP concentration with time by using the firefly luciferase assay.

The ATP-monitoring reagent (purified firefly luciferase) was purchased from LKB (Turku, Finland). Oligomycin was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [γ-^{32}P]ATP was obtained from New England Nuclear (Boston, MA, U.S.A.). Chemicals and biochemicals were of the highest purity commercially available.

Results

Fig. 1A shows that a limited but significant synthesis of ATP occurs under the conditions of respiratory State 4(a), i.e., in the absence of added ADP [12]. This synthesis is linear with time only during the very first period of incubation; then an equilibrium concentration of 6–8 nmol ATP per mg protein is reached in about 1 min. In the presence of either KCN or 2,4-dinitrophenol ATP synthesis is completely inhibited. In both cases, the block of synthesis is associated with a progressive decrease in ATP concentration; the rate of this decrease is more pronounced in the presence of 2,4-dinitrophenol than in the presence of KCN. An essentially similar synthesis of ATP occurs also under the conditions of respiratory State 4(b), i.e., immediately after the State 3–State 4 transition (Fig. 1B). Also, in this case, a net synthesis is observed during the first minute of incubation, after which the level of ATP remains unmodified over time. Addition of KCN causes inhibition of ATP synthesis and progressive decrease in ATP concentration.

These results suggest that State 4 respiration is coupled to phosphorylation. To test further this conclusion, the effect of oligomycin, a specific inhibitor of respiration tightly coupled to phos-

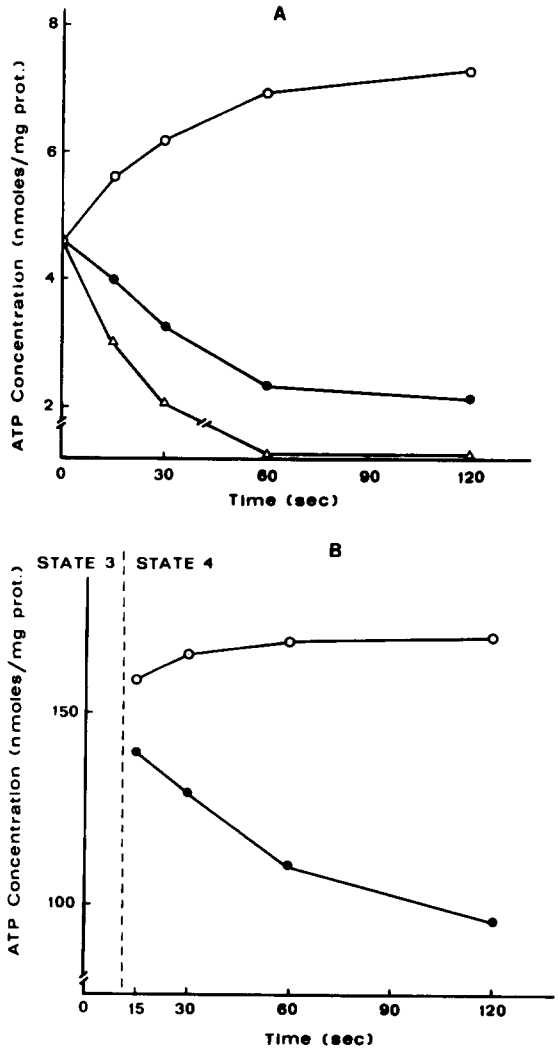

Fig. 1. Time course of ATP concentration during incubation of mitochondria under State 4 conditions. Mitochondria (3.1 mg/ml) were incubated in State 4. The composition of the metabolic medium and ATP measurement are described in Materials and Methods. (A) (○———○) State 4(a) conditions; (●———●) plus 2 mM KCN; (△———△) plus 25 μM 2,4-dinitrophenol. (B) (○———○) State 4(b) conditions; (●———●) plus 2 mM KCN, added at the end of State 3. State 3 was attained by the addition of 0.5 mM ADP. The data are from one of four identical experiments in which the results were within 5% of each other.

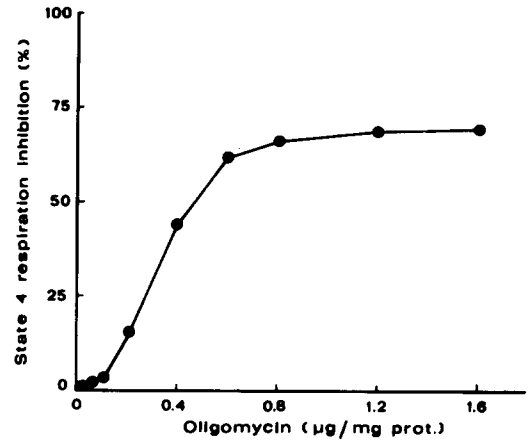

Fig. 2. Inhibition of the State 4 respiratory rate by oligomycin in rat liver mitochondria. Mitochondria (3.3 mg/ml) were incubated for 1 min in the metabolic medium with pyruvate as substrate. 0.5 mM ADP was then added to initiate State 3 respiration. 2 min after the State 3-State 4 transition, increasing concentrations of oligomycin were added in a constant volume of 10 μl. Control State 4 respiration = 14.1 ngatom oxygen/mg protein per min. All other conditions as in Fig. 1.

phorylation [16,17], on State 4 respiration was studied. The results are reported in Fig. 2. It appears that the oxygen uptake in State 4 is inhibited by oligomycin and the inhibition-concentration curve is sigmoidal, similar to that reported by other authors under different metabolic conditions [17,18]. The maximal inhibition observed is of the order of 70% and is reached in the presence of 0.8 μg per mg protein; higher concentrations do not enhance the inhibition. The inhibition of the oxygen uptake by this antibiotic is completely reversible by 2,4-dinitrophenol or FCCP (results not shown).

Since the active functioning of oxidative phosphorylation is an essential requirement for the ATP levels to be maintained during State 4, it seems reasonable to suppose that the apparently constant concentrations of ATP in steady-state 4 result from a balanced equilibrium between ATP synthesis and ATP utilization. To test this working hypothesis, a possible ATP hydrolysis was investigated under the conditions of State 4, i.e., in the presence of substrate, oxygen and P_i. The ATPase activity was measured as $^{32}P_i$ liberated from [γ-^{32}P]ATP. The results are reported in Fig. 3. It appears that a significant hydrolysis of ATP occurs under the conditions of State 4. The rate of the hydrolysis, which is rather high in the very first period of incubation, tends to decrease with time and reaches a steady state of about 40 nmol ATP hydrolysed/min per mg protein within 2 min.

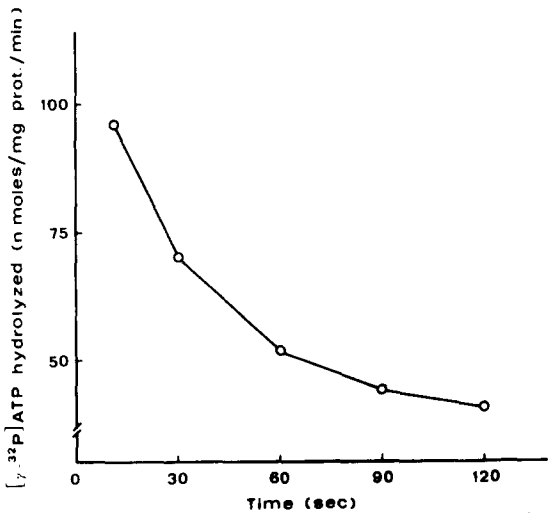

Fig. 3. Time course of ATPase activity during incubation of mitochondria under State 4 conditions. Mitochondria (3.0 mg/ml) were incubated for 1 min in State 4. Then the ATPase reaction was started by the addition of 0.5 mM [γ-^{32}P]ATP. The ATPase activity was measured as described in Materials and Methods. All other conditions as in Fig. 1.

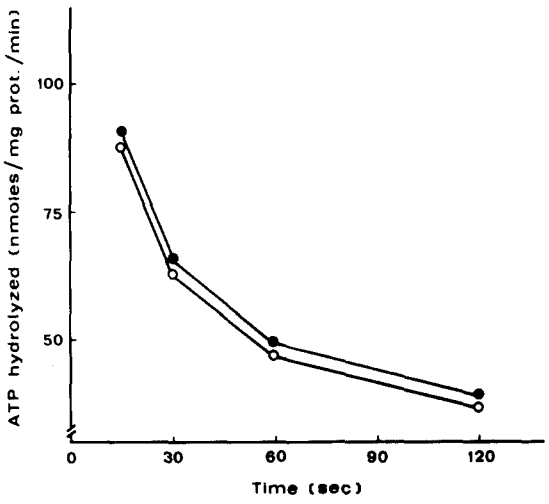

Fig. 4. Comparison of the rates of endogenous and exogenous ATP hydrolysis in mitochondria respiring in State 4. Mitochondria (3.1 mg/ml) were incubated for 1 min (○——○) under State 4(a) conditions in the presence of 0.50 mM ATP and (●——●) under state 4(b) conditions, i.e., following the exhaustion of the added 0.50 mM ADP. 2 mM KCN was then added to both systems. Measurement of the ATPase reaction was started following the addition of 2 mM KCN as described in Materials and Methods. All other conditions as in Fig. 1.

In order to ensure that the rate of ATP hydrolysis is the same in the presence and absence of added ATP, i.e., that the influx of ATP through the adenine nucleotide translocator is not rate limiting for the measured rate of ATP hydrolysis when added ATP is used, a series of experiments was performed in which the hydrolysis of endogenous ATP was compared with that of equal amounts of added ATP. The ATPase activity was estimated, in both cases, from the decay in ATP concentration following the addition of 2 mM KCN when the steady-state level of ATP was reached (see Fig. 1). These results are reported in Fig. 4. It appears that the rate of hydrolysis of endogenous ATP is as high as that of exogenous ATP. Therefore, it may be concluded that the adenine nucleotide translocator is not rate limiting for the measurement of ATP hydrolysis. The results also exclude the possibility that the measured rate of ATP hydrolysis may be misleading due to the operation of the ATP-P_i exchange reaction catalyzed by the mitochondrial ATPase. In fact, the contribution of the latter reaction could not be excluded by simply measuring the rate of ATP hydrolysis as $^{32}P_i$ released from [γ-^{32}P]ATP.

Fig. 5 illustrates the kinetic properties of State 4 ATPase. It appears that by plotting the reciprocal of the initial velocities against the reciprocal of the substrate concentrations, the ATPase activity is characterized by an apparent K_m(ATP) = 0.088 mM. It also appears from the same figure that the enzyme activity is competitively inhibited by the product, i.e., by ADP. It is noteworthy that ADP inhibition was found to be strictly competitive with ATP also in membrane-bound ATPase of submitochondrial particles [19,20].

As shown in Fig. 6, the ADP inhibition observed with intact mitochondria incubated in State 4 is proportional to ADP concentration only up to a value of about 50% of inhibition; no further effect by ADP is seen when the ATPase is inhibited by 75%. KCN does not affect the rate of this ATPase (results not shown).

By comparing the results illustrated in Fig. 1 with those shown in Fig. 3, it appears that during steady-state 4 the equilibrium levels of ATP are maintained in spite of the fact that ATP is hydro-

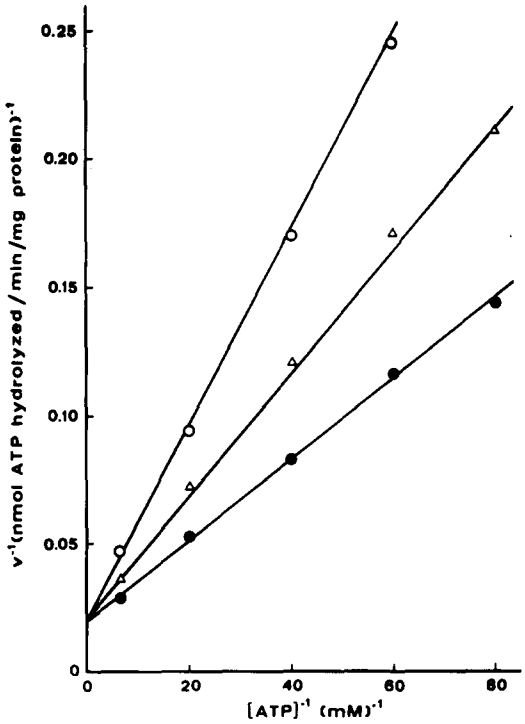

Fig. 5. Kinetic analysis of the mitochondrial ATPase. Double-reciprocal plots were constructed from initial rate data. The lines represent a weighted least-squares analysis of the data. Mitochondria (2.0 mg/ml) were incubated as described in Fig. 3. (●———●) State 4(a) conditions; (△———△) plus 12.5 μM ADP; (○———○) plus 25 μM ADP. ADP was added at the same time with ATP.

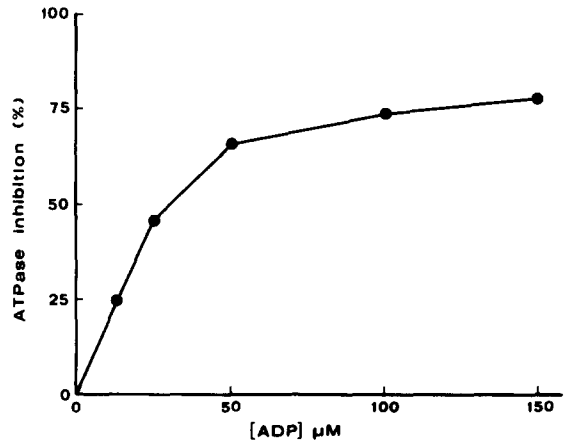

Fig. 6. Inhibition of the mitochondrial ATPase by ADP. Mitochondria (2.0 mg/ml) were incubated as described in Fig. 2. The concentration of [γ-^{32}P]ATP was 25 μM. All other conditions as in Fig. 1.

TABLE I

OXYGEN UPTAKE AND ATPase ACTIVITY UNDER STEADY-STATE 4 CONDITIONS IN RAT LIVER MITOCHONDRIA

ATPase activity was determined as $^{32}P_i$ liberated from 0.50 mM [γ-^{32}P]ATP in respiratory State 4. The reaction was started by the addition of ATP. Aliquots of mitochondrial suspension were taken up from the polarographic chamber during the registration of oxygen uptake and used for the determination of $^{32}P_i$ liberated. The ATPase activity was measured after equilibrium conditions were reached, i.e., after 1 min of incubation in State 4 after ATP addition. The ADP/O ratio was determined in the same mitochondrial suspension by adding 0.2 mM ADP after an additional minute of incubation. All other conditions as in Fig. 1.

State 3 ADP/O	State 4		
	ATPase (nmol P_i/mg protein per min)	Oxygen uptake (nmol $1/2O_2$/mg protein per min)	ATP hydrolysed/O taken up
2.56	36	14.8	2.43

lysed at a constant rate. It seems therefore necessary to assume that the amount of ATP hydrolysed during steady-state 4 is balanced by an equal amount of ATP synthesized and that the energy for this synthesis is provided by the operations of oxidative phosphorylation. A comparison between the rate of ATP hydrolysis in steady-State 4 with the rate of respiration during the same state is reported in Table I. It appears that the amount of oxygen consumed in State 4 can be correlated with the phosphorylation of the amount of ADP formed from the ATP hydrolysed during the same state. The table shows that the ratio ATP hydrolysed/O taken up in respiratory State 4 is of the same order as the ADP/O ratio measured in State 3 in the same mitochondrial preparation.

Discussion

The results presented show that the respiration observed in State 4 is, to a large extent, coupled to phosphorylation. Two lines of evidence support this conclusion:

(a) A synthesis of ATP occurs under the conditions of State 4, which appears to depend strongly on respiration: in fact, the synthesis is prevented in the presence of KCN or 2,4-dinitrophenol.

(b) State 4 respiration is inhibited up to at least

70% by oligomycin, a reagent that specifically inhibits only that portion of oxygen uptake which is tightly coupled to phosphorylation.

The effect of oligomycin on State 4 respiration leads to the conclusion that respiration in this state arises for the most part because of the driving of ATP synthesis rather than because the H^+ electrochemical gradient is subjected to a steady-state dissipation owing to a leakage of H^+ across the membrane [21]. The H^+ leakage may possibly account for the remaining 30% of respiration, although other energy-utilizing reactions cannot be ruled out on the basis of the present results.

The present findings also show that the accumulation of ATP in State 4 is prevented by a concomitant hydrolysis of this compound. It is worthy of note that this ATPase operates in respiring mitochondria under the very precise conditions of steady-State 4, i.e., in the presence of oxygen, an oxidizable substrate and P_i, and thus it appears a remarkable feature of mitochondria respiring under controlled conditions. Since ATP synthesis and ATP hydrolysis are concomitant and clearly of the same order, as shown by the maintenance of a constant level of ATP under steady-state conditions, it may be concluded that the ATP hydrolysed is continuously resynthesized by the operations of coupled respiration. When calculated on this basis (see Table I), the phosphorylating efficiency of respiring mitochondria in State 4 is as high as that in State 3, as assumed by Cockrell et al. [22] and Slater [23–25].

These conclusions do not appear to be in agreement with those of Ernster and co-workers [3,26]. These authors found a diminution of phosphorylation efficiency by making the phosphorylation system rate limiting, i.e., they found a decrease in the P/O ratios by reducing, in an ADP-regenerating system (hexokinase plus glucose), the amounts of hexokinase added to the system. In this regard it should be remarked that the ATPase activity, observed in our experiments in mitochondria respiring in State 4, is competitively inhibited by ADP. In the experiments with hexokinase plus glucose as ADP-regenerating system, carried out by Ernster and co-workers, when high concentrations of enzyme were used, high concentrations of ADP were accumulated [27]; therefore, inhibition of the ATPase activity should be expected to occur and thus high P/O ratios can be measured. On the other hand, in the presence of low amounts of hexokinase, the ADP concentrations are quite small [27] and therefore the ATPase is expected to operate fully; under these conditions the measured ATP synthesized may well be masked by the concomitant ATP hydrolysis and thus low P/O ratios are found.

As to the possible significance of State 4 ATPase, it may be speculated that the ATP hydrolysis is needed to maintain an ion gradient between the matrix space and the surrounding medium under the conditions of State 4. In fact, when respiring under these steady-state conditions, mitochondria exhibit an expanded configuration [11,28,29], which is characterized by a greater amount of water within the matrix space [30,31]. Since the inner membrane is freely permeable to water [32], the larger amount of water is most probably taken in to equilibrate an increased uptake of ions [33], which in turn is energy dependent and requires energy to be maintained [34,35]. Should this proposed role of State 4 ATPase in determining the structural steady state, characteristic of this metabolic state, be proved by direct evidence, it would appear remarkable that the same factor, i.e., ADP concentration, controls the respiratory [1,27] as well as the structural transition from State 4 to State 3.

Acknowledgement

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