REGULATION OF MITOCHONDRIAL ATPase. EFFECT OF ELECTRON FLOW*

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1. Introduction

It has been reported [1–6] that the inhibition of electron flux through the respiratory chain results in a decrease of the rate of uncoupler stimulated and calcium induced ATP hydrolysis. These findings point to the regulation exerted by the operation of respiratory chain on the ATPase system. However, in contrast with some assumptions [5] that the extent of inhibition observed during different redox state of the respiratory carriers is essentially the same, we have reported that the initial rate of calcium activated and FCCP stimulated ATP hydrolysis progressively decrease by about 30, 50 and 65% in the presence of rotenone, antimycin A, and cyanide, respectively. On the other hand, somehow similar results have emerged from a recent paper by Weiner and Lardy [6].

Since these events are central to the understanding of the architecture of mitochondrial energy transforming machinery, we wish to report in the present paper more detailed studies on the relationships between the activity of mitochondrial ATPase and the different redox states of respiratory carriers. It is clearly shown that under appropriate conditions the rate of ATP hydrolysis can be reduced by 30–35% by rotenone, 55–60% by antimycin A, and over 70% by cyanide. It may be concluded that there is a close correlation between the activity of mitochondrial reversible ATPase and the electron flux in the respiratory chain; precisely, the electron transport through the *entire* respiratory chain is required to maintain the maximum rate of mitochondrial ATPase. It is also shown that three functionally distinct ATP forming systems are operative in mitochondria, each connected to one energy conserving site.

2. Materials and methods

Rat liver mitochondria were isolated in 0.25 M sucrose essentially as described by Schneider [7]. ATP hydrolysis was followed by measuring the H^+ ejected into the suspending medium with a glass electrode and an expanded scale pH meter linked to a strip chart recorder. The absolute amount of H^+ ejected was determined by addition of internal standard of HCl in each experiment. The reaction system contained 5 mM Tris, pH 7.4, 50 mM NaCl, 50 mM KCl, and rat liver mitochondria, 5 mg protein, in a total vol of 2 ml. Temperature was 21°C. ATP was 4 mM, and FCCP 0.3 μM. When present, rotenone was 0.4 μg per mg protein, antimycin A 0.2 μg per mg protein, and cyanide was 1.5 mM.

3. Results and discussion

In the experiments described below the uncoupler induced ATP hydrolysis was examined under two sets of conditions. In the first, ATP hydrolysis was measured

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** *Abbreviations*: FCCP: Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD: *N,N,N',N'*, tetramethyl *p*-phenylenediamine.

Table 1
ATPase activity as a function of the time of preincubation

Exp.	Preincubation Time (min)	H^+ ejected nions · min^{-1} · mg^{-1}
A	no	150
B	2	250
C	4	369
D	7	369

The standard system (2 ml) contained 5 mM Tris, pH 7.4, 50 mM KCl, and 50 mM NaCl. In the experiment A the system contained 4 mM ATP and 0.3 μM FCCP; rat liver mitochondria (5 mg protein) were then added to start the reaction. In B, C, and D after preincubation of mitochondria for the time periods indicated, ATP and FCCP were added to start the reaction and H^+ ejection during the hydrolysis of ATP was measured.

by adding mitochondria to a medium containing 50 mM NaCl, 50 mM KCl, 5 mM Tris pH 7.4, 4 mM ATP, and 0.3 μM FCCP. As it can be seen in table 1, under these conditions the initial rate of ATPase is relatively low (about 150 nions H^+ per min per mg protein). In the second type of system, mitochondria were incubated for various periods of time in the absence of ATP and FCCP; the nucleotide and the uncoupler were then added to the system and the initial rate of ATP hydrolysis was measured. The representative data collected in table 1 show that the initial rate increases with the time of preincubation, until a plateau is reached after 4–7 min. The time of preincubation necessary to get maximum rate of ATP hydrolysis varies considerably with mitochondrial preparation employed. The maximum levels of activity which have been observed in a long series of experiments lie in the range 350 to 400 nions H^+ per min per mg protein.

When respiratory inhibitors, rotenone, antimycin A and cyanide, were present in the preincubation medium, the rate of ATP hydrolysis was reduced in comparison with the control carried out in the absence of inhibitors. Moreover, when the time of preincubation was such (2 min) that ATPase activity was not at its maximum rate, the extent of inhibition was proportional to the number of energy conserving spans of respiratory chain kept in the reduced state (see table 2, exp. A).

As described above, by increasing the time of preincubation, the rate increased to reach its maximum (about 360 nions H^+ per min per mg); under these

Table 2
Effect of respiratory inhibitors on the rate of ATP hydrolysis

Exp.	Respiratory inhibitor	H^+ ejected nions · min^{-1} · mg^{-1}
A.	2 min of preincubation	
	none	250
	rotenone	160
	antimycin A	95
	cyanide	85
B.	4 min of preincubation	
	none	370
	rotenone	82
	antimycin A	76
	cyanide	79

The system as in table 1. When added, rotenone was 0.4 μg per mg protein, antimycin A 0.2 μg per mg protein, and cyanide 1.5 mM. After the preincubation periods indicated, 4 mM ATP and 0.3 μM FCCP were added to start the reaction, and the H^+ ejection during the hydrolysis of ATP was measured.

conditions no correlation whatsoever between the reduction of specific respiratory spans and the inhibition of ATPase activity was observed. In other words the rate of ATP hydrolysis was depressed at the same extent (about 80%) when either rotenone, or antimycin A, or cyanide were alternatively added to the system (see table 2, exp. B). Furthermore, the experiment illustrated in table 3 shows that the

Table 3
Restoration of ATPase activity by electron flow through the respiratory chain

Respiratory inhibitor	Respiratory substrate	H^+ ejected nions H^+ · min^{-1} · mg^{-1}
None	none	360
None	succinate	330
Rotenone	none	82
Rotenone	succinate	201
Antimycin A	none	80
Antimycin A	ascorbate (+TMPD)	105

The system as in table 1. After four min of preincubation in the presence of respiratory inhibitors, 1 mM succinate, or 10 mM ascorbate (+ 0.1 mM TMPD) were alternatively added, the incubation continued for 1 min; 4 mM ATP and 0.3 μM FCCP were then added to start the reaction.

inhibition induced by rotenone was removed by the addition of succinate. In the absence of rotenone, succinate did not affect significantly the rate of ATP hydrolysis, except in few experiments where the maximum rate of ATP hydrolysis was preceeded by a very short phase (10–20 sec) of slower rate. Finally, when antimycin A was the respiratory inhibitor, only a poor reactivation was obtained by restoring the electron flux through site III (addition of ascorbate plus TMPD).

The bulk of results presented in this paper fully support our previous findings that preincubation of mitochondria in the absence of externally added substrates, induced a stimulation of the uncoupler evoked ATP hydrolysis [2]. Presumably, as it has been assumed by Bertina [5], the electron flux from endogenous substrates through the respiratory carriers during the preincubation, induces the dissociation of the polipeptide inhibitor of F_1. In line with this interpretation, we confirm that the lack of electron flow, as induced by the respiratory inhibitors, results in a severe depression of ATPase activity.

What emerges striklingly from our experiments are the different results obtained in the two sets of conditions, that is: 'short' and 'long' preincubation. During the short preincubation endogenous substrates are still available, and the extent of ATPase inhibition is proportional to the number of respiratory spans blocked. Thus, in the presence of rotenone, the electron flux through site I is stopped, whereas sites II and III are still receiving electrons; as a consequence the ATPase inhibition is only of the order of 30%. Antimycin A, by abolishing the electron transport through sites I and II reduces the ATPase activity by 50%. Finally, the maximum inhibition is observed with cyanide. On the contrary, when preincubation is continued for a long period of time in the presence of rotenone, all the endogenous substrates have been already consumed so that electrons are no longer available for the carriers; under these conditions the extent of ATPase inhibition by rotenone is the same as that induced by antimycin A or cyanide. It follows then that the inhibition of ATPase activity is manifested only when electron transport is interrupted, independently of the redox state of the carriers. This conclusion is confirmed by the experiment described in table III: during 'long preincubation' experiments the addition of succinate (plus rotenone) which feeds with electrons sites II and III of the chain, resulted in a restoration of ATPase activity to about 60–70% as compared to the control (absence of rotenone). Similarly, ascorbate (plus TMPD) by feeding the terminal part of the respiratory chain restored the ATPase activity, almost completely inhibited by antimycin A, to about one third of the control.

The most significant point of difference between these results and those of Bertina [5] involves the extent of reactivation induced by succinate on the rotenone inhibited ATPase. Our results show that, in the absence of rotenone, the rate of ATP hydrolysis is the same in the presence and in the absence of succinate; however, as said before, in some experiments, the high rate of ATP hydrolysis was preceded by a short phase of lower rate (about 30% slower). A possible explanation for this early phase can be the uncoupler stimulated electron flux through site I, which was previously kept in a reduced state by succinate supported reversal of electron transport. Increasing inhibitions of ATPase following progressive reduction of the respiratory chain carriers have also been recently reported by Weiner and Lardy [6] who however utilized different experimental conditions and much longer times of incubation.

4. Conclusions

The data reported in this paper clearly show that in mitochondria the uncoupler stimulated ATPase is inhibited whenever the electron flux through the respiratory chain is interrupted. Conversely, so long as coupled electron flow occurs through specific spans of the chain, the ATPase activity is stimulated. In addition, the ATPase complex can be functionally discriminated into three fractions, each connected to one energy conserving site. Each one of these fractions, or units, can be inhibited by cutting off electron transport at the level of one specific energy transducing site, and, viceversa can be activated by switching on coupled electron flow at the same site.

Our results may have a broader significance with the respect to oxidative phosphorylation on the light of the postulation that native F_1 is an oligomer built up by three promoter units [8]. In our tentative interpretation each promoter is functionally connected to one energy conserving site. This interpretation is

not merely based on the results reported in this paper, but derives also from the following lines of evidence: a) the binding of adenine nucleotides (ATP and ADP) to submitochondrial particles exhibits three distinct saturation curves which nicely parallel three distinct kinetics of the uncoupler induced ATP hydrolysis [2]; b) the binding of calcium to mitochondrial structure during respiration driven calcium uptake [9] appears to be related to the number of energy conserving sites in operation, thus indicating the existence of three distinct components of the energy transducing machinery.

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