OLIGOMYCIN-INSENSITIVE ATPase AND CALCIUM TRANSPORT IN RAT KIDNEY CORTEX MITOCHONDRIA

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Received 5 August 1974

1. Introduction

During metabolic studies on rat kidney cortex mitochondria (RKCM) it was found that they never showed proper respiratory control, i.e. oxygen uptake did not return to state 4 rate after exhaustion of added ADP (fig. 1). This suggested the presence of ATPase activity in the preparations of RKCM. Therefore, attempts were undertaken either to purify the mitochondria from possible microsomal contaminations, or to characterise more closely the ATPase, if it proved to be the mitochondrial enzyme. The results suggest that oligomycin-insensitive Mg–ATPase is localised in the outer membrane of RKCM, and that it is involved in calcium transport in intact mitochondria.

2. Methods

Mitochondria from kidney cortex of Wistar rats were prepared and suspended in 0.25 M sucrose – 5 mM Tris–Cl – 0.01 mM EGTA by the method of Hogeboom [1] or, when indicated, of Johnson and Lardy [2], except that the mitochondrial pellet was washed three times. Zonal centrifugation was performed on MSE 65 ultracentrifuge using No. XV zonal rotor and ficoll gradient. ATPase activity was measured by proton release method of Nishimura et al. [3] in standard medium containing 0.23 M sucrose, 10 mM Tris–Cl, 3 mM $MgCl_2$, 0.1 mM EGTA and 0.3–0.5 mg/ml mitochondrial protein, pH 7.2. Reaction was started by addition of 5 mM ATP-Mg, and proton release was followed with glass–calomel electrode, pH-meter and recorder. ATP-Mg solution was standardized spectrofotometrically before use ($A_{260\,nm}$ = 15.0 cm^2 μmole). Inner and outer membranes of RKCM were prepared by digitonon treatment according to Brdiczka et al. [4]. Calcium uptake was measured with specific calcium electrode (Philips IS-562 Ca), pH-meter and recorder. Protein was estimated by a biuret method [5].

3. Results and discussion

Fig. 1 shows the respiratory control of RKCM in Mg-containing and Mg-free medium. In the presence of Mg^{2+} the rate of oxygen uptake after exhaustion of added ADP was nearly twice that before ADP addition (trace A). Addition of ATP in the presence of Mg^{2+} and substrate produced twofold stimulation of respiration. On the other hand, good respiratory control was observed in Mg^{2+}-free medium, but it was released by subsequent addition of Mg^{2+} (traces C and D). This indicated the presence of Mg^{2+}-stimulated ATPase in RKCM preparations. The activities of Mg–ATPase in RKCM and, for comparison, in identically prepared rat liver mitochondria are shown in table 1. Contrary to liver mitochondria, freshly prepared RKCM showed considerable Mg–ATPase activity which was only slightly inhibited by oligomycin. DNP-stimulated ATPase activities were almost identical in mitochondria from both tissues. Atractyloside did not inhibit the Mg–ATPase activity in RKCM, suggesting its localization outside the atractyloside barrier (i.e. in the outer membrane or intermembrane space).

In attempt to get rid of the possible microsomal contamination, RKCM were washed five times and

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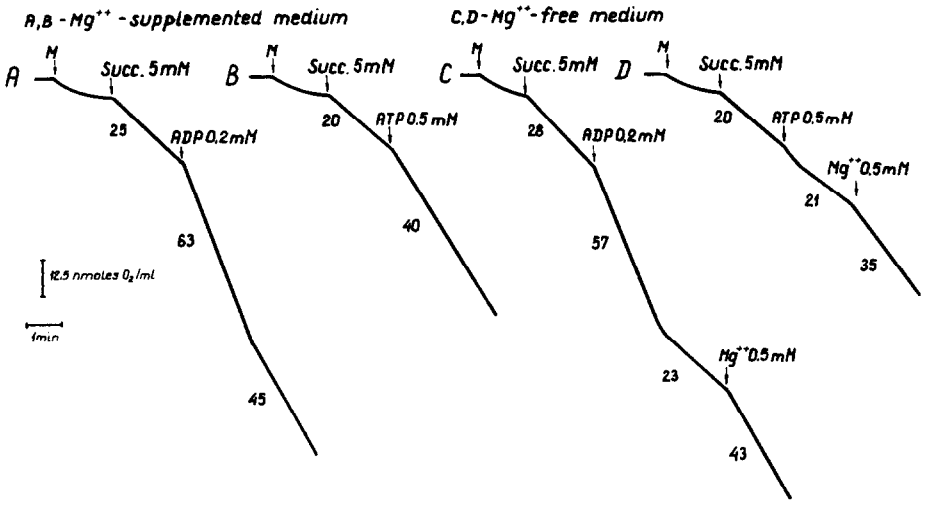

Fig. 1. Effects of Mg^{2+} on respiratory control in rat kidney cortex mitochondria. Incubation medium contained: sucrose 180 mM, Tris–Cl 10 mM, KCl 10 mM, Pi 5 mM, EGTA 0.1 mM and, in experiments A and B, $MgCl_2$ 3 mM; pH 7.2, mitochondrial protein 0.6 mg/ml.

Table 1
Mg-ATPase activity in freshly prepared rat kidney cortex and rat liver mitochondria

	nmoles ATP/min × mg protein
A. Kidney cortex mitochondria	
Control	82
+ DNP 0.1 mM	127
+ oligomycin 10 μg/ml	65
+ DNP + oligomycin	65
+ atractyloside 80 μM	71
B. Liver mitochondria	
Control	0
+ DNP 0.1 mM	55

ATPase activity was measured as described in Methods. Mitochondria from rat kidney cortex and rat liver were prepared simultaneously by the method of Hogeboom [1].

Mg–ATPase activity was measured after each washing (table 2). Na–K–ATPase activity, which is present in both plasma membranes and microsomes of rat kidney cortex [6,7,8], was used as an index of contamination by these fractions. Na–K–ATPase activity disappeared after fourth washing, but Mg–ATPase reached a steady level of about 80 nmoles ATP/mg protein per min. This already suggested that the oligomycininsensitive Mg–ATPase was tightly bound to rat kidney mitochondria. Further purification of RKCM was obtained by means of zonal centrifugation on continuous ficoll gradient (fig. 2). RKCM were pre-prepared for zonal centrifugation by the method of Johnson and Lardy [2], which gave higher yeld of mitochondrial protein, but also much higher contamination with microsomes. Clear separation of two peaks of protein with corresponding two peaks of Mg–ATPase activity were obtained:

Table 2
Mg-ATPase and Na, K-ATPase activities in rat kidney cortex mitochondria: effects of washing

Washing No.	Mg-ATPase	Na, K-ATPase
	nmoles ATP/min × mg protein	
–	128	23
1	117	8
2	101	11
3	90	10
4	92	0
5	85	0

Mg, Na, K-ATPase activity was measured by proton release method in medium containing: sucrose 10 mM, Tris–Cl 10 mM, KCl 10 mM, NaCl 100 mM, EGTA 0.1 mM and ATP-Mg 5 mM, pH 7.2. Na, K-ATPase activity was calculated as difference between Mg, Na, K-ATPase and Mg-ATPase activities.

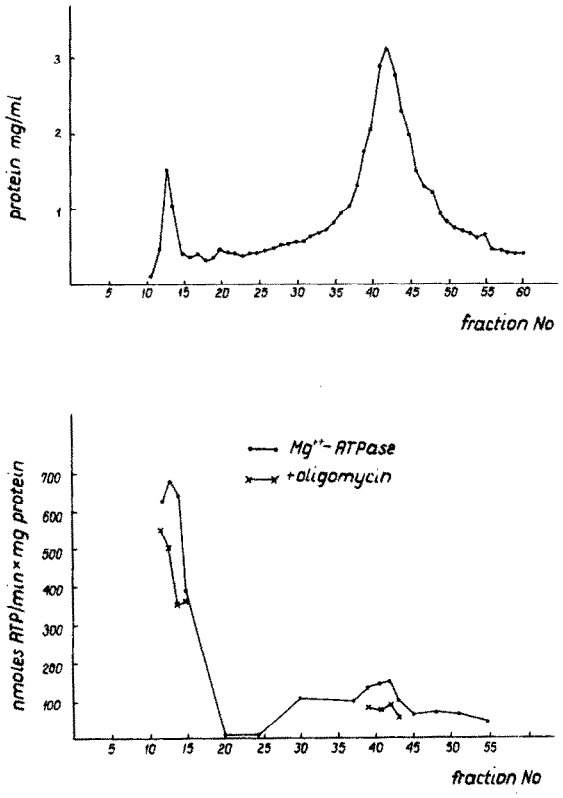

Fig. 2. Mg-ATPase activity in rat kidney cortex mitochondria separated by zonal centrifugation on continuous ficoll gradient. Separation conditions: cushion – 150 ml 45% sucrose; gradient – 350 ml 20–50% ficoll in 0.25 M sucrose; sample – 12 ml kidney cortex mitochondria, 33 mg protein/ml; overlay – 150 ml 0.25 M sucrose; centrifugation at 165 000 *g* for 90 min on MSE-65 ultracentrifuge. 10 ml fractions were collected for estimations of protein and Mg-ATPase. In samples indicated in the figure Mg-ATPase activity was measured in the presence of 10 μg/ml oligomycin. Mitochondria were pre-prepared for separation by the method of Johnson and Lardy [2].

first one at $d = 1.06–1.10$, corresponding to microsomes and microsomal ATPase, and second one at $d = 1.18–1.24$, corresponding to mitochondria and mitochondrial ATPase. No mitochondrial fraction free of Mg–ATPase activity was recovered. These data suggest strongly that the oligomycin-insensitive Mg– ATPase is an inherent constituent of RKCM. Small but significant inhibition of Mg–ATPase by oligomycin in both microsomal and mitochondrial fractions suggested that some mitochondria were damaged to various degrees

Table 3
Mg-ATPase activity / oligomycin-insensitive / in the inner and outer membranes of rat kidney cortex mitochondria

	nmoles ATP/min × mg protein
Intact mitochondria	60
Inner membranes	15
Outer membranes	95

Rat kidney cortex mitochondria were incubated with digitonin (2.1 mg/10 mg protein) for 20 min at 0°C, and centrifuged for 10 min at 5000 *g*. Sediment (inner membranes) was washed once. Supernatant was centrifuged for 90 min at 105 000 *g*, resulting supernatant (outer membranes) was suspended in small volume of preparation medium and taken for estimations. Mg-ATPase activity was measured in the presence of 10 μg/ml oligomycin.

during preparation, presumably during homogenization of the tissue.

To localize further the Mg–ATPase within the mitochondria inner and outer membranes of RKCM were separated by digitonin treatment and subsequent differential centrifugation. Mg–ATPase activities in fractions obtained are shown in table 3. Highest activity was recovered in outer membrane fraction, with some activity still in the inner membranes, probably due to incomplete separation. This suggests that the oligomycin-insensitive Mg–ATPase is localized in the outer membrane of RKCM, although certainly more detailed studies with use of marker enzymes are necessary to strengthen this conclusion.

In search for the possible role of the Mg–ATPase in the outer membrane, calcium accumulation supported by succinate and ATP was studied in RKCM. The results are shown in fig. 3. For comparison, calcium accumulation in identically prepared liver mitochondria is also shown. Succinate-supported Ca^{2+} accumulation was identical in mitochondria from both tissues. In liver mitochondria, ATP was as effective as succinate in supporting Ca^{2+} uptake. In kidney cortex mitochondria, however, Ca^{2+} uptake in the presence of ATP was slow and incomplete , coincident with high ATPase activity. Addition of succinate in the presence of ATP did not increase neither the rate nor extent of Ca^{2+} accumulation. The rates of succinate- or ATP-supported Ca^{2+} uptake in liver and kidney mitochondria are presented in table 4. It may be

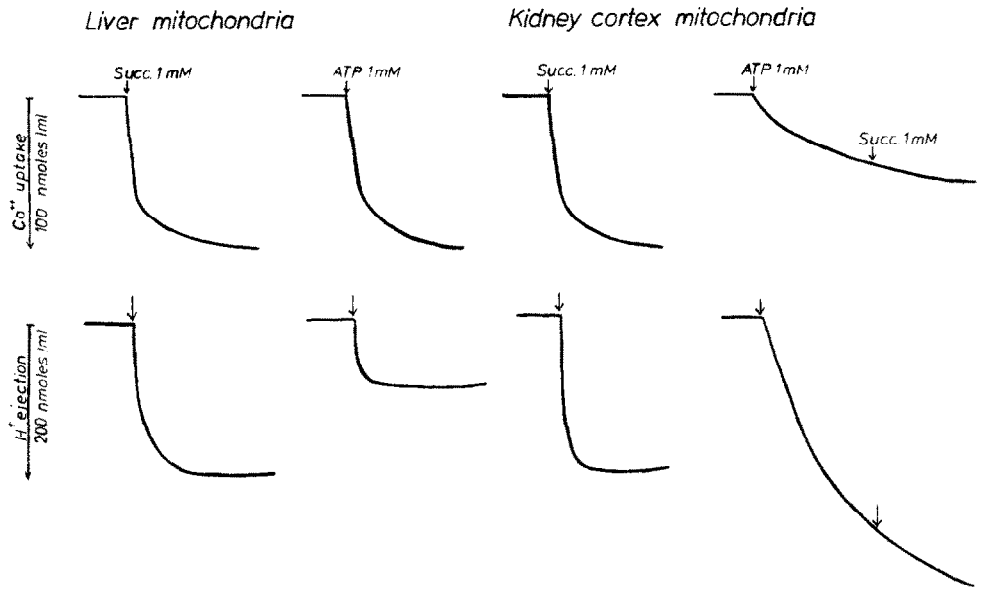

Fig. 3. Ca^{2+} accumulation and proton ejection supported by succinate or ATP in liver and kidney cortex mitochondria. Incubation medium contained sucrose 170 mM, Tris–Cl 20 mM, KCl 10 mM, $MgCl_2$ 10 mM, $CaCl_2$ 0.1 mM, rotenone 5 μg/ml and 1.5 mg/ml mitochondrial protein. Reaction was started by addition of succinate or ATP. Ca^{2+} accumulation and proton ejection were followed with Ca^{2+}-sensitive and pH-sensitive electrodes.

seen that in RKCM that rate of Ca^{2+} uptake is about 4 times slower in the presence of ATP than when succinate is the energy donor. Since it was observed that the oligomycininsensitive Mg–ATPase decreased, and ATP-supported Ca^{2+} uptake increased upon storage of RKCM, it is possible that even slower rates could be observed in very quickly prepared RKCM.

Table 4
Rates of Ca^{++} accumulation supported by ATP or succinate in kidney cortex and liver mitochondria

	Ca^{2+} accumulation rate nmoles/min × mg protein
A. Kidney cortex mitochondria	
+ succinate 1 mM	70
+ ATP 0.5 mM	14
+ ATP 1.0 mM	17
+ ATP 5.0 mM	28
B. Liver mitochondria	
+ succinate 1 mM	72
ATP 1 mM	64

Incubation medium contained: sucrose 170 mM, Tris–Cl 20 mM, KCl 10 mM, $MgCl_2$ 10 mM, rotenone 5 μg/ml, $CaCl_2$ 0.1 mM and mitochondrial protein 1.5 mg/ml, pH 7.2. Reaction was started by addition of succinate or ATP. Accumulation was followed with calcium-sensitive electrode. Rates were calculated from accumulation during initial 30 sec.

The presented data suggest that in renal tubular cells, which carry out bulk transcellular transport of calcium, the mitochondria are equipped with an ATP-dependent system, localized in the outer membrane, which prevent their massive loading with calcium. They also suggest that under physiological conditions outer membrane of rat kidney mitochondria may not be freely permeable to all substances of low molecular weight.

References

[1] Hogeboom, G. H. (1955) in: Methods in Enzymology, (S. P. Colowick and N. O. Kaplan, eds.), Vol. I, p. 16, Academic Press, New York.
[2] Johnson, D. and Lardy, H. (1967) in: Methods in Enzymology, (R. W. Estabrook and M. E. Pullman, eds.), Vol. X, p. 94, Academic Press, New York.

[3] Nishimura, M., Ito, T. and Chance, B. (1962) Biochim. Biophys. Acta 59, 177.
[4] Brdiczka, D., Gerbitz, K. and Pettke, D. (1969) European J. Biochem. 11, 234.
[5] Szarkowska, L. and Klingenberg, M. (1963) Biochem. Z. 338, 474.
[6] Katz, A. I. and Genant, H. K. (1971) Pflugers Arch. 330, 136.
[7] Manitius, A., Bensch, K. and Epstein, F. H. (1968) Biochim. Biophys. Acta 150, 563.
[8] Hendler, E. D., Torretti, J. and Epstein, F. H. (1971) J. Clin. Invest. 50, 1329.