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Kinetic Properties of Mitochondrial H^+-Adenosine Triphosphatase in Morris Hepatoma 3924A[1]

Ferdinando Capuano, Riccardo Stefanelli, Emanuela Carrieri, and Sergio Papa[2]

Institute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy

ABSTRACT

A study of kinetic properties of mitochondrial ATPase in Morris hepatoma 3924A is reported. The results show that submitochondrial particles isolated from the tumor tissue exhibited a three-fold increase in both the K_m for ATP hydrolysis and K_i for the competitive inhibitor [β,γ-*imido*]ATP with regard to normal rat liver. Eadie-Hofstee analysis of the kinetics of ATP hydrolysis show that both the high and the low affinity constants for ATP were enhanced in the hepatoma with respect to the rat liver enzyme.

Kinetic analysis of passive proton conduction through the F_0 sector of ATPase does not reveal any difference between Morris hepatoma and rat liver. In Morris hepatoma particles, 50% inhibition of the hydrolase activity required 10 times more oligomycin than in control particles. On the contrary, 50% inhibition of proton conduction occurred in both hepatoma and rat liver particles at the same concentration of oligomycin.

It is concluded that in Morris hepatoma the catalytic process in F_1 and the functional connection between F_1 and F_0 of the ATP synthase are altered with regard to control rat liver.

INTRODUCTION

The mitochondrial H^+-ATPase complex, H^+-ATP synthase, is a membrane-associated enzyme which utilizes the electrochemical proton gradient generated by the respiratory chain to produce most the ATP necessary to cellular metabolism.

The ATPase complex can be resolved into two functionally distinct multipeptide moieties: the water soluble catalytic sector F_1, which participates directly in ATP hydrolysis, and the F_0 sector which spans the inner mitochondrial membrane and functions as an H^+ translocator (for review see Refs. 1 and 2).

In rapidly growing tumor cells, Na^+-dependent dissipation of the ΔpH component of the aerobic proton motive force in mitochondria has been observed (3), with consequent depression of proton-coupled uptake by mitochondria of pyruvate (4) and phosphate (5), which are needed to support oxidative phosphorylation. Furthermore, data have been presented indicating altered hydrolytic activity of mitochondrial H^+-ATPase in certain tumor cells (6–9).

In this paper a study is presented of the kinetic properties of mitochondrial H^+-ATP synthase in Morris hepatoma 3924A, a rapidly growing tumor, poorly differentiated and characterized by high rate of aerobic glycolysis. The results show that the enzyme from tumor mitochondria presents altered characteristics of the catalysis of ATP hydrolysis in F_1 and alterations in the F_1-F_0 functional connection.

MATERIALS AND METHODS

Materials. Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, β-nicotinamide-adenine dinucleotide, reduced form, [β,γ-*imido*]-ATP[3], and catalase from Boehringer (Mannheim, FRG). Oligomycin and valinomycin were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity grade available.

Preparation of Mitochondria and Submitochondrial Particles. Morris hepatoma 3924A tumors were transplanted into both hind legs of inbred ACI/T rats and harvested 3 to 4 weeks after transplantation.

Mitochondria were isolated from the tumors after removal of necrotic tissue according to (10). Rat liver mitochondria were prepared as described in (11).

"Inside out" submitochondrial particles were obtained by exposure of mitochondria, that had been stored frozen at −70°C for 24 h, to ultrasonic energy in the presence of EDTA at pH 8.5 (ESMP) (12, 13).

Mg-ATPSMP were prepared by sonication of mitochondria in the presence of ATP (0.1 mM) and $MgCl_2$ (0.16 mM) as in Ref. 14.

Determination of ATPase Activity. ATPase activity was determined in the presence of added pyruvate kinase, phosphoenolpyruvate, and lactate dehydrogenase by following NADH oxidation spectrophotometrically at 340 nm in a termostatically controlled reaction cell at 30°C. The reaction mixture consisted of 250 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Tris/HCl (pH 7.5), 0.025 mM NADH, 0.5 μg rotenone, 1 mM phosphoenolpyruvate, 2.5 units lactate dehydrogenase, 2 units pyruvate kinase in a final volume of 1 ml. The reaction was started by the addition of ATP at the concentrations reported in the legends to the figures.

Measurement of Proton Translocation. ESMP (3 mg protein/ml) was incubated in a reaction mixture containing: 250 mM sucrose, 30 mM KCl, 0.5 μg valinomycin/mg protein, 0.2 mg/ml purified catalase, and 20 mM succinate as respiratory substrate; final volume 1.5 ml (pH 7.5). Incubation was carried out in a glass vessel, under a constant stream of N_2, termostated at 25 °C. Respiration-driven proton translocation was activated by repetitive pulses of 1–3% H_2O_2 (5 μl/ml). The pH of the suspension was monitored potentiometrically with a Beckman combination electrode (No. 39030; Beckman Instruments International, Geneve, Switzerland) connected to a Keithley differential electrometer amplifier (Model 604; Keithley Instruments) and from this to a strip chart recorder. The overall response time (10–90% change) of the pH recording systems used was 300 ms at 25°C (15). For kinetic analysis of the anaerobic proton release from submitochondrial particles, the potentiometric traces were converted into proton equivalents by double titration with standard HCl and KOH and treated by a double-exponential equation as in Ref. 16.

Protein was determined by the method of Lowry (17).

RESULTS

The ATP hydrolase activity of submitochondrial particles (ESMP) from control rat liver and Morris hepatoma 3924A was calculated from saturation curves (Fig. 1), the line of best fit was obtained by the method of least squares. The mean values for V_{max} and K_m measured in different ESMP preparations are reported in Table 1. No significant difference in the V_{max} values for ATP hydrolysis between ESMP from control rat-liver and hepatoma was found, whereas the K_m value for ATP was considerably higher in hepatoma than in control ESMP. This difference was also seen in submitochondrial particles prepared in the presence of ATP and Mg (Mg-ATPSMP) (see Table 1), where the ATPase activity, for both control and

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[2] To whom requests for reprints should be addressed, at Institute of Medical Biochemistry and Chemistry, University of Bari, Piazza Giulio Cesare, 70124 Bari, Italy.

[3] The abbreviations used are: [β,γ-*imido*]ATP, adenosine 5'[β,γ-imido]triphosphate; CCCP, carboxylcyanide *m*-chlorophenylhydrazone; Mg-ATPSMP, magnesium-ATP submitochondrial particles; ESMP, energy-transfer system of submitochondrial particles.

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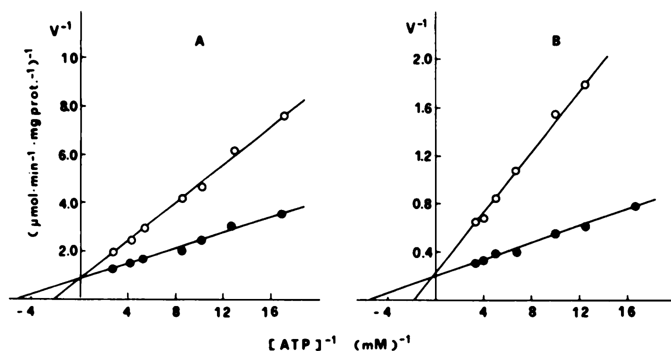

Fig. 1. Double reciprocal plots of ATP hydrolase activities of coupled (*A*) and uncoupled (*B*) EDTA submitochondrial particles from control rat liver and Morris hepatoma 3924A. ESMP (50 μg of protein/ml) were incubated in the sucrose medium described in "Materials and Methods." Final volume 1 ml, pH 7.5. Temperature 30°C. The concentration of protonophoric agent CCCP was 0.2 μM. ●, rat liver; ○, Morris hepatoma 3924A.

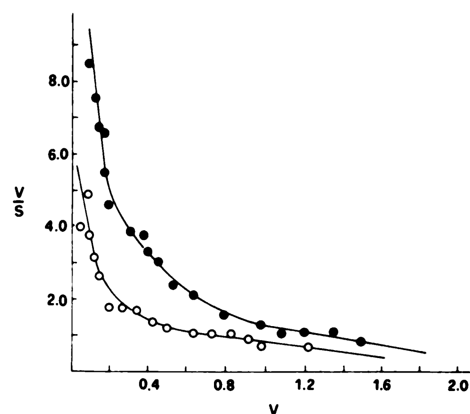

Fig. 2. Eadie-Hofstee plots of the kinetics of ATP hydrolysis by ESMP from control rat liver and Morris hepatoma 3924A. ESMP (100 μg of protein/ml) were incubated as described under "Materials and Methods." The ATP concentration ranged from 0.01 to 1.8 mM. ●, control rat liver; ○, Morris hepatoma.

Table 1 *ATPase activity of submitochondrial particles from rat liver and Morris hepatoma 3924 A*

Submitochondrial particles prepared in the presence of EDTA (ESMP) or in the presence of Mg-ATP (Mg-ATPSMP) were incubated as described in "Materials and Methods," at 50 μg of protein/ml or 30 μg of protein/ml, respectively. Where indicated, ESMP were frozen 24 h at −70°C then thawed and assayed for ATPase activity.

	V_{max} (μmol min⁻¹ mg protein⁻¹)	K_m (mM)
Rat liver		
ESMP	(6) 1.13 ± 0.12	(6) 0.17 ± 0.01
ESMP after freeze-thaw	2.55	0.12
ESMP + 0.2 μM CCCP	4.79	0.18
Mg-ATPSMP	1.43	0.22
Morris hepatoma		
ESMP	(6) 1.08 ± 0.10	(6) 0.49 ± 0.02
ESMP after freeze-thaw	3.03	0.69
ESMP + 0.2 μM CCCP	4.44	0.56
Mg-ATPSMP	1.38	0.56

hepatoma, was slightly higher than that of ESMP. The same situation was also found for the ATPase activity of ESMP uncoupled as a consequence of freeze-thaw treatment (18) or by the addition of the protonophoric agent CCCP (Fig. 1 and Table 1).

The fact that the V_{max} measured for ATP hydrolysis in coupled and uncoupled particles is equal in hepatoma and normal rat liver (see also Ref. 18) indicates that the content of F_1 in the particles from Morris hepatoma is the same as that in particles from rat liver.

The catalytic process in F_1 involves positive and negative cooperativity between two (or three) catalytic sites located on the β subunits or at the α-β interface (19–23). Thus, double-reciprocal plots do provide only overall approximate information for the kinetic properties of the ATP hydrolase activity.

To analyze the cooperative behavior of the ATPase activity, a wide range of substrate concentrations were used in submitochondrial particles from control rat liver and Morris hepatoma and the data analyzed by Eadie-Hofstee plots (Fig. 2). The plot shows the reported cooperative behavior of the ATP hydrolase activity.

The curvilinear data of Eadie-Hofstee plots were computer analyzed for the least number of straight lines which when combined best fitted the experimental points. It was found that data best fitted two slopes. The values of the kinetic constants derived from the two lines, further refined using an iterative computer program, are presented in Table 2. It can be noted that the K_m values for the high affinity site (K_{m_1}) and for the low affinity site (K_{m_2}) were both higher in hepatoma than in control ESMP.

[β,γ-*imido*]ATP, an analogue of adenosine triphosphate, is a nonhydrolyzable competitive inhibitor of the ATPase complex. Measurement of ATPase activity at different ATP and [β,γ-*imido*]ATP concentrations allowed to calculate, using Dixon plots, the dissociation constant K_i. The observed K_i for [β,γ-*imido*]ATP was considerably higher in ESMP from hepatoma than from control, the values being 3.7 μM and 1.2 μM, respectively (Fig. 3).

Oligomycin inhibits the catalytic activity of F_1 by blocking specifically the proton conduction through F_0 (24). Titration curves for the inhibitory effect of oligomycin on the ATP hydrolase activity in ESMP from control and hepatoma are shown in Fig. 4. It can be noted that much more oligomycin was required to cause half-maximal inhibition in hepatoma with respect to control particles. In control ESMP 50% inhibition of hydrolytic activity occurred at 0.21 μg of oligomycin/mg particle protein (Fig. 4*A*) whereas in hepatoma the same extent of inhibition was reached at 2.7 μg of oligomycin/mg of protein (Fig. 4*B*).

In ESMP the anaerobic relaxation of transmembrane proton gradient set up by respiration, occurs through the membrane F_0 sector of the H^+-ATPase, the process consisting of two apparent first-order phases characterized by the kinetic constants k_1 and k_2 (12, 16, 25). Statistical evaluation of proton translocation in ESMP is reported in Table 3. It can be noted that no significant difference in the steady state extent of anaerobic proton uptake, in the overall rate of passive proton back flow, expressed as $1/t_{1/2}$, and in the first-order kinetic constants of the two phases of this process was found between control and hepatoma ESMP.

A titration of the effect of oligomycin on proton backflow through F_0 in hepatoma and rat-liver ESMP is shown in Fig. 5. The oligomycin titer for half-maximal depression of the overall rate of anaerobic relaxation of the respiratory proton gradient was, in both type of particles, close to 0.1 μg/mg of protein. These observations are consistent with the data of Nelson *et al.* (26) showing a content of F_0 in hepatoma mitochondria equivalent to that of rat-liver mitochondria.

DISCUSSION

The alterations in the catalytic activity of the mitochondrial H^+-ATPase of Morris hepatoma as compared to rat liver, revealed in this study, can be summarized as follows: (*a*) decreased affinity of the enzyme for ATP; (*b*) altered interaction of the catalytic sector F_1 with the proton-conducting F_0 sector

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Table 2 *Kinetic constants of ATP hydrolysis by ESMP from rat liver and Morris hepatoma 3924A*

The assay conditions were the same as those described under "Materials and Methods" and Legend to Fig. 2. Kinetic constants were computer derived from the curvilinear data of Eadie-Hofstee plots of the type shown in Fig. 2. Values are means ± SE for four determinations.

	μmol min^{-1} mg protein^{-1}		mM	
ESMP	V_1	V_2	K_{m_1}	K_{m_2}
Rat liver	0.15 ± 0.02	1.45 ± 0.1	0.014 ± 0.003	0.36 ± 0.05
Morris hepatoma	0.16 ± 0.01	1.6 ± 0.1	0.033 ± 0.002	0.95 ± 0.07

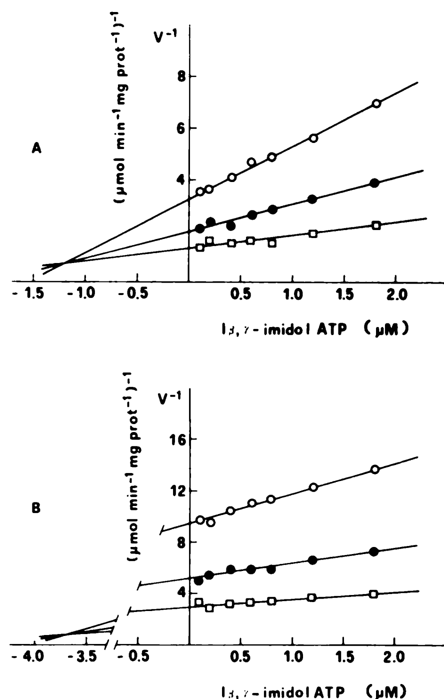

Fig. 3. Dixon plots of [β,γ-*imido*]ATP inhibition of ATPase activity in ESMP isolated from rat liver and from Morris hepatoma. Submitochondrial particles (50 μg of protein/ml) were preincubated 5 min in the presence of the inhibitor before ATP additions. ATPase activity was measured as described in "Materials and Methods." The ATP concentrations used were: (○) 0.05 mM, (●) 0.1 mM, and (□) 0.2 mM. *A*, control rat liver; *B*, Morris hepatoma 3924A. The dissociation constant K_i calculated from this graph was 1.2 μM for rat liver and 3.7 μM for Morris hepatoma.

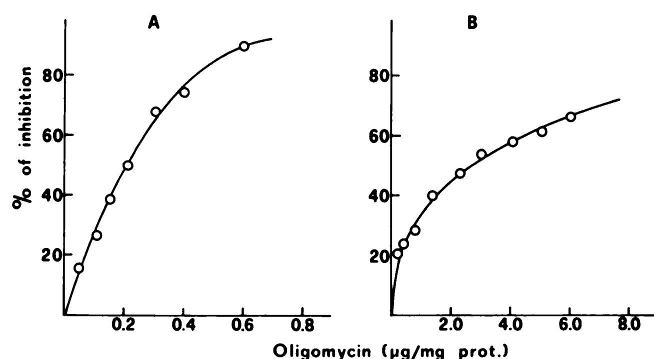

Fig. 4. Titration of the inhibitory effect of oligomycin on ATPase activities in ESMP from rat liver (*A*) and Morris hepatoma 3924A (*B*). *Abscissa*, ESMP were preincubated 5 min with oligomycin at the concentration given. For experimental conditions see under "Materials and Methods." Half-maximal inhibition occurred at 0.21 μg of oligomycin/mg of protein for rat liver and 2.7 μg/mg of protein for Morris hepatoma.

as revealed by the much lower sensitivity of ATP hydrolysis to inhibition by oligomycin. The decreased affinity for ATP is documented by the marked enhancement of the apparent K_m for ATP, as determined from the kinetics of ATP hydrolysis in everted particles of the inner mitochondrial membrane in the presence or absence of Mg^{2+} and in the coupled or uncoupled state. Thus it is independent of possible effects involving translocation across the membrane of adenine nucleotides (27), loss of Mg^{2+} (7, 27), or different binding of the ATPase inhibitor protein (9), whose functional association of F_1 is controlled by $\Delta\mu H^+$. ESMP from hepatoma exhibit a significantly higher K_i for the competitive inhibitor [β,γ-*imido*]ATP as compared to ESMP from rat liver. This provides further evidence for an alteration of the catalytic domain of F_1 resulting in a lower affinity for ATP.

The activity of mitochondrial ATPase is known to be affected by phospholipids (28). In Morris hepatoma 3924A, as well as in other hepatoma mitochondria, the cholesterol-to-phospholipid ratio is higher than in normal liver mitochondria (29). It has, however, been reported (30) that cholesterol enrichment of the inner mitochondrial membrane causes an increase in the ATPase activity and a decrease in the K_m value for ATP which are just opposite to the changes described here.

The observation that the V_{max} of ATP hydrolysis referred to the protein content of the membrane, is in hepatoma particles equal to that of rat liver particles, both in coupled and uncoupled state, indicates that the F_1 content in hepatoma mitochondria is not decreased with respect to normal rat liver. Direct immunochemical analysis by Nelson *et al.* (26) have, in fact, shown that in Zajdela hepatoma mitochondria the content of both F_1 and F_0 is equivalent to that of rat liver mitochondria.

Also in the presence of a normal content of ATP synthase, the decreased affinity of the enzyme for ATP will result in a significant depression of the rate of ATP hydrolysis and possibly favor ATP synthesis driven by $\Delta\mu H^+$. These changes in the catalytic characteristics of the H^+-ATPase, and the reported (31) enhancement of the content of the ATPase inhibitor protein may represent an important defense attribute of tumor cells. In tumors since the inhibition of mitochondrial utilization of pyruvate (4) and phosphate (5) and depression of respiratory $\Delta\mu H^+$ (3), the ATPase complex would tend to rapidly hydrolyse glycolytic ATP in the absence of the inhibitory factors just described.

The catalytic cycle in F_1 is compulsorily coupled to proton conduction in F_0 (32) , the overall rate of proton backflow through the F_0 was, however, the same in ESMP from rat liver and hepatoma, this being in agreement with the observation of a normal F_0 content in these tumors (26).

Oligomycin is a specific inhibitor of the proton-conducting pathway in the F_0 sector of ATPase and the extension of the inhibitory effect to ATP hydrolysis (or synthesis), appears to involve one (oligomycin sensitivity conferring factor) (33) or more proteins at the F_1-F_0 junction (34). The oligomycin concentration required in hepatoma particles for half-maximal inhibition of the ATP hydrolase activity was one order higher than that required in control particles. On the other hand, 50% inhibition of passive proton conduction in F_0 occurred in hepatoma particles at the same concentration causing 50% inhibition of proton conduction in control particles. Thus, the change observed in hepatoma particles in the inhibitory titre of oligomycin for the ATP hydrolase activity cannot be ascribed to alterations of F_0 subunits directly involved in oligomycin sensitive proton conduction (35). These observations would there-

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Table 3 *Kinetic parameters of anaerobic relaxation of respiratory $\Delta\mu H^+$ in ESMP from rat liver and Morris hepatoma 3924A*

ESMP (3 mg/ml) were incubated in the reaction medium described under "Materials and Methods." Final volume 1.5 ml, pH 7.4. Temperature 25°C. $t_{1/2}$ and the first order kinetic constants were calculated as described in (16). The values are means ± SE for six experiments.

ESMP	H^+ uptake (ng ion/mg prot.)	$1/t_{1/2}$	k_1 (s^{-1})	k_2
Rat liver	2.8 ± 0.4	2.59 ± 0.14	3.35 ± 0.9	0.55 ± 0.16
Morris hepatoma	2.4 ± 0.6	2.28 ± 0.20	2.72 ± 0.5	0.54 ± 0.10

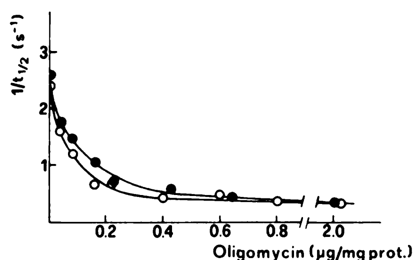

Fig. 5. Effect of oligomycin on the overall rate of proton conduction in EDTA submitochondrial particles from rat liver and Morris hepatoma 3924A. For experimental conditions see Fig. 4, *legend*, and "Materials and Methods." Half-maximal inhibition occurred at 0.1 µg of oligomycin/mg of protein for rat liver and 0.08 µg/mg of protein for Morris hepatoma. ●, rat liver; ○, Morris hepatoma.

fore suggest that in H^+-ATPase complex from hepatoma, whereas subunits involved in the proton conducting pathway are not affected, there is an alteration of one or more of the subunits (oligomycin sensitivity conferring factor, F6, γ, δ, and ϵ), which are thought to be involved in the functional interaction between the two sectors of the complex.

Further work is in progress to identify the subunits involved in the observed changes and the role they may play in the energy metabolism of neoplastic cells.

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