

The effect of the natural protein inhibitor on H^+ -ATPase in hepatoma 22^a mitochondria

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The uncoupler-induced inactivation of H^+ -ATPase in hepatoma 22^a and mouse liver mitochondria has been studied. The dependence of this process on $\Delta\mu H$, and pH and ATP was established. The inactivated ATPase could be reactivated at alkaline pH values in the absence of ATP. These data indicate that the inactivation is apparently caused by the natural protein inhibitor. ATP- and pH-dependent decrease of ATPase activity is also observed after Lubrol-WX disruption of mitochondria. It can be proposed that practically all ATPase molecules in hepatoma mitochondria are in a catalytically active complex with the protein inhibitor. At low $\Delta\mu H$ this complex is inactivated via reversible pH-dependent and irreversible ATP-dependent rearrangements. The pH-dependent rearrangement of the isolated protein inhibitor from hepatoma mitochondria is also observed.

Mitochondria; ATPase inhibitor protein; Lubrol-WX disruption; (Submitochondrial particle, Hepatoma)

1. INTRODUCTION

The effect of uncouplers on ATPase activity is one of the most clearly expressed differences between mitochondria from a number of tumors and normal tissues [1-3]. ATPase activity decreases in tumor mitochondria after preincubation with uncouplers (but not with valinomycin) and does not change in mitochondria of different normal tissues. In our previous works [4,5] this phenomenon was also demonstrated in mitochondria of mouse hepatoma 22^a. It was shown that this effect is a consequence of at least two processes.

The first is the slowing of the ATP transport rate through an ATP/ADP-carrier as a result of ADP release from the matrix at low $\Delta\mu H$ values, and the second one is the pH-dependent inactivation of ATPase. The data obtained in the present work provided us with strong evidence that this latter process is caused by ATPase interaction with the natural protein inhibitor. This protein with an M_r of approx. 10000 blocks the ATPase at low $\Delta\mu H$, acidic pH values and in the presence of MgATP [6]. Preliminary results of the present work were published in [5].

2. MATERIALS AND METHODS

A rapidly growing hepatoma 22^a (11 days) was transplanted into adult females of C3HA mice [7]. Mitochondria and particles (SMP) lubrolyzed (at pH 8.0) from mouse liver and hepatoma 22^a were isolated according to Pedersen et al. [8]. The preincubation medium contained 70 mM sucrose, 220 mM mannitol, 10 mM KCl, 10 mM Mes (pH

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Abbreviations: SMP, submitochondrial particle; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; BSA, bovine serum albumin

6.0) or 5 mM Hepes (pH 7.0) or 20 mM Tris-HCl (pH 8.0–8.7), 10 mM succinate, 1 mg/ml BSA. The following medium was used to measure ATPase activity: 70 mM sucrose, 220 mM mannitol, 30 mM KCl, 10 mM KHSO_3 , 0.1% Lubrol-WX, 20 mM Tris-HCl, 3 mM MgSO_4 , 2 mM ATP, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 3 μM rotenone, 5 U/ml pyruvate kinase, 5 U/ml lactate dehydrogenase, pH 8.7. This medium was preincubated with myokinase (1 U/ml) to convert the AMP present in the chemicals and to prevent the kinase reaction during the measurement. Separation of the mitochondria disrupted by Lubrol-WX from external nucleotides was performed in columns with Sephadex G-50 (fine) equilibrated with an incubation medium, pH 8.0, containing Lubrol-WX (0.1%), according to Penefsky [9]. The protein inhibitor was extracted from mitochondria with KOH as described in [10] and used without additional purification.

3. RESULTS

3.1. $\Delta\bar{\mu}H^+$ -dependent inactivation of ATPase in mitochondria

In order to exclude the effect of transport pro-

cesses, ATPase activity was determined in the presence of a detergent, Lubrol-WX. ATPase activity became insensitive to carboxyatractyloside (inhibitor of the ATP/ADP-carrier) but could be blocked by oligomycin (inhibitor of intact F_0F_1 -ATPase) after this treatment. Lubrol-WX did not affect ATPase activity or the interaction of ATPase with the added protein inhibitor in sub-mitochondrial particles (not shown).

Preincubation of hepatoma mitochondria with an H^+ -conducting uncoupler, CCCP, or with combination of valinomycin and nigericin in the presence of MgATP resulted in a pH-dependent inactivation of ATPase (fig.1A, curve 1). Neither valinomycin ($\Delta\psi$ -dissipating agent), nor nigericin (ΔpH -dissipating agent) alone induced the inactivation at any pH within the range of 8.7–6.0, so it is $\Delta\bar{\mu}H$ that seems to be crucial for this effect. It must be noted, however, that the effect of valinomycin was not observed, possibly because the pH value in the matrix was increasing under this condition and thus prevented inhibition.

Inactivation with a similar $\Delta\bar{\mu}H$ and pH-dependence but to a lesser extent was observed with mitochondria from mouse liver (fig.1A, curve 3).

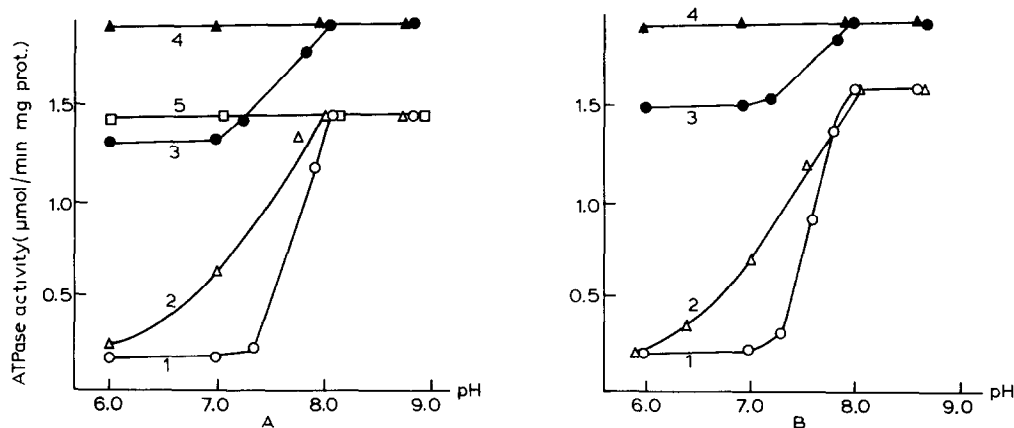


Fig.1. pH-dependence of ATPase inactivation in intact (A) and disrupted (B) mitochondria. (A) Mitochondria (0.2 mg protein/ml) from mouse liver (close symbols) or hepatoma 22^a (open symbols) were preincubated at various pH values for 2 min at 25°C in the medium described in section 2. The additions in preincubation were the following (curves; symbols): (1,3; $\circ, \bullet$) CCCP (2×10^{-6} M) plus MgATP (2 mM); (2,4; $\Delta, \blacktriangle$) CCCP (2×10^{-6} M); (5; $\square$) nigericin (10^{-7} M) plus MgATP (2 mM) (the activity in hepatoma mitochondria preincubated without additions was equal). (B) Mitochondria (0.2 mg protein/ml) were preincubated at pH 8.7 with Lubrol-WX (0.1%) for 5 min at 25°C and passed through Sephadex G-50 columns (see section 2). Thus treated mitochondria were preincubated exactly as in A (curves; symbols): (1,3; $\circ, \bullet$) with MgATP (2 mM); (2,4; $\Delta, \blacktriangle$) without additions.

These data indicate that the uncoupler-induced inactivation of ATPase is apparently a result of protein inhibitor action.

3.2. *MgATP-dependence of ATPase inactivation and reactivation*

The uncoupler-induced inactivation of ATPase in mouse liver mitochondria was observed only in the presence of MgATP (fig.1A, curves 3,4). On the other hand, ATPase in hepatoma 22^a mitochondria was inactivated without added MgATP (fig.1A, curve 2). If hepatoma mitochondria were preincubated for 5 min with valinomycin at pH 8.0 when ATP was hydrolyzed [4] and the protein inhibitor did not block the enzyme (see above), the addition of CCCP resulted in 55% inhibition of ATPase at pH 7.0; the addition of CCCP plus MgATP, in 90% inhibition. Therefore, the extent of inactivation did not depend on the endogenous ATP level.

If hepatoma mitochondria, preincubated with CCCP at pH 6.0–7.0 in the absence of MgATP, were disrupted by Lubrol-WX (0.1%) and transferred to pH 8.7, the reactivation was observed (fig.2, curve 1). The reactivation was com-

pletely blocked by MgATP (2 mM) and was not detectable at pH values lower than 8.0 (fig.2, curve 2). When hepatoma mitochondria were preincubated with CCCP (or valinomycin plus nigericin) in the presence of MgATP, no reactivation was observed (fig.2, curve 3).

These findings gave us further evidence that the uncoupler-induced inactivation of ATPase in the absence of MgATP is a result of protein inhibitor action. These data also indicate that different enzyme-inhibitor complexes are formed in the presence or in the absence of MgATP.

3.3. *Rearrangement of the complex of ATPase with protein inhibitor*

To study the effect of the protein inhibitor bound to the enzyme, hepatoma mitochondria treated with Lubrol-WX (0.1%) (at pH 8.7 to prevent inactive complex formation) were passed through columns with Sephadex G-50 (see section 2). The control tests show that the protein inhibitor isolated from hepatoma mitochondria does not pass through these columns. When thus treated mitochondria were transferred to pH 6.0–7.0, the ATPase was inactivated to the same extent as in intact organelles (fig.1B, curve 2). MgATP-dependences of inactivation (curves 1,2) and reactivation also coincide in both cases. Partial inactivation of ATPase in mouse liver mitochondria was also observed after the disruption. Inactivation occurred only in the presence of MgATP (fig.1B, curves 3,4) and was irreversible.

The data obtained indicate that in mitochondria from hepatoma 22^a nearly all of the ATPase molecules are in an active complex with the protein inhibitor which can be converted to the inactive state.

3.4. *Interaction of the protein inhibitor isolated from hepatoma mitochondria with submitochondrial particles*

From the experiments described in section 3.2, it was not clear if the protein inhibitor from hepatoma mitochondria could block ATPase without MgATP hydrolysis. The added protein inhibitor did not affect ATPase in the SMP from hepatoma or normal liver in the absence of MgATP (table 1). However, if the inhibitor was preincubated at pH 4.5 (according to [11,12]), the inhibition (at pH 6.0) was observed without added

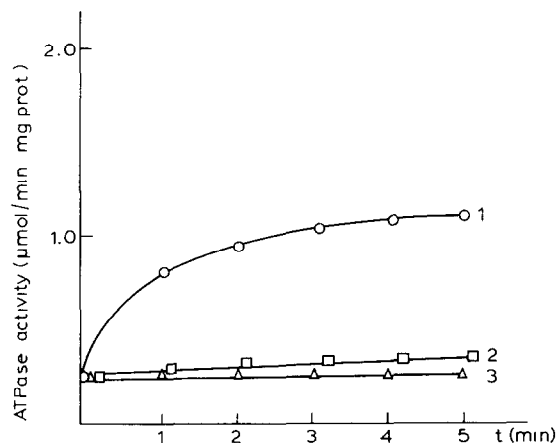


Fig.2. Reactivation of ATPase in hepatoma 22^a mitochondria preincubated with uncoupler. Mitochondria were preincubated at pH 6.0 with CCCP (curves 1,2) or with CCCP plus MgATP (curve 3) as described in fig.1A. Then Lubrol-WX (0.1%) was added and mitochondria were passed through Sephadex G-50 columns (see section 2) to avoid MgATP action in the reactivation medium. The reactivation was studied in incubation medium (with 0.5 mM EDTA added) at pH 8.7 (curves 1,3) or pH 7.4 (curve 2).

Table 1

Effect of the protein inhibitor from hepatoma 22^a mitochondria on ATPase in SMP from hepatoma and mouse liver

Conditions of SMP preincubation	ATP hydrolysis rate ($\mu\text{mol}/\text{min}$ per mg protein)	
	Hepatoma 22 ^a SMP	Mouse liver SMP
No additions	4.05	5.11
2 mM MgATP	4.00	5.11
Protein inhibitor	3.90	5.03
Protein inhibitor + 2 mM MgATP	0.97	1.14
Acidic protein inhibitor ^a	0.80	1.11
Acidic protein inhibitor + 0.5 mM EDTA ^a	4.00	4.90

^a Protein inhibitor was incubated for 5 min at pH 4.5 in a medium containing 250 mM sucrose, 10 mM KCl and 20 mM Mes to the obtain acidic form

SMP (0.05 mg protein/ml) were preincubated with the protein inhibitor (0.2 units) in 100 μl incubation medium, pH 6.0. ATPase activity was measured at pH 8.7. The inhibitor amount that suppresses 0.2 U ATPase by 50% at pH 6.0 in the presence of 5 mM MgATP was designated as 1 unit

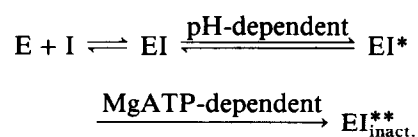
MgATP. This effect was prevented by the addition of 0.5 mM EDTA, so it seems probable that the hydrolysis of trace amounts of MgATP was sufficient for inhibition of ATPase with the 'acidic form' of the protein inhibitor. The same effects were observed with the protein inhibitor from mouse liver (not shown), so there is no qualitative difference between the normal and the hepatoma enzyme-inhibitor complexes if reconstructed from isolated components. The difference described in section 3.3 is probably caused by some mitochondrial matrix factors in the course of complex formation.

4. DISCUSSION

The data on $\Delta\mu\text{H}$, pH and MgATP-dependence of ATPase inactivation strongly support the suggestion that this effect is a result of the protein inhibitor's action [6]. Partial (30–40%) uncoupler-induced inactivation (only in the presence of

MgATP) was observed in mitochondria of mouse liver. These data are in good agreement with the results of Luciakova and Kůzela [13] who showed that the protein inhibitor content is higher in mitochondria of different hepatomas than in liver mitochondria.

In the present work it was shown that in hepatoma mitochondria practically all the ATPase molecules are in a tight active complex with the protein inhibitor (see section 3.3). At pH lower than 8.0 and in the presence of MgATP this complex is irreversibly converted to the inactive state. The hypothetical scheme for this process is:



In this sequence the main pH-dependent step seems to be the reversible rearrangement $\text{EI} \rightleftharpoons \text{EI}^*$ without inactivation. As shown recently [11,12], the protein inhibitor from yeast or bovine heart mitochondria is capable of a slow pH-dependent conformational change. The acidic form of inhibitor blocks ATPase much more effectively than the 'alkaline' form. An analogous effect was observed for the inhibitor from hepatoma mitochondria in the present work. It can be proposed that rearrangement of EI includes a similar change in the protein inhibitor conformation. The consequent ATP-dependent conversion of the acidic complex appears to be pH-independent and rapid. According to this mechanism, the uncoupler-induced pH-dependent inactivation of ATPase observed in hepatoma mitochondria in the absence of MgATP is the result of rapid blocking during the course of activity measurements. The extent of inactivation in this case correlates with the fraction of ATPase-inhibitor complex in the acidic form in intact mitochondria.

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