Regulation of ATP hydrolysis in liver mitochondria from ground squirrel

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The uncoupler-induced inactivation of H^+-ATPase in liver mitochondria from ground squirrel has been studied. The dependence of this process on $\Delta\tilde{\mu}H^+$, pH and ATP indicates that it is caused by the protein inhibitor. This conclusion is also supported by the protective effect of Zn^{2+} and Cu^{2+}. The inactivation can be induced by Ca^{2+} at low concentrations in the presence of phosphate. It is shown that the protein inhibitor inactivates ATPase almost completely under optimal conditions while its effect in mice or rat liver mitochondria does not exceed 30%. The potential efficiency of the inhibitor's action does not depend on either the season or the state of animals (hibernating or active). At the same time, the sensitivity of this system to Ca^{2+} is significantly lower in active (summer) animals.

ATPase inhibitor protein; Uncoupling; Calcium ion; Hibernation; Liver mitochondria; Ground squirrel

1. INTRODUCTION

ATP synthase (H^+-ATPase) localized in inner mitochondrial membrane catalyzes both the synthesis and the hydrolysis of ATP. At physiological concentrations of ATP and ADP a direction of the reaction is determined by the value of transmembrane difference of electrochemical potentials of protons ($\Delta\tilde{\mu}H^+$). Regulation of the enzyme activity is organized in such a fashion that futile ATP hydrolysis in de-energized mitochondria (at decreased $\Delta\tilde{\mu}H^+$) is minimized [1]. The natural protein inhibitor of ATPase plays the key role in the regulatory mechanism [2,3]. This 9.5 kDa protein forms a tight complex with the catalytic component of ATPase (F_1) and blocks ATP hydrolysis at low $\Delta\tilde{\mu}H^+$ and acidic pH values. At the same time, protein inhibitor does not inhibit the stationary rate of oxidative phosphorylation as a result of dissociation of enzyme-inhibitor complex at high $\Delta\tilde{\mu}H^+$ [2].

The content of the inhibitor and its efficiency vary in different cell types and animal species. Its content in mitochondria from rat liver is several-fold lower than in heart, brain, skeletal muscles and is only 20–30% of ATPase molar content [4]. The efficiency of the inhibitor in mitochondria from rapidly growing mice hepatoma was shown to be significantly higher than in mitochondria from mouse liver [5]. De-energization of hepatoma mitochondria at pH 6.0–7.0 leads to the almost complete inactivation of ATPase. These data are in good agreement with the results of immunochemical analysis that show high content of protein inhibitor in hepatoma mitochondria [4].

The data presented in this work indicate the high efficiency of ATPase protein inhibitor in mitochondria from ground squirrel liver. The regulation of ATPase in these mitochondria is suggested to be of great importance under conditions of hibernation and arousal of the animals.

2. MATERIALS AND METHODS

Ground squirrels (*Citullus undulatus*) from Yakutia were housed in individual cages at 20–22°C, natural daylight and were supplied with satisfactory food, water and nest material. In November they were transferred to the dark room with temperature 2–4°C where they stayed in hibernation until April. Hibernation consists of two repeating phases: 10–14 days of deep sleep (torpid phase) followed by 1–2 days of arousal active period. The animals in torpid phase were used when body temperature was 3–5°C.

Liver mitochondria were isolated in the following medium: 0.3 M sucrose, 0.5 mM $MgSO_4$, 1 mM EGTA, 10 mM Tris-SO_4, pH 7.4. The preincubation medium contained 0.25 M sucrose, 10 mM KCl, 5 mM succinate, 20 mM Mes (pH 6.0–6.5) or 20 mM Hepes (pH 6.9–7.5) or 20 mM Tris (pH 8.3). ATPase activity was measured in the medium containing 0.25 M sucrose, 20 mM KCl, 20 mM Tris-HCl, 3 mM $MgCl_2$, 5 mM $KHSO_3$, 1 mM phosphoenolpyruvate, 0.4 mM NADH, 2 mM ATP, 3 μM rotenone, 5 U/ml pyruvate kinase, 5 U/ml lactate dehydrogenase, 0.2% Lubrol-WX, pH 8.3. 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.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethyl benzimidazole

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3. RESULTS AND DISCUSSION

3.1. *Uncoupler-induced inactivation of ATPase*

The activity of ATPase in mitochondria from ground squirrel liver was determined by the method developed for liver and hepatoma mitochondria [5]. In order to exclude the effect of transport processes, ATPase activity was measured after disruption of mitochondria with detergent Lubrol-WX. Under these conditions, ATP hydrolysis became insensitive to carboxyatractyloside (inhibitor of ATP/ADP-carrier) but could be blocked by oligomycin (inhibitor of intact F_0F_1-ATPase). Control experiments show that Lubrol-WX did not affect ATPase activity or the interaction of ATPase with added protein inhibitor in submitochondrial particles from mice liver [5].

Preincubation of mitochondria from ground squirrel liver with H^+-conducting uncouplers CCCP (5×10^{-6} M) and TTFB (8×10^{-6} M) resulted in a pH-dependent inactivation of ATPase (Fig. 1). The inactivation is nearly complete in 1–2 min at pH 6.1 and is practically absent at pH higher than 8.0. The K^+/H^+ exchanger nigericin (5×10^{-7} M) dissipates ΔpH but does not induce inactivation at any pH studied. So, both the complete deenergization (dissipation of $\Delta\tilde{\mu}H^+$) and the low pH in the matrix are crucial for this effect.

Addition of Mg-ATP increases efficiency of the uncoupler-induced inactivation of ATPase (Fig. 1). The properties of inactivated enzyme being formed in the presence or in the absence of Mg-ATP are also different. If mitochondria preincubated with CCCP at pH 6.1 in the absence of Mg-ATP were disrupted by Lubrol-WX (0.2%) and transferred to the medium containing 5 mM EDTA at pH 8.6, reactivation was observed that reached 60–70% in 10 min. If Mg-ATP was added to the preincubation medium, the subsequent reactivation was not observed.

The data on $\Delta\tilde{\mu}H^+$, pH and Mg-ATP-dependence of ATPase inactivation indicate that this effect is a result of action of the protein inhibitor. This conclusion is strongly supported by the effect of such divalent cations as Zn^{2+} and Cu^{2+}, that prevent the inactivation (Table I). It was shown earlier that these cations prevent the effect of the protein inhibitor on ATPase in submitochondrial particles as well as on F_1-ATPase [6]. The effect of the cations is probably caused by interaction with functionally significant histidine residue in the molecule of the inhibitor [7]. In intact mitochondria from ground squirrel liver, Zn^{2+} and Cu^{2+} prevent the uncoupler-induced inactivation of ATPase but fail to reactivate the enzyme. Probably the effect of these cations depends on their potential-driven accumulation in energized mitochondria.

Inactivation of ATPase is induced also by rotenone, inhibitor of the respiratory chain (not shown). The effect of rotenone is observed at pH 6.4 but not at pH higher than 8.0 and takes 5–10 min to reach maximum.

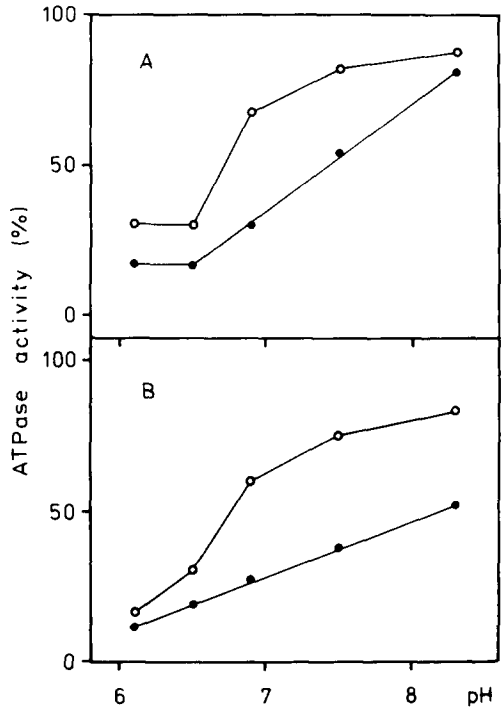

Fig. 1. pH-dependence of ATPase inactivation in liver mitochondria from arousal (A) and hibernating (B) ground squirrels. Mitochondria (0.2–0.4 mg protein/ml) were preincubated at various pH values for 2 min at 25°C in the medium described in section 2. The additions to the preincubation mixture: (○) 5 μM CCCP, (●) 5 μM CCCP and 2 mM Mg-ATP. Control activities (100%) measured after preincubation without CCCP were equal to 1.27 ± 0.11 μmol/min·mg protein (7 arousal animals) and 0.96 ± 0.18 μmol/min·mg protein (5 torpid animals). They do not significantly change with pH of preincubation medium.

Mg-ATP completely prevents the inactivation. These data indicate that the main factor determining the interaction of ATPase with the protein inhibitor in intact mitochondria is the $\Delta\tilde{\mu}H^+$ value.

The efficiency of uncoupler-induced protein inhibitor action in liver mitochondria of the ground

Table I

The effect of Zn^{2+} and Cu^{2+} on the uncoupler-induced inactivation of ATPase in liver mitochondria from arousing ground squirrel

Preincubation[a]	ATPase activity[b] (nmol/min·mg protein)
Without additions	720
100 μM $ZnSO_4$	650
5 μM CCCP	110
5 μM CCCP + 100 μM $ZnSO_4$	605
5 μM CCCP + 12.5 μM $ZnSO_4$	430
5 μM CCCP + 12.5 μM $CuSO_4$	510
5 μM CCCP + 2.5 μM $CuSO_4$	270

[a] Mitochondria were preincubated as described in caption to Fig. 1 at pH 6.5

[b] The medium for ATPase activity measurements additionally contained 50 μM EDTA

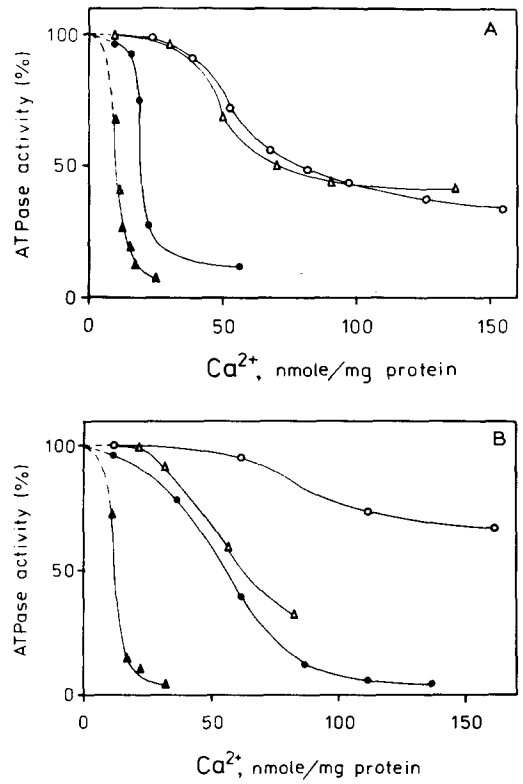

Fig. 2. Ca^{2+}-induced inactivation of ATPase in liver mitochondria from hibernating (A) and active (B) ground squirrels. (A) Mitochondria from arousal (○,●) and hibernating (△, ▲) animals were preincubated as described in caption to Fig. 1 at pH 6.5 without additions (open symbols) or with 2 mM Mg-ATP (closed symbols). (B) Mitochondria from active animals (isolated in May) were preincubated at pH 6.5 without additions (△), with 2 mM Mg-ATP (○), with 5 mM phosphate (▲), with 2 mM Mg-ATP and 5 mM phosphate (●). Inactivation was not observed in all these cases in the absence of Ca^{2+} (in the presence of 1 mM EGTA). Control activity (100%) measured after preincubation with EGTA was equal to 1.17 μmol/min·mg protein for arousal animal, 0.87 μmol/min·mg protein for torpid one and 1.57 μmol/min·mg protein for completely active ground squirrel.

squirrels studied in January-March is not significantly different in hibernating state and immediately after arousal (Fig. 1A and B). A similar effect was revealed in May-June, when ground squirrels were in the active state.

3.2. *Ca^{2+}-induced inactivation of ATPase*

Not only uncouplers and respiratory poisons but also other agents deenergizing mitochondria induce the inactivation of ATPase. Ca^{2+} is one of these agents that probably plays a physiological role. Ca^{2+} alone induces the inactivation of ATPase at relatively high concentrations but this effect is observed at low levels of Ca^{2+} (10–20 nmol/mg protein) provided that ATP is present (Fig. 2). This probably is a result of ATP hydrolysis and phosphate accumulation in the medium. Actually addition of 0.5 mM phosphate enhances the Ca^{2+}-induced inactivation so it is practically complete at concentrations lower than 10 nmol Ca^{2+}/mg protein (not shown). It seems probable that phosphate does not only enhance Ca^{2+} accumulation but also dissipates ΔpH on mitochondrial membrane. The effect of Ca^{2+} is not observed at pH higher than 8.0. It is completely prevented by ions of Zn^{2+} (50 μM). Ca^{2+}-induced inactivation can be partially reversed by EGTA (0.2 mM), but only in the first 1–3 min after the addition of Ca^{2+}. Prolonged incubation of mitochondria with Ca^{2+} leads to irreversible deenergization and inactivation of ATPase. These data provide us with strong evidence that Ca^{2+}-induced inactivation of ATPase as well as uncoupler-induced one is a result of action of the protein inhibitor.

The parameters of Ca^{2+}-induced inactivation are not significantly different in hibernating and arousal states (Fig. 2A), but change drastically in active animals studied in May-June (Fig. 2B). The sensitivity to Ca^{2+} is significantly lower in these mitochondria even in the presence of phosphate. In the presence of Mg-ATP, the inactivation is observed at significantly higher concentrations of Ca^{2+} and only in the presence of phosphate (Fig. 2B). Possibly Mg-ATP switches on the additional $\Delta\tilde{\mu}H^+$ generator, H^+-ATPase, and partially prevents deenergization induced by transport of Ca^{2+}.

Thus the high potential efficiency of the protein inhibitor in liver mitochondria from ground squirrel is probably the peculiarity of this animal, but the sensitivity of this system to physiological stimuli may be different under different conditions and changes with the season.

4. CONCLUSION

The regulation of mitochondrial ATPase can be suggested to play a significant role in metabolic transitions in hibernators. The effect of the protein inhibitor can be induced by $\Delta\tilde{\mu}H^+$ decrease and acidification of tissues during hibernation [8], that probably is a result of low activity of the respiratory chain in this state [9,10]. Inhibition of ATPase by the protein inhibitor is probably crucial in the transition period from hibernating to active state also. Partial uncoupling of liver mitochondria of hibernators during this period was originally observed by Neifakh and Daudova [11]. As was recently shown by Brustovezky et al. [12], hibernation and especially arousal are accompanied by the free fatty acid-induced uncoupling in liver mitochondria of ground squirrel. Under these conditions the effect of the protein inhibitor may be essential for prevention of the exhaustion of cellular ATP caused by its hydrolysis in deenergized mitochondria.

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