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Energization of the membrane prevents the formation of tight inactive complexes of ATPase with MgADP in submitochondrial particles

B.V. Chernyak and E.Yu. Khodjaev

A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR

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Mitochondrial ATPase binds ADP tightly, forming inactive complexes. Dissociation of the complexes is blocked by azide. Azide inhibits neither ATP hydrolysis at high $\Delta\tilde{\mu}H^+$ nor ATP-dependent NAD^+ reduction, provided that the ADP concentration is higher than 10^{-4} M. At lower ADP levels, azide is inhibitory. These data suggest that in the presence of ATP $\Delta\tilde{\mu}H^+$ prevents the fromation of one of the inactive complexes. In the absence of ATP at high $\Delta\tilde{\mu}H^+$, azide-sensitive complexes are not formed at any ADP concentrations tested (5×10^{-7}–5×10^{-4}M). The inactive E·ADP complexes can play a significant role in the regulation of ATPase in mitochondria, preventing futile ATP hydrolysis at low $\Delta\tilde{\mu}H^+$.

Mitochondrial enzyme; ATPase, H^+-; Enzyme-MgADP complex

1. INTRODUCTION

Mitochondrial H^+-ATPase catalyzes both synthesis and hydrolysis of ATP. At physiological [ATP]/([ADP] + [P_i]) ratios, the direction of the reaction is determined by the value of the transmembrane electrochemical potential difference of H^+ ($\Delta\tilde{\mu}H^+$). Regulation of the enzyme's activity is directed mainly at preventing futile ATP hydrolysis at decreased $\Delta\tilde{\mu}H^+$ [1]. One of the regulatory mechanisms is an interaction of ATPase with the natural protein inhibitor (review [2]).

Vinogradov and co-workers have previously shown (review [3]) that at low ADP concentrations an inactive E · MgADP complex is formed. Its rate of dissociation is low ($k_{-1} = 0.2\ min^{-1}$) and increases 10-fold in the presence of ATP. At ADP concentrations above 10^{-4} M formation of another inactive complex (of possible composition E · $(MgADP)_2$) proceeds [4].

The present data suggest that energization prevents formation of these complexes. A possible role of the complexes in the regulation of ATPase is discussed. Some preliminary results of the current work have been published [5].

Correspondence address: B.V. Chernyak, A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR

Abbreviations: Mg,Mn-SMP and S-SMP, different types of submitochondrial particles; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone

2. MATERIALS AND METHODS

Mg,Mn-SMP were isolated from beef heart mitochondria according to [6]. Isolation media contained 1 mM ATP and 1 mM succinate. S-SMP were prepared from Mg,Mn-SMP as in [7]. The rate of ATP hydrolysis was determined in a coupled ATP-regenerating system [8] or pH-metrically in a medium containing 250 mM sucrose, 50 mM KCl, 2 mM Tris-HCl, 5 mM succinate, 2 mM ATP, 2 mM $MgSO_4$ (pH 8.3). ATP-dependent NAD^+ reduction was assayed in the following medium: 250 mM sucrose, 50 mM Tris-HCl, 10 mM KCl, 5 mM succinate, 1 mM NAD^+, 2 mM $MgSO_4$, 2 mM ATP, 1 mM KCl (pH 7.5). In experiments with pyruvate kinase the medium additionally contained 1 mM phospho*enol*pyruvate (PEP). Stationary concentrations of ADP were determined in control assays in the same medium with 0.3 mM NADH and 5 U/ml of lactate dehydrogenase by addition of pyruvate kinase in great excess (20 U/ml).

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SMP were incubated for 15 min in the presence of 10 mM malonate.

3. RESULTS

3.1. *Azide fails to inhibit ATP hydrolysis at high $\Delta\tilde{\mu}H^+$*

Direct measurements of ATP hydrolysis at high $\Delta\tilde{\mu}H^+$ on SMP membranes are difficult to perform, since SMP preparations contain fractions of differing $\Delta\tilde{\mu}H^+$ values. Dissipation of $\Delta\tilde{\mu}H^+$ by the uncoupler CCCP results in a 4–5-fold rise in the rate of hydrolysis in Mg,Mn-SMP which possess low initial ATPase activity (fig.1, curve 1). Stimulation by CCCP is significantly lower (approx. 30%) in S-SMP depleted of the protein inhibitor (fig.1, curve 3). S-SMP hydrolyse ATP at rates 15–20-fold higher vs Mg,Mn-SMP. On the other hand, both types of SMP catalyze ATP-dependent NAD^+ reduction and oxidative phosphorylation at almost equal rates and contain similar quantities of 'coupled' particles (20–25%, assuming that only two populations of SMP occur: 'coupled' and 'uncoupled') [9].

Thus many-fold stimulation of ATPase by uncouplers in Mg,Mn-SMP (despite the low content of coupled fraction) is probably caused by inactivation of the enzyme by the protein inhibitor in uncoupled particles. In fact, $\Delta\tilde{\mu}H^+$ generation results in dissociation of the inhibitor [2], therefore coupled but not uncoupled SMP could lose it during the course of isolation in the presence of substrates of respiration.

Our data allowed comparison of the properties of ATPase at high and low values of $\Delta\tilde{\mu}H^+$. In the presence of CCCP azide blocks ATPase in Mg,Mn-SMP to an extent of up to 95% for 1–2 min (fig.1, curve 1). In the absence of the uncoupler, inhibition does not exceed 40–50%. Addition of the uncoupler to SMP preincubated with azide (fig.1, curve 2) leads initially to a sharp rise in ATPase activity which subsequently changes to a slow decrease, reaching an identical final level to that of curve 1. Thus, azide blocks uncoupled ATPase but not the coupled enzyme (at high $\Delta\tilde{\mu}H^+$). The inhibitory effect in the absence of CCCP (approx. 50%) is possibly determined by the contribution of uncoupled particles. Consequently, the actual stimulation of coupled ATPase by CCCP can be calculated to be nearly 15-fold. A similar value for stimulation (approx. 14-fold) is observed immediately after addition of the uncoupler to SMP preincubated with azide. This provides independent confirmation that azide does not block coupled ATPase.

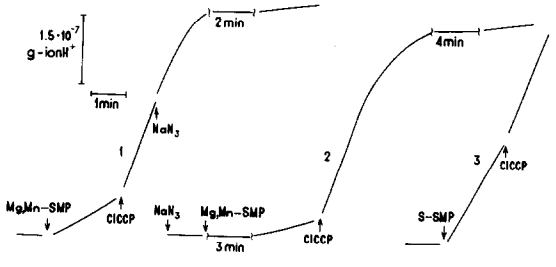

Fig.1. Effect of uncoupler (CCCP) on inhibition of ATPase by sodium azide. Final concentrations: Mg,Mn-SMP, 0.08 mg protein/ml (curves 1,2); S-SMP, 0.015 mg protein/ml (curve 3); CCCP, 10^{-6} M; sodium azide, 0.2 mM.

It was shown earlier that ATPase in SMP and isolated F_1-ATPase form inactive complexes with MgADP (review [3]). Prevention of the dissociation of these complexes determines the inhibitory effect of azide. Our data show that the complexes are not formed at high $\Delta\tilde{\mu}H^+$.

3.2. *Effect of azide on ATP-dependent reduction of NAD^+*

Measurement of ATP-dependent NAD^+ reduction in the presence of succinate and cyanide is a commonly used probe for ATP hydrolysis at high $\Delta\tilde{\mu}H^+$. This process is not blocked by azide (fig.2A, curves 1,2). Decrease in $\Delta\tilde{\mu}H^+$ as a result of partial ATPase inhibition by oligomycin or addition of CCCP at low concentrations promotes the inhibition of NAD^+ reduction by azide (fig.2A, curves 3,4). The value of the $\Delta\tilde{\mu}H^+$ preventing the effect of azide on ATPase is probably near threshold for ATP-dependent NAD^+ reduction.

The data in fig.2B show that azide fails to inhibit NAD^+ reduction only at relatively high ADP concentrations in the medium (> 100 μM). Addition of pyruvate kinase and PEP to the reaction medium leads to blocking of ATP hydrolysis by azide. The rate of inactivation increases with rising ADP stationary concentration.

3.3. *$\Delta\tilde{\mu}H^+$ prevents formation of tight inactive E·MgADP complexes in the absence of ATP*

Mg,Mn-SMP were preincubated with various

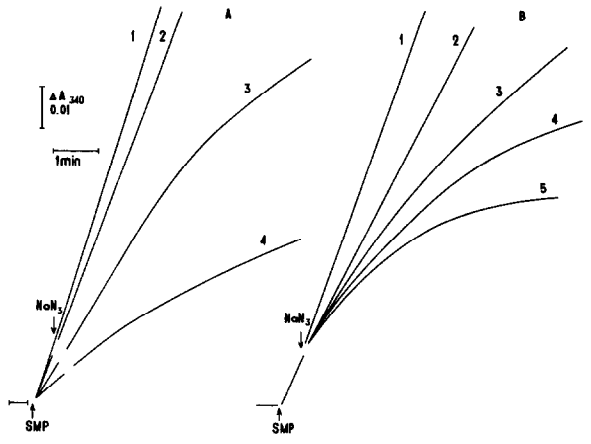

Fig.2. Effect of sodium azide on ATP-dependent NAD^+ reduction in Mg,Mn-SMP. Final concentrations: SMP, 0.03 mg protein/ml; sodium azide, 0.2 mM. (A) Curves: 1, without azide; 2–4, azide added 1 min after initiation of the reaction; 3, SMP preincubated for 15 min with oligomycin (0.07 μg/mg protein); 4, in the presence of 2×10^{-7} M CCCP. (B) 2 mM PEP added to the medium in the absence (curve 1) or presence of pyruvate kinase at (curves 2–5, respectively): 0.25, 0.75, 1.25, 12.5 U/ml. Stationary concentration of ADP: 109 (2), 41 (3), 20 (4), 3 μM (5).

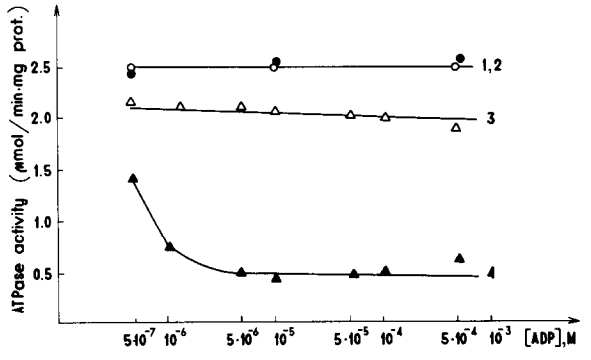

Fig.3. Formation of azide-sensitive complexes of ATPase with MgADP. Mg,Mn-SMP (0.1 mg protein/ml) were preincubated for 5 min in medium contained 250 mM sucrose, 10 mM KCl, 1 mM $MgSO_4$, 50 μM P^1,P^5-di(adenosine-5')pentaphosphate, 5 mM succinate, 10 mM Tris-HCl (pH 8.0) and ADP as indicated. Additions to the medium: 5 mM EDTA (curves 1,2), 2×10^{-6} M CCCP (curve 4). The initial rate of ATP hydrolysis was measured during the first 30 s in coupled ATP-regenerating system in the absence (curve 1) or presence (curves 2–4) of 0.2 mM azide.

concentrations of ADP in the presence or absence of CCCP (fig.3). ATPase activity was assayed in an ATP-regenerating system containing CCCP and azide which prevented reactivation of the complex. The initial rate of ATP hydrolysis correlated with the quantity of inactive E·MgADP complexes formed during preincubation. SMP preincubated with EDTA were used to determine the activity of the MgADP-free enzyme. The initial rate of hydrolysis in SMP thus treated did not depend on the presence of azide in the reaction medium. Energization completely prevents the formation of inactive azide-sensitive complexes over the range of ADP concentrations 5×10^{-7}–5×10^{-4} M (fig.3).

4. DISCUSSION

The above data show that energization of SMP membranes prevent formation of tight inactive azide-sensitive E·MgADP complexes. Data on the existence of different inactive complexes at varying ADP concentrations [4] have been confirmed here. In the presence of ATP, energization prevents formation of these complexes only at high ADP concentrations (fig.2). In the absence of ATP none of the complexes are formed (fig.3). One may suggest that formation of such complexes constitutes part of the regulatory mechanism preventing ATP hydrolysis in mitochondria at decreased $\Delta\tilde{\mu}H^+$. It is probably analogous to ADP-dependent regulation of ATPase in chloroplasts [10] and some bacteria [11].

Our experiments on intact rat liver mitochondria [12] showed that nearly 90% of the ATPase molecules are in the tight inactive complex with MgADP in the presence of uncouplers. We suppose that residual ATPase activity is an artifact resulting from damage to the regulatory system during the course of isolation of organelles. Formation of the inactive complex of ATPase with MgADP can also play a significant role in the regulation of oxidative phosphorylation, determining the ratio of active and inactive forms of ATPase at different $\Delta\tilde{\mu}H^+$ values.

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