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Nucleotide/H^+-dependent change in Mg^{2+} affinity at the ATPase inhibitory site of the mitochondrial F_1-F_0 ATP synthase

V.V. Bulygin, A.V. Syroeshkin and A.D. Vinogradov

Department of Biochemistry, School of Biology, Moscow State University, 119899 Moscow, Russian Federation

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The interactions between ADP and Mg^{2+} that result in the slowly reversible inhibition of the mitochondrial F_1-F_0 ATPase were studied. The K_i for the inhibitory Mg^{2+} is shown to be strongly dependent on the occupation of the nucleotide-binding sites. The inhibitory binding site for Mg^{2+} is not seen unless a stoichiometric amount of ADP is added [Biochem. J. 276 (1991) 149–156]; it appears ($K_i = 2 \cdot 10^{-6}$ M) in the presence of stoichiometric ADP and the affinity for inhibitory Mg^{2+} decreases to a K_i value of $7 \cdot 10^{-5}$ M when the second nucleotide binding site with $K_d = 5 \cdot 10^{-6}$ M is loaded with ADP. The binding of the inhibitory Mg^{2+} is competitively inhibited by H^+ ions within the pH interval 6.8–8.2. The nucleotide-dependent affinity transition of the Mg^{2+}-specific site suggests that H^+/Mg^{2+} exchange may play an important role in the catalytic mechanism of ATP synthesis/hydrolysis at the active site(s) of F_1-F_0 ATP synthase.

F_1-F_0 ATPase; Nucleotide-binding sites; Mg^{2+} binding

1. INTRODUCTION

Three out of six nucleotides which can be bound to one mol of the mitochondrial oligomeric proton translocating F_1-F_0 ATPase are rather rapidly exchangeable with the medium ATP or ADP [1–4], thus indicating that three sites are potentially capable of participation in the catalytic turnover during ATP hydrolysis/synthesis, or in a short-term control of the enzyme activity by the ATP/ADP ratio. The arguments for the equipotency of three β-subunit-associated nucleotide-binding site in the $\alpha_3\beta_3\gamma\delta\varepsilon$ structure of F_1 during the cooperative binding change mechanism has been extensively discussed [4], although the models with one [5] or two [6] catalytic sites participating in the catalysis have also been advanced.

The vast majority of experimental data on F_1 or F_1-F_0 ATPases has been concerned with the interplay of the nucleotide-binding sites and the effects of their loading on the catalytic and other properties of the enzyme [4]. Following the pioneering observation of Moyle and Mitchell on the Mg^{2+}-dependent slow active/inactive enzyme transition [7] it has been well established that low concentrations of ADP in the presence of Mg^{2+} result in a formation of inactive soluble [8] or membrane-bound [9–11] F_1 capable of ATP-dependent reactivation of the ATPase activity. The ADP(Mg^{2+})-inhibited form of the enzyme was shown to be an immediate target for the inhibitory effect of azide and the stimulatory effect of sulphite on ATP hydrolysis by the F_1-type ATPases [12]. Neither binding of ADP alone ($K_d \sim 10^{-8}$ M) nor Mg^{2+} alone produce the slowly reversible inhibition of the ATPase activity [13] whereas ADP-preloaded enzyme is rather rapidly inactivated in the presence of saturating ($K_i = 2 \cdot 10^{-6}$ M) Mg^{2+}. Thus the presence of a single Mg^{2+}-specific, ADP-dependent inhibitory site on F_1-F_0 ATPase has been recognized [13,14].

The intriguing property of ADP(Mg^{2+})-induced deactivation is that the ATPase inhibited by low concentrations of ADP (in the presence Mg^{2+}) is activated via an ATP-dependent mechanism at a slower rate than that inhibited by higher concentrations of ADP [15]. In this report we will show that the pH-dependent inhibitory site for Mg^{2+} changes its affinity from a zero to high-affinity state and then to a low-affinity state when the concentration of added ADP is varied from zero to the millimolar range.

2. MATERIALS AND METHODS

Bovine heart submitochondrial particles free from protein ATPase inhibitor were prepared and stored as described [16]. ATPase activity was measured at 25°C as a decrease of NADH in the presence of lactate dehydrogenase and ATP-regenerating system (phospho*enol*-pyruvate and pyruvate kinase) in the standard mixture containing: 0.25 M sucrose, 10 mM HEPES, 3 mM $MgCl_2$, 50 μM EDTA, 2 mM phospho*enol*pyruvate, 200 μM NADH, 1 mM ATP, 3 μM rotenone, 10 μM ClCCP, pyruvate kinase (5.5 units/ml), lactate dehydrogenase (5 units/ml) and 200 μM sodium azide (pH 7.4). The reaction was started by the addition of the particles (about 20 μg on the basis of protein content determined by the biuret method). All the activities

Correspondence address: A.D. Vinogradov, Department of Biochemistry, School of Biology, Moscow State University, 119899 Moscow, Russian Federation.

Abbreviations: ClCCP, carbonylcyanide *m*-chlorophenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid.

indicated are expressed in the relative units; one unit (control, 4.2 μmol of ATP hydrolyzed per min per mg of protein) corresponds to the initial rate of ATP hydrolysis by the particles preincubated for 1 h at 20°C in the mixture containing 0.25 M sucrose, 10 mM HEPES and 3 mM EDTA (potassium salts, pH 7.4).

ATP, NADH, phospho*enol*pyruvate, pyruvate kinase and lactate dehydrogenase were from Reanal (Hungary), ADP was from Serva (Germany), rotenone was from Ferak (Germany) and ClCCP was from Sigma (USA). Other chemicals were of the purest grade commercially available.

3. RESULTS

The ADP (Mg^{2+})-deactivated ATPase is rapidly trapped in its deactivated form if azide is present in the assay system [12,13]. The residual ATPase activity measured as the initial rate of ATP hydrolysis in the presence of azide is, therefore, proportional to the fraction of the enzyme remaining in the catalytically competent state after preincubation with ADP and Mg^{2+}. Fig. 1 shows such residual activity as a function of added ADP in the presence of different (relatively low) concentrations of Mg^{2+}. When substoichiometric amounts of ADP were added, the residual activity gradually decreased, as expected [9], to the level only slightly dependent on Mg^{2+} within the concentration range used (note that the equilibrium K_i for Mg^{2+} for the ADP-loaded enzyme is $2 \cdot 10^{-6}$ M [13]). An increase of ADP in the preincubation mixture unexpectedly resulted in the increase of the active enzyme fraction which was dependent on the Mg^{2+} concentration. The most conceivable model which accounts for the behavior depicted in Fig. 1 is that the binding of free ADP at the site with a K_d of about 10^{-8} M makes the enzyme susceptible to inhibitory Mg^{2+} with K_i of about 10^{-6} M and an occupation of the second ADP-specific site (K_d of about $5 \cdot 10^{-6}$ M) results in a decrease of the specific site affinity to Mg^{2+}. For such a model it might be expected that the inhibitory effect of Mg^{2+} would compete with ADP varied in the micromolar range. Fig. 2 demonstrates that this is indeed the case and an apparent K_i for Mg^{2+} increased from 10^{-6} M at 1 μM ADP [13] to 10^{-5} at 5 μM ADP and saturates at a value of $7 \cdot 10^{-5}$ M at 150 and 200 μM ADP. The K_i for Mg^{2+} was pH-dependent suggesting that some deprotonated group(s) is involved in coordination of Mg^{2+} at the nucleotide dependent inhibitory site (Fig. 3).

4. DISCUSSION

The results presented in this report unambiguosely show that strong negative/positive cooperativity exists between binding of two molecules of free ADP and one inhibiting Mg^{2+} at their specific sites. The data provide a simple explanation for the ADP-dependency of the ATP-promoted reactivation of the ADP(Mg^{2+})-deactivated enzyme [15]. It appears that the reactivation of the inhibited enzyme in the assay system [10,11] is, at least partially, due to the transition of the Mg^{2+} inhibitory site from its tight state (low ADP is present) or from its looser state (high ADP is present) to the complete absence of the inhibition when the ATP–Mg complex is

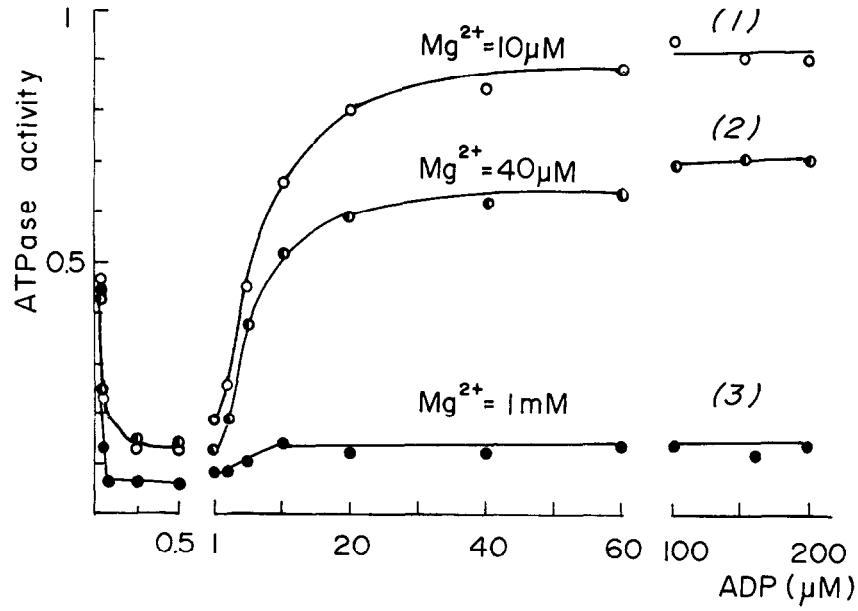

Fig. 1. Dependence of the inhibitory effect of Mg^{2+} on the ADP concentration in the preincubation medium. Submitochondrial particles (0.5 mg/ml) were incubated at 20°C for 1 h in a mixture containing: 0.25 M sucrose, 10 mM HEPES-KOH (pH 7.4), 10 μM (curve 1), 40 μM (curve 2), 1 mM (curve 3) $MgCl_2$ and ADP (concentrations are indicated). The initial rate of ATP hydrolysis was measured in the presence of azide as described in section 2.

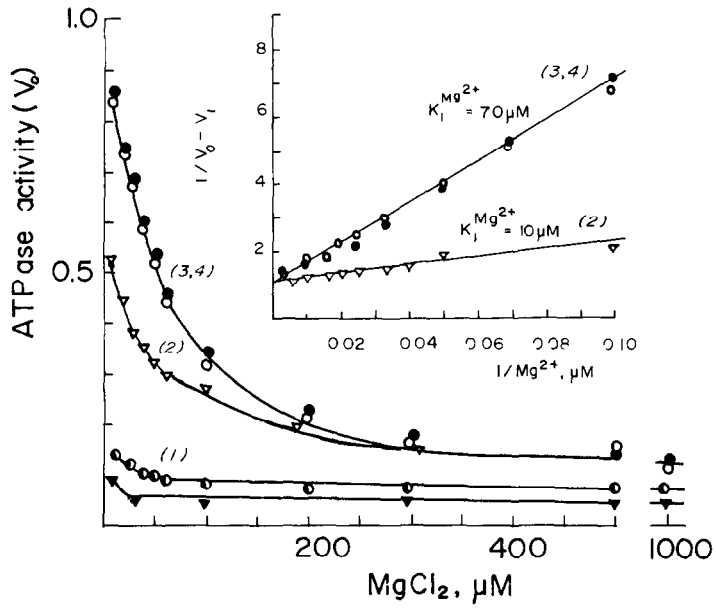

Fig. 2. Effect of ADP on the equilibrium between active (azide-insensitive, V_0) and Mg^{2+}-deactivated (azide-stabilized, V_i) ATPase as a function of the Mg^{2+} concentration. Submitochondrial particles (0.5 mg/ml) were preincubated and assayed as described in Fig. 1. The concentrations of ADP in the preincubation medium were: 0.5 μM (curve 1), 5 μM (curve 2), 150 μM (curve 3) and 200 μM (curve 4). 200 μM sodium azide and 200 μM ADP were present in the preincubation medium (lowest curve, ▼). Insert, the double reciprocal anamorphoses of curves 2–4.

bound at the catalytic site. Obviously, numerous enzyme–substrate (product) complexes may exist in a system composed of oligomeric F_1 and the specific ligands (free ADP, ATP and their Mg^{2+} complexes, P_i, free Mg^{2+}). The central question yet to be answered is what are the kinetically significant intermediates during the steady-state H^+-translocating ATP hydrolysis or $\Delta\bar{\mu}_{H^+}$-dependent ATP synthesis. Many years ago

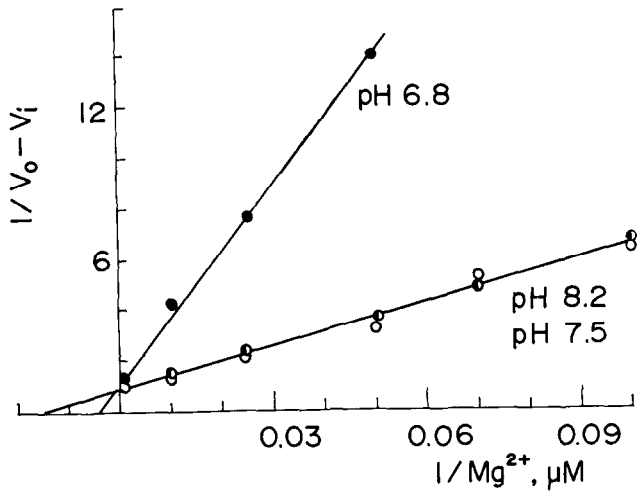

Fig. 3. pH dependence of the inhibitory effect of Mg^{2+} on ATPase activity. The preincubation conditions (except for pH) were as described in Fig. 1. 200 μM ADP was added. The control values V_0 (all measured at pH 7.4) were the same for the samples preincubated with EDTA (see section 2) at different pH.

it was shown that the ADP(Mg^{2+})-inhibited form of F_1-F_0 ATPase of the coupled submitochondrial-particles is capable of $\Delta\bar{\mu}_{H^+}$-dependent ATP synthesis [17]. This form is likely to be present under the conditions of oxidative phosphorylation (high ADP, low ATP, high free Mg^{2+} [18]). There are strong indications that free ADP and inhibitory Mg^{2+} bind to the enzyme separately to form deactivated ATPase [13] whereas the Mg–ADP complex is likely to be the substrate for ATP synthesis and free ADP has little inhibitory effect on chloroplast ATP synthase [19]. Whether the ADP(Mg^{2+})-inhibited form of F_1-type ATPases serves as the catalytically competent form of the ATP synthase reaction [17] or this form may arise just as a laboratory artifact [20] remains to be established.

An important point relevant to the presented findings is that binding of ADP (this paper), ATP and P_i [13,21], H^+ (this paper) and ATP–Mg, i.e. all the possible substrates (products) of oxidative phosphorylation, strongly affect the Mg^{2+}-specific inhibitory site. Because of thermodynamic reasons it is expected that the binding and release of Mg^{2+} should in turn influence the binding properties of the enzyme to those specific ligands. It is also worth noting that the Mg^{2+}-induced deactivation is the only strongly pH-dependent property of the mitochondrial H^+-ATPase which is known so far. Thus, it seems likely that Mg^{2+}/H^+ exchange at the ATPase inhibitory site, directly or indirectly linked with the proton motive force across the coupling membrane, is an important step in the molecular events during ATP synthesis.

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