

Three adenine nucleotide binding sites in F_1 - F_0 mitochondrial ATPase as revealed by presteady-state and steady-state kinetics of ATP hydrolysis

Evidence for two inhibitory ADP-specific noncatalytic sites

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Preincubation of submitochondrial particles with ADP in the presence of Mg^{2+} results in the complete inhibition of ATPase which is slowly reactivated in the assay mixture containing ATP and the ATP regenerating system. Significantly, the rate of activation increases as the concentration of ADP in the preincubation mixture rises from 1 μM to 20 μM and reaches a constant value at higher ADP concentrations. The first-order rate constant for the activation process in the assay mixture is ATP-dependent at any level of inhibitory ADP. The data obtained strongly suggest that two ADP-specific inhibitory sites and one ATP-specific hydrolytic site are present in F_1 - F_0 ATPase. Taking into account the $(3\alpha \cdot 3\beta) \cdot \gamma \cdot \delta \cdot \epsilon$ structure of F_1 , it is concluded that the synchronous discharge of ADP from two inhibitory sites during the activation occurs after ATP binds to the ATPase catalytic site.

F_1 -ATPase; Steady-state ATP hydrolysis; Nucleotide-binding site

1. INTRODUCTION

The oligomeric mitochondrial F_1 - F_0 ATP synthase complex couples ATP synthesis or hydrolysis with proton translocation across the membrane during reversible oxidative phosphorylation. Data on the structure of F_1 [1] and numerous studies on nucleotide binding ([2-6] and references cited therein) strongly suggest the presence of six adenine nucleotide-binding sites on the solubilized F_1 component from mitochondria and *E. coli*. The function of three non-exchangeable sites [7] is unclear, although some structural role of these sites

has been proposed [8]. Three other sites readily exchange bound nucleotides for medium ones [7,9,10], thus being able to participate in the catalytic turnover and/or regulation of the enzyme activity. At least ten enzyme-nucleotide complexes with different catalytic and regulatory properties may exist, if an enzyme, which has three identical nucleotide-binding sites, is gradually equilibrated with increasing nucleotide concentrations at different ATP/ADP ratios. It may thus be expected that the extremely complex kinetic behaviour of ATP hydrolysis or synthesis would be observed for the soluble F_1 or membrane bound F_1 - F_0 . Indeed, several laboratories have reported a complex steady-state activity, i.e. substrate concentration dependence [11-14]. However, simple Michaelis-Menten kinetics for ATP hydrolysis [15,16], or synthesis (our unpublished observation) has been demonstrated under the conditions where the inhibition by the reaction product and the hysteretic enzyme behaviour were excluded [17-19].

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Abbreviation: CICCIP, carbonylcyanide *m*-chlorophenylhydrazine

We have reported, several years ago, on the presence of a Mg^{2+} -dependent high affinity inhibitory site for ADP on the membrane-bound [16,20] and soluble F_1 [21], which is clearly different from the site to which ATP is bound during its steady-state hydrolysis. Starting from the original observation on the slow ATP/ADP-dependent activation/deactivation kinetics of F_1 ATPase, a concept of pseudoreversibility of ATP hydrolysis/synthesis has gradually been developed [22–26]. Here we describe the kinetic properties of the membrane-bound F_1 - F_0 ATPase which demonstrate the presence of two ADP-specific inhibitory sites different from the site involved in the steady-state ATP hydrolysis. Interestingly, the kinetics of ATP-dependent activation of the ADP-inhibited enzyme suggest a synchronous discharge of two ADP molecules from the inhibitory sites after ATP has been bound at the ATPase catalytic site.

2. MATERIALS AND METHODS

Bovine heart submitochondrial particles free of the protein ATPase inhibitor (AS particles) were prepared and stored as described [16]. The ATPase activity was measured spectrophotometrically as a decrease of $NAD \cdot H$ in a system containing ATPase, pyruvate kinase and lactate dehydrogenase [16,17]. To achieve a better time resolution, the stopped-flow apparatus attached to Hitachi-557 spectrophotometer was used. Equal volumes of AS particle suspension, equilibrated with the appropriate ligands, and the reaction mixture, containing all the components of the assay system, were mixed (mixing time < 6 ms) and the decrease of absorption $\Delta(A_{340} - A_{366})$ was measured as a function of time (for details see the legends to the figures).

ATP, $NAD \cdot H$, phosphoenolpyruvate (potassium salts), pyruvate kinase, lactate dehydrogenase were from Reanal (Budapest, Hungary). ADP was from Sigma (USA). The enzymes were extensively dialyzed before use. Other chemicals were of the purest grade commercially available.

3. RESULTS

In accord with our previous results [16,20], we

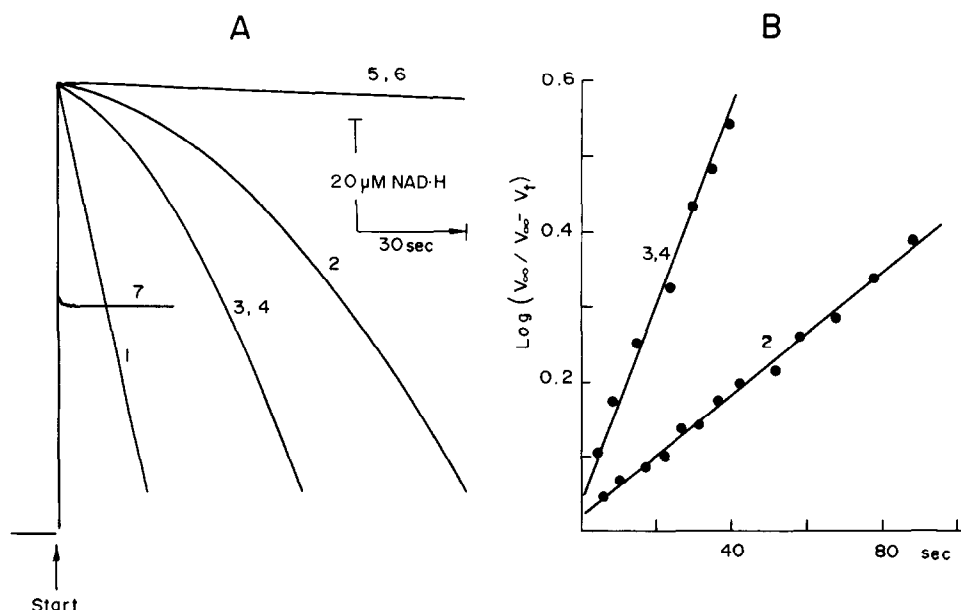


Fig.1. Kinetics of ATP-dependent activation of ADP-inhibited ATPase. (A) Time course of ATP hydrolysis. AS particles (0.25 mg/ml) were preincubated for 5 min at 25°C in one syringe of stopped-flow apparatus in a mixture containing 0.25 M sucrose, 10 mM Hepes, pH 7.4, 0.1 mM EDTA, 2 mM $MgCl_2$, 3 μM CICCIP and various concentrations of ADP. Curve 1, ADP and $MgCl_2$ were omitted; curve 2, 0.5 μM ADP was added; curve 3, 20 μM ADP was added; curve 4, 200 μM ADP was added; curves 5 and 6, the same as 2 and 4, 100 μM sodium azide was added to another syringe; curve 7, AS particles were omitted and 40 μM ADP was added to assure that the ATP-regenerating system was never limiting. Another syringe contained a mixture comprising 0.25 M sucrose, 0.2 M KCl, 10 mM Hepes, pH 7.4, 0.1 mM EDTA, 6 μM CICCIP, 4 mM phosphoenolpyruvate, 0.6 mM $NAD \cdot H$, 6 μM rotenone, pyruvate kinase (60 units/ml), lactate dehydrogenase (60–70 units/ml) and ATP to give a final concentration of 0.1 mM in the reaction chamber. The reaction was started by rapid mixing (< 6 ms) of equal volumes of the contents of two syringes, as indicated by the arrow. (B) Linear anamorphoses of curves 2, 3 and 4 of panel A. V_{∞} , the constant rate of ATP hydrolysis by the particles preincubated without ADP and $MgCl_2$ (curve 1, panel A).

observed that when AS particles were preincubated with low concentrations of ADP, their initial ATPase activity measured in the presence of ATP-regenerating system was inhibited, and slow activation of the enzyme occurred during the assay (fig.1A, curves 1 and 2). In our original publication on these phenomena, we have claimed that the inhibition of ATPase by ADP was prevented, when the concentration of the latter in the preincubation mixture was increased up to 1 mM [20]. This unusual property might be due to either true reactivation in the presence of high inhibitor concentrations, or to the increase of the enzyme-inhibitor dissociation rate. To get a more close insight into

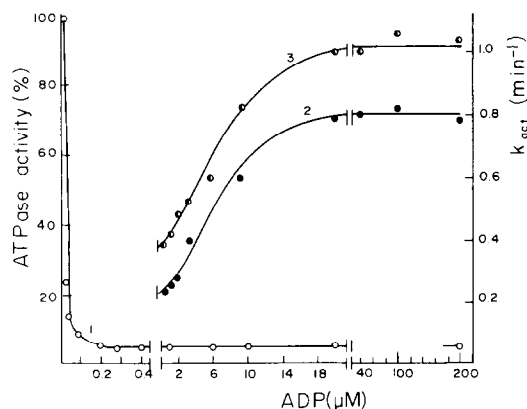


Fig.2. Dependence of the initial rate of ATPase (curve 1, left ordinate) and first-order rate constant for activation (curves 2 and 3, right ordinate) on the ADP concentration in the preincubation mixture. Curve 1: AS particles (1 mg/ml) were preincubated for 5 min at 25°C in a mixture containing 0.25 mM sucrose, 10 mM Hepes, pH 7.4, 0.1 mM EDTA, 2 mM MgCl₂ and ADP as indicated. The initial rate of ATP hydrolysis was measured at 340 nm in a mixture containing the same component and 0.1 mM ATP, 10 μM CICCIP, 4 μM rotenone, 0.16 mM NAD·H, 1 mM phosphoenolpyruvate, 0.1 mM sodium azide [18], pyruvate kinase and lactate dehydrogenase (5 units/ml of each). The reaction was started by the addition of 20 μl of the suspension of preincubated particles. 100% activity corresponds to 2.6 μmol of ATP hydrolyzed per min per mg of protein. Curves 2 and 3: the experiments were performed exactly as described in fig.1A; AS particles were preincubated with different concentrations of ADP (indicated on the abscissa). The reactions were measured in the presence of 0.1 (curve 2) and 1.0 (curve 3) mM ATP. The first-order rate constants for activation (right ordinate) were determined as shown in fig.1B. Slight deviations from the first-order pattern were observed as expected for longer times of reaction, when 'intermediate' concentrations of ADP (3–7 μM) were used to inhibit ATPase. The initial slopes of the semi-logarithmic functions were plotted in those cases.

the problem, we followed the kinetics of the ATPase reactivation with a better time resolution. It was found that the initial rates of ATP hydrolysis by the particles preincubated with low ($\leq 1 \mu\text{M}$) and high ($> 20 \mu\text{M}$) concentrations of ADP appeared to be inhibited by more than 90%; however, the rate of the enzyme activation was higher in the latter case (fig.1, curves 2, 3 and 4). The reactivation process was prevented by azide [18,23] independently of the ADP concentration used (fig.1A, curves 5 and 6) and followed the first-order kinetics (fig.1B).

When the first-order rate constant for the activation process was plotted as a function of ADP concentration in the preincubation medium, the sigmoidal curves shown in fig.2 were obtained. The ratio between the first-order rate constants measured at 0.1 and 1.0 mM ATP was about 1.5 independently of the ADP concentration used to inhibit the ATPase activity. This value is close to that expected (1.7), derived on the assumption that the same site should be occupied by ATP for ATP-dependent reactivation of the enzyme and for ATP hydrolysis ($K_m^{\text{ATP}} = 1 \times 10^{-4} \text{ M}$ [16,20]).

4. DISCUSSION

The presence of three exchangeable adenine nucleotide-binding sites in F₁ is well documented [7,9,10]. The results presented in this report allow the description of the kinetical significance of at least three nucleotide-binding sites in membrane-bound F₁ (fig.2). The binding of ADP in one site ($K_d \sim 10^{-8} \text{ M}$) which is different from the catalytic site for ATP hydrolysis [16,20,21] is evident from curve 1. When concentration of ADP increases, another inactive complex apparently containing two ADP molecules with a half-maximal saturation at $\sim 7 \mu\text{M}$ ADP is formed (fig.2, curve 2). Both inactive complexes can still bind ATP at the ATP hydrolysis catalytic site, as evident from the ATP dependence of the reactivation process.

Paradoxically, the ATPase inhibited by low concentrations of ADP is activated via an ATP-dependent mechanism at a slower rate than that inhibited by higher concentrations of ADP. Assuming the symmetry between at least two α - β pairs of subunits in the oligomeric F₁, the only conceivable explanation for such a behaviour is that the synchronous discharge of two ADP bound at the in-

hibitory sites occurs faster than the dissociation of one ADP bound at either of these sites. It certainly cannot be excluded that some isomeric kinetically unstable complex(es) different from the 'slow' $F_1 \cdot ADP$ complex [17,25] is (are) an intermediate(s) of the reactivation process; however, such a mechanism would be kinetically identical to the postulated synchronous discharge within the time resolution of the experiments.

It should be emphasized that neither of the ADP-specific ATPase inhibitory sites can be identified as the site, where ADP is formed during the steady-state ATP hydrolysis. The highest k_{off} value for dissociation of the ADP-inhibited enzyme ($\sim 1 \text{ min}^{-1}$) is much lower than the turnover of ATPase ($\sim 10^4 \text{ min}^{-1}$). Thus, some asymmetry between the ATP hydrolytic site and two ADP-specific inhibitory sites should be considered. It has been shown that a single copy of the γ -subunit of F_1 is associated with one of the three α - β pairs [27,28]. This gives a clue for the structural basis of functional asymmetry. We propose that one α - β pair bound to a single copy of smaller subunits carries a catalytic site which operates as ATP hydrolase or ATP synthetase depending on the occupation of the other two identical α - β pairs with ATP or ADP, respectively. This proposal is somehow similar to one of the models discussed by Williams et al. [6] and can be substantiated by a number of experimental observations earlier reported by our group [22–26].

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