# Adenine Nucleotide Binding at a Noncatalytic Site of Mitochondrial $F_1$-ATPase Accelerates a $Mg^{2+}$- and ADP-Dependent Inactivation during ATP Hydrolysis†

Marat B. Murataliev

*Molecular Biology Institute and Department of Chemistry and Biochemistry, University of California at Los Angeles, Los Angeles, California 90024-1570*

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ABSTRACT: The evidence is presented that the ADP- and $Mg^{2+}$-dependent inactivation of $MF_1$-ATPase during MgATP hydrolysis requires binding of ATP at two binding sites: one is catalytic and the second is noncatalytic. Binding of the noncatalytic ATP increases the rate of the inactive complex formation in the course of ATP hydrolysis. The rate of the enzyme inactivation during ATP hydrolysis depends on the medium $Mg^{2+}$ concentration. High $Mg^{2+}$ inhibits the steady-state activity of $MF_1$-ATPase by increasing the rate of formation of inactive enzyme–ADP–$Mg^{2+}$ complex, thereby shifting the equilibrium between active and inactive enzyme forms. The $Mg^{2+}$ needed for $MF_1$-ATPase inactivation binds from the medium independent from the MgATP binding at either catalytic or noncatalytic sites. The inhibitory ADP molecule arises at the $MF_1$-ATPase catalytic site as a result of MgATP hydrolysis. Exposure of the native $MF_1$-ATPase with bound ADP at a catalytic site to 1 mM $Mg^{2+}$ prior to assay inactivates the enzymes with $k_{inact}$ 24 $min^{-1}$. The maximal inactivation rate during ATP hydrolysis at saturating MgATP and $Mg^{2+}$ does not exceed 10 $min^{-1}$. The results show that the rate-limiting step of the $MF_1$-ATPase inactivation during ATP hydrolysis with excess $Mg^{2+}$ precedes binding of $Mg^{2+}$ and likely is the rate of formation of enzyme with ADP bound at the catalytic site without bound $P_i$. This complex binds $Mg^{2+}$ resulting in inactive $MF_1$-ATPase. Acceleration of the $MF_1$-ATPase inactivation by noncatalytic MgATP binding could reflect the promotion of $P_i$ release from the catalytic site to give the E·ADP complex required for inactivation by $Mg^{2+}$. The interplay of $Mg^{2+}$ and ADP inhibitory effects can account for previous suggestions of more than one apparent $K_m$ above micromolar ATP concentrations.

$MF_1$-ATPase is well known to show nonlinear kinetics of ATP hydrolysis [for example, see Mitchell and Moyle (1971), Caterall and Pedersen (1972), Hackney (1979), and Fitin et al. (1979)]. When added from a $Mg^{2+}$-free solution to an assay mixture with MgATP present, the enzyme shows an initial rapid phase of hydrolysis followed by a decrease to a lower steady-state level (Vasilyeva et al., 1982a,b; Vulfson, et al., 1984). Exposure of $F_1$-ATPase containing tightly bound ADP to $Mg^{2+}$ prior to assay results in an inactive enzyme which slowly regains ATPase activity in the presence of MgATP (Hackney, 1979; Vasilyeva et al., 1982a; Vulfson et al., 1984; Feldman & Boyer, 1985). The activity reaches the same steady-state level as that without prior exposure to $Mg^{2+}$. The steady-state activity of $F_1$-ATPase under usual assay conditions is determined by the slow (compared to catalytic turnover) pseudoequilibrium between active and inactive enzyme forms (Vasilyeva et al., 1980, 1982a; Vulfson et al., 1984, 1986b; Guerrero et al., 1990b).

An understanding of the mechanism of ATP synthesis by $MF_1$-ATPase requires knowledge of the number of catalytic sites participating in catalysis and how they interact throughout the catalytic cycle. When the formation of the inactive complex is excluded, $MF_1$-ATPase shows simple Michaelis–Menten kinetics above micromolar ATP with a single $K_m$ of about $10^{-4}$ M (Mitchell & Moyle, 1972; Vasilyeva et al., 1980; Vulfson et al., 1986b). The complex ADP and $Mg^{2+}$-dependent inhibition of $MF_1$-ATPase complicates kinetic analyses and interpretations of the results. Both the rate and the extent of the $MF_1$-ATPase inactivation during ATP hydrolysis depend on the MgATP concentration (Fitin et al., 1979; Vasilyeva et al., 1980; Vulfson et al., 1986b; Milgrom & Murataliev, 1989). Since both inactivation and reactivation rates depend on MgATP concentration, the extent of $MF_1$-ATPase inactivation at steady state varies with changing of substrate concentration (Vulfson et al., 1986b). As a result, measurements of the steady-state rates as a function of MgATP concentration give nonhyperbolic dependence with more than one apparent $K_m$ above micromolar ATP (Ebel & Lardy, 1975; Gresser et al., 1982; Grubmeyer et al., 1982; Roveri & Calcaterra, 1985; Wong et al., 1984; Muneyuki & Hirata, 1988). As noted herein, those deviations from Michaelis–Menten dependence, originally attributed to the cooperative interactions of the multiple catalytic sites, can be accounted for by the inhibitory $Mg^{2+}$ and ADP effect.

Only 1 mol of ADP is required for the complete inactivation of the nucleotide-depleted $MF_1$-ATPase (Drobinskaya et al., 1985). Single-turnover hydrolysis of the stoichiometric amount of ATP by $MF_1$-ATPase (uni-site) results in an inactive enzyme as well. The presence of an ATP-regenerating system during uni-site ATP hydrolysis by the nucleotide-depleted $MF_1$-ATPase prevents the formation of an inactive complex (Milgrom & Murataliev, 1987). The interactions of the multiple nucleotide-binding sites are required for $Mg^{2+}$- and ADP-induced inactivation of the $MF_1$-ATPase during ATP hydrolysis. Several findings indicate that noncatalytic nucleotide-binding sites could be involved in the inactivation. Bulygin and Vinogradov (1988) have reported that binding of ADP at a noncatalytic site accelerated the ATP-dependent reactivation of $MF_1$-ATPase of submitochondrial particles inhibited by ADP and $Mg^{2+}$. Binding of $PP_i$ at noncatalytic sites of the $MF_1$-ATPase prevents the $Mg^{2+}$ and ADP-induced inactivation (Kalashnikova et al., 1988). Recently, the

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activation of the MgATPase activity of $CF_1$-ATPase by ATP binding at a noncatalytic site has been demonstrated (Xue & Boyer, 1989; Milgrom et al., 1990, 1991). This activation is due to the increased rate of the ATP-dependent reactivation of the inactive enzyme–ADP–$Mg^{2+}$ complex (Murataliev & Boyer, 1992).

Studies reported here were motivated by a need for a better understanding of the above and other factors governing the unusual inhibition and their impact on kinetic studies of $MF_1$-ATPase. The results obtained show that an ATP binding at a noncatalytic site of $MF_1$-ATPase accelerates the inactivation of the enzyme during ATP hydrolysis. Inhibitory ADP arises at a catalytic site from ATP while the $Mg^{2+}$ needed for inactivation binds from medium. The binding of $Mg^{2+}$, MgATP at a noncatalytic site, and MgATP as a substrate occur independent of each other. The rate-limiting step of the $MF_1$-ATPase inactivation during ATP hydrolysis precedes the step of inhibitory $Mg^{2+}$ binding. The $MF_1$-ATPase forms inhibited the enzyme–ADP–$Mg^{2+}$ complex which has very low or no initial activity, but MgATP reactivates enzyme within a few seconds.

## MATERIALS AND METHODS

*Enzyme Preparation.* $MF_1$-ATPase was isolated by the Knowles and Penefsky procedure (1972). The ammonium sulfate precipitate was dissolved in a buffer containing 250 mM sucrose, 50 mM Tris-HCl, pH 8.0, and 1.0 mM $MgCl_2$ and 10 mM $P_i$ and then desalted by passing through a Sephadex centrifuge column (Penefsky, 1977) equilibrated with the same buffer. For studies of the enzyme inhibition by the exposure to $Mg^{2+}$, the enzyme was prepared as described above except a buffer containing 250 mM sucrose, 50 mM Tris-HCl, pH 8.0, and 1.0 mM EDTA was used. The specific activity of the enzyme as measured in the presence of 3 mM MgATP and 1 mM free $Mg^{2+}$ at 25 °C was 100–110 μmol/(min·mg) corresponding to 600–650 $s^{-1}$.

*Activity Assay.* The ATPase assay was carried out at 25 °C in a medium, containing at pH 8.0 25 mM Tris-HCl, 40 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 0.1 mg/mL pyruvate kinase, 0.1 mg/mL lactate dehydrogenase, 300 μM NADH, indicated concentrations of MgATP, 5–15 nM $MF_1$-ATPase, and where indicated 500 μM sodium azide. A Beckman DU-7 spectrophotometer working in absorbance measurement mode (120 readings/min) was used for the assay. Recording of the sample began 2–3 s after addition of $MF_1$-ATPase and an error in determination of "zero time" did not exceed 2 s.

*Calculations of the Initial Velocities and Rate Constants of Inactivation.* When added into assay mixture from $Mg^{2+}$-free solution, $MF_1$-ATPase shows high initial velocity (Vasilyeva et al., 1982a,b; Vulfson et al., 1984). Within a minute, the reaction rate declined reaching a constant steady-state level. The addition of the azide results in an almost complete inactivation of the enzyme because of the prevention of $MF_1$-ATPase reactivation (Vasilyeva et al., 1982a,b). As shown by Vasilyeva et al. (1982a,b), azide has no effect on the initial rate of ATP hydrolysis by $MF_1$-ATPase and on the $K_m$ for ATP obtained from initial velocities. In the presence of azide, a time course of ATP hydrolysis by $MF_1$-ATPase follows a first-order course and can be used for calculations of the initial velocities and inactivation rate constants.

Initial velocities of ATP hydrolysis and rate constants of $MF_1$-ATPase inactivation were obtained from a semilogarithmic plot as described elsewhere (Vasilyeva et al., 1982b; Vulfson et al., 1984). Briefly, traces of the time course of ATP hydrolysis in the presence of azide were replotted in semilogarithmic coordinates (ln $(v_t - v_{oo})$) vs time, where $v_t$ and $v_{oo}$ are the reaction rates at the time moment $t$ and the residual steady-state activity in the presence of azide, respectively. An extrapolation of a straight line obtained to zero time gives the initial velocity and the slope gives a value of the rate constant of $MF_1$-ATPase inactivation. It should be noted that both initial ATPase and inactivation rates at given concentration of ATP were obtained from the single sample.

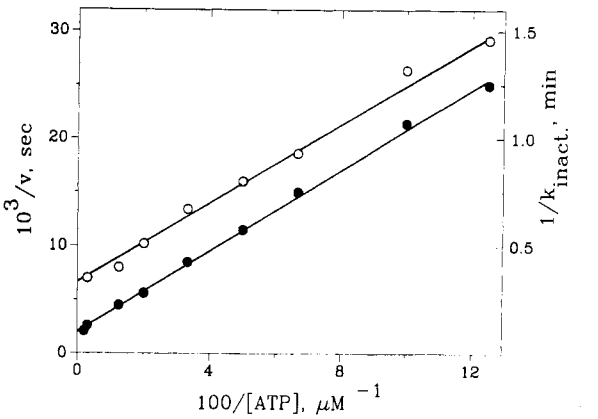



FIGURE 1: Dependencies of initial rate of ATP hydrolysis and $MF_1$-ATPase inactivation rate in the presence of azide on ATP concentration. The initial rates of MgATP hydrolysis (●) and inactivation rate constants (○) were measured as described in Materials and Methods. The figure gives the reciprocal of the rate constant of inactivation (right orginate) and of the initial rate of ATP hydrolysis (left ordinate) versus the reciprocal of the ATP concentration.

*Nucleotide Binding.* Binding of [$^3$H]nucleotide was measured in the medium containing at pH 8.0 50 mM Tris-HCl, 40 mM KCl, 1.0 mM $MgCl_2$, 10 mM phosphoenolpyruvate, 0.1 mg/mL pyruvate kinase, 1.2 mg/mL bovine serum albumin, indicated concentrations of [$^3$H]ATP, and 50–150 nM $MF_1$-ATPase. The relatively high enzyme concentrations needed for binding studies required the presence of a high concentration of phosphoenolpyruvate and a limited time of experiments. Aliquots (100 μL) of the reaction mixture were passed through Sephadex centrifuge columns (Penefsky, 1977) equilibrated with a buffer containing 50 mM Tris–HCl, pH 8.0, 1.0 mM $MgCl_2$, and 1.2 mg/mL bovine serum albumin.

*Protein Measurement.* Protein concentration was measured by the Lowry procedure (Lowry et al., 1951) using lyophilized $MF_1$-ATPase as the standard. The molecular weight of $MF_1$-ATPase was taken as 371 000 (Walker et al., 1985).

## RESULTS

*Participation of the Noncatalytic Nucleotide Binding Site(s).* Experiments were undertaken to find out if noncatalytic sites are involved in $MF_1$-ATPase inactivation by $Mg^{2+}$ and catalytic site ADP. The procedures described in the Material and Methods section were used for determinations of the inactivation rate constants and the initial velocities of the ATPase reaction. The dependencies on MgATP concentration of $MF_1$-ATPase inactivation in the presence of azide and of the initial velocity of ATP hydrolysis are shown in Figure 1. Both dependencies are hyperbolic. The results demonstrate the important point that the inactivation of the enzyme during MgATP hydrolysis requires a binding of MgATP with a concentration of 25 μM for the half-maximal inactivation rate. This value is 4–5 times lower than the $K_m$ for MgATP hydrolysis (125 μM, Figure 1). Similar results were obtained with the membrane-bound enzyme of submi-

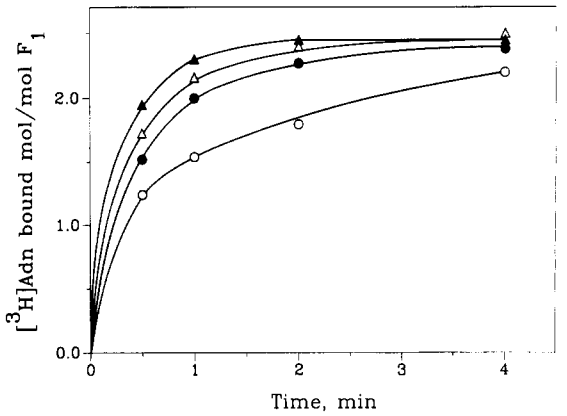



FIGURE 2: Time course of the binding of [$^3$H]nucleotides by $MF_1$-ATPase during hydrolysis of [$^3$H]ATP. The binding of [$^3$H]-nucleotide was measured in a medium containing at pH 8.0 50 mM Tris–HCl, 40 mM KCl, 1.0 mM $MgCl_2$, 10 mM phosphoenolpyruvate, 0.1 mg/mL pyruvate kinase, 1.2 mg/mL bovine serum albumin, 50–125 nM $MF_1$-ATPase, and 5 (○), 15 (●), 30 (△), or 50 (▲) $\mu$M [$^3$H]ATP; 100-$\mu$L aliquots of the reaction mixture were passed through Sephadex centrifuge columns equilbrated with a buffer containing 50 mM Tris–HCl, pH 8.0, 1.0 mM $MgCl_2$, and 1.2 mg/mL bovine serum albumin.

tochondrial particles. The $K_m$ for ATP hydrolysis of 140 $\mu$M was measured, and the rate of enzyme inactivation was half-saturated at 30 $\mu$M of ATP. The maximal rate of inactivation of the membrane-bound enzyme was 4–5 times slower compared to that of soluble $MF_1$-ATPase (data not shown). The results suggest that the ATP hydrolysis and the ATP-dependent inactivation depend on the MgATP binding to different sites.

To determine the nature of these sites, the binding of nucleotides during [$^3$H]ATP hydrolysis was measured (Figure 2). In a 4-min exposure the native $MF_1$-ATPase binds up to 2.0–2.5 mol of AdN as measured by the Sephadex centrifuge column procedure. Less than 1 mol of bound nucleotide can be chased by an excess of cold ATP (Figure 3B), indicating that the rest of the nucleotides are bound at a noncatalytic site(s). The results of Figures 2 and 3 show that under the conditions of ATP hydrolysis followed by the $MF_1$-ATPase inactivation enzyme binds one [$^3$H]ATP at a catalytic site and 1.0–1.5 mol of nucleotide at a nonexchangeable site(s). The first catalytic site of $F_1$-ATPase is known to have high affinity for ATP ($K_m \approx 10^{-9}$–$10^{-8}$ M; Milgrom & Murataliev, 1987; Cunningham & Cross, 1988). The binding of the catalytic ATP is complete in fractions of a second at the concentrations employed, $k_{on} \approx 5 \times 10^6$ $M^{-1}$ $s^{-1}$ (slope of the ATPase activity curve, Figure 1; see also Cross et al. (1982), Milgrom and Murataliev (1987), and Cunningham and Cross (1988)). Binding of the nonexchangeable nucleotide occurs considerably slower. The results of Figure 2 show that [$^3$H]-ATP is bound at a nonexchangeable site(s) within 30 s in the same range of ATP concentration ($[ATP]_{1/2}$ = 20–30 $\mu$M) as required for the inactivation ($K_{ATP}$ = 25 $\mu$M, Figure 1).

The centrifuge column procedure (Penefsky, 1977) for the ligand binding assay does not allow quantitative measurement of the kinetics of the relatively rapid ($t_{1/2}$ < 1 min) binding. Thus, another approach was used to reveal further the nature of the MgATP-binding sites accelerating enzyme inactivation.

Exposure of $MF_1$-ATPase to $PP_i$ is known to result in the binding of $PP_i$ at a noncatalytic site(s) (Kalashnikova et al., 1988). $PP_i$ binding prevented $Mg^{2+}$- and ADP-dependent inactivation of $MF_1$-ATPase without an effect on the MgATP binding at the catalytic sites and on the initial ATPase activity (Kalashnikova et al., 1988). Figure 3 shows the effect of $PP_i$

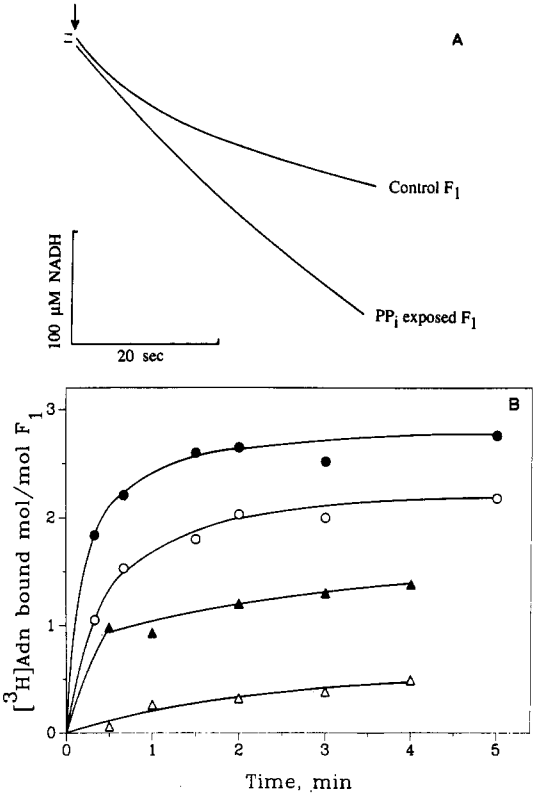



FIGURE 3: Effect of the prior exposure to $PP_i$ on the time course of MgATP hydrolysis (A) and nucleotide binding (B) by $MF_1$-ATPase. The $MF_1$-ATPase at 5 $\mu$M was incubated in the buffer containing 250 mM sucrose, 50 mM Tris–HCl, pH 8.0, 1 mM $Mg^{2+}$, and 10 mM $P_i$ (control) or in the same buffer containing 5 mM $PP_i$ for 15 min. (A) The kinetics of 50 $\mu$M MgATP hydrolysis in the absence of azide was measured at 1 mM free $Mg^{2+}$ concentration as described in the Materials and Methods section. The arrow indicates the moment of the $MF_1$-ATPase addition. (B) The binding of 20 $\mu$M [$^3$H]ATP by control $MF_1$-ATPase (○, ●) or by the enzyme exposed to $PP_i$ (△, ▲) was measured before (●, ▲) or after (○, △) cold ATP chase. For the cold ATP chase experiments, samples were incubated for an additional 20 s with 2 mM unlabeled ATP.

binding on ATPase activity (A) and nucleotide binding by $MF_1$-ATPase (B). $PP_i$ binding does not change the initial activity of the $MF_1$-ATPase but prevents the formation of the inactive enzyme–ADP–$Mg^{2+}$ complex (Figure 3A; see also Kalashnikova et al. (1988)). At the same time, the exposure of the $MF_1$-ATPase to $PP_i$ decreases the binding of [$^3$H]ATP during ATP hydrolysis (Figure 3B). $PP_i$-exposed $MF_1$-ATPase rapidly binds only one nucleotide. The bound label is almost completely chased by an excess of unlabeled ATP and, therefore, is bound at a catalytic site. Exposure of the $MF_1$-ATPase to $PP_i$ prevents binding of adenine nucleotides at noncatalytic sites. The noncatalytic nucleotide binding was initially near zero and increased slowly over the 4-min period (Figure 3B). Slow binding of noncatalytic nucleotide and consequent inactivation of $MF_1$-ATPase after exposure to $PP_i$ (Figure 3) are likely limited by the release of $PP_i$ from the noncatalytic site(s).

The results of Figures 1 and 2 show that maximal rate of $Mg^{2+}$- and ADP-dependent inactivation of the $F_1$-ATPase during ATP hydrolysis depend upon MgATP binding at a site(s) with $K_{ATP}$ = 25 $\mu$M (Figure 1). Both binding of MgATP at this site and exchange of bound nucleotide with the medium occur considerably slower than catalytic turnover (Figures 2 and 3B). Exposure of $MF_1$-ATPase to $PP_i$ prior to the assay prevents both the inactivation of the $MF_1$-ATPase (Figure 3A) and the binding of [$^3$H]AdN at noncatalytic sites (Figure 3B). The data of Figures 1–3 give evidence that

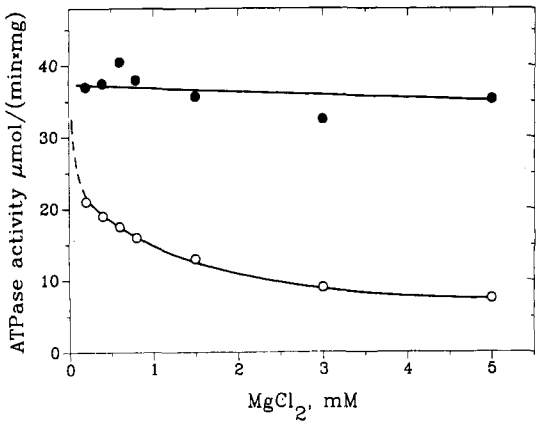



FIGURE 4: Effect of free $Mg^{2+}$ concentration on the initial and steady-state rates of MgATP hydrolysis by $MF_1$-ATPase. The initial ATPase activities (●) were measured as described in the Materials and Methods section. The steady-state activities (○) were measured 3 min after the reaction was started. The reaction mixture contained 100 μM MgATP, no azide, and indicated concentrations of added $Mg^{2+}$.

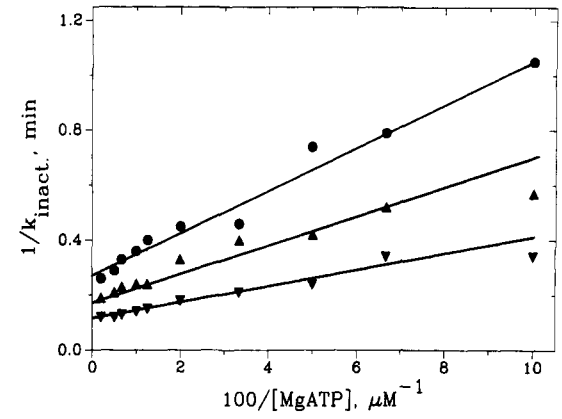



FIGURE 5: ATP dependence of $MF_1$-ATPase inactivation during ATP hydrolysis in the presence of azide at different concentrations of $MgCl_2$. The rate constants of the $MF_1$-ATPase inactivation were measured as described in the Materials and Method section, except the reaction mixtures contained 0.2 (●), 1.0 (▲), or 5.0 (▼) mM $Mg^{2+}$. The figure gives the reciprocal of the rate constant of inactivation versus the reciprocal of the ATP concentration.

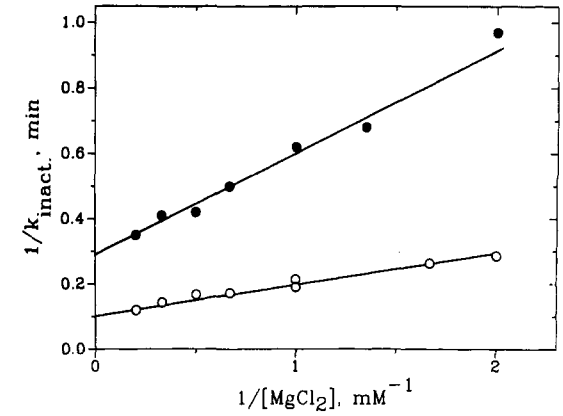



FIGURE 6: Dependence of the rate of $MF_1$-ATPase inactivation in the presence of MgATP on the $Mg^{2+}$ concentration. The rate constants of the $MF_1$-ATPase inactivation were measured as described in the Materials and Methods section in the presence of 15 (●) or 200 (○) μM MgATP and the indicated concentrations of added $MgCl_2$. The figure gives the reciprocal of the rate constant of inactivation versus the reciprocal of the ATP concentration.

the $Mg^{2+}$- and ADP-dependent inactivation of $F_1$-ATPase during ATP hydrolysis occurs with a significant rate only if the enzyme binds two or more nucleotides and one of them binds at the noncatatlytic site.

Results qualitatively similar to those presented above have been obtained with the nucleotide-depleted $MF_1$-ATPase preparations (data not shown), except that the nucleotide-depleted enzyme bound one more noncatalytic nucleotide, reaching a total of 3.0–3.5 mol of [$^3$H]AdN/enzyme. $PP_i$ exposure blocked binding of [$^3$H]AdN at all noncatalytic sites.

*Participation of Free $Mg^{2+}$ in Inactivation of $MF_1$-ATPase.* Free $Mg^{2+}$ was found to increase the extent of $CF_1$-ATPase inactivation at steady state but had no effect on the initial reaction rate (Guerrero et al., 1990b). The experiments in this section were undertaken to find if free $Mg^{2+}$ influenced inactivation of $MF_1$-ATPase by modifying ATP binding at a noncatalytic site or by other $Mg^{2+}$ binding to the enzyme.

Figure 4 shows that free $Mg^{2+}$ has little if any effect on the initial ATPase rate although the steady-state activity decreased significantly with $Mg^{2+}$ increased. The increased $Mg^{2+}$ concentrations had no effect on the $K_m$ for an ATP of 125 μM for initial reaction rate but increased the apparent $K_m$ for steady-state ATPase activity. Apparent $K_m$ values of 300 and 550 μM for ATP were obtained for steady-state activities in the presence of 0.5 and 5.0 mM free $Mg^{2+}$, respectively (data not shown). The result is as expected if the binding of $Mg^{2+}$ increased the extent of ADP-dependent inactivation. To check on this, the effect of free $Mg^{2+}$ on GTP hydrolysis was studied. It is known that $MF_1$-ATPase does not form an inactive complex during hydrolysis of GTP (Vulfson et al., 1984; Drobinskaya et al., 1985). The MgGTPase activity of $MF_1$-ATPase was linear and insensitive to the free $Mg^{2+}$ concentration (data not shown) confirming that medium $Mg^{2+}$ inhibits the steady-state ATPase via ADP-dependent inactivation, probably by increasing the rate of inactive complex formation.

Figures 5 and 6 show that $Mg^{2+}$ indeed increases the rate of the inactive enzyme–ADP–$Mg^{2+}$ complex formation but has no effect on the binding of ATP that accelerates the inactivation of $MF_1$-ATPase ($[ATP]_{1/2}$ = 25 μM). The dependence on the free $Mg^{2+}$ concentration of the rate of $MF_1$-ATPase inactivation (Figure 6) shows that half-maximal inactivation occurs at $[Mg]_{1/2}$ = 1.0 mM in the presence of both 15 and 200 μM ATP. The results of Figures 4–6 show that binding of $Mg^{2+}$ required for inactivation occurs independently of the MgATP binding at both noncatalytic and catalytic sites.

*Rate of Inhibitory $Mg^{2+}$ Binding.* The results of Figure 6 show that the rate of $MF_1$-ATPase inactivation saturates at a relatively high $Mg^{2+}$ concentration. This could result either from a relatively slow inactivation step following $Mg^{2+}$ binding or from a limited concentration of the $MF_1$-ATPase form that binds $Mg^{2+}$. These possibilities were assessed in further experiments.

As has been shown earlier (Vasilyeva et al., 1982b; Vulfson et al., 1986a,b; Murataliev et al., 1991), azide stabilizes the inactive enzyme–ADP–$Mg^{2+}$ complex. The initial velocity measured in the presence of azide as described in Materials and Methods gives a measure of the fraction of the active $MF_1$-ATPase (i.e., $MF_1$-ATPase without inhibitory ADP and $Mg^{2+}$ bound).

The results of Figure 7A show that in accord with earlier data (Vasilyeva et al., 1982b; Vulfson et al., 1984) azide had no effect on the initial activity of the control enzyme. A 10-min exposure to $Mg^{2+}$ (Figure 7B) results in enzyme with very low initial activity. $MF_1$-ATPase regains activity slowly in the presence of MgATP and azide prevents reactivation. The maximal reactivation rate for this complex at saturating

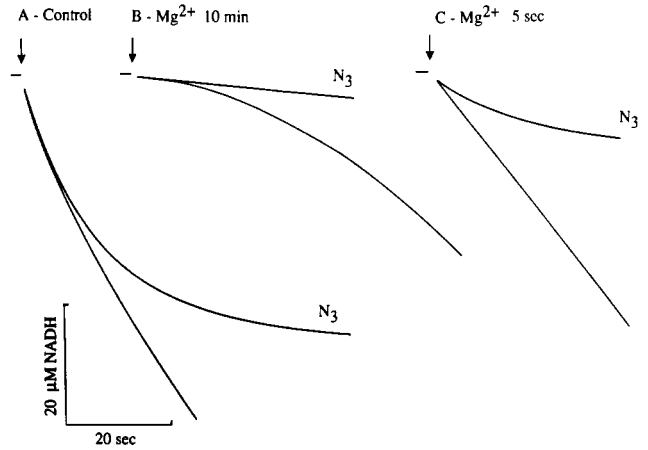



FIGURE 7: Effect of the time of $MF_1$-ATPase exposure to $Mg^{2+}$ on the time course of MgATP hydrolysis. $MF_1$-ATPase was added to an assay medium from the buffer (see Materials and Methods) containing 250 mM sucrose, 50 mM Tris–HCl, pH 8.0, and 1 mM EDTA (A) or 10 min (B) or 5 sec (C) after the addition of 2 mM $MgCl_2$ to give 1 mM free $Mg^{2+}$. Where indicated, the assay media contained 500 $\mu$M azide. The arrows indicate the moment of the $MF_1$-ATPase addition.

ATP is about 2 $min^{-1}$ and $K_{ATP}$ is about 150–200 $\mu$M (data not shown; see also Vasilyeva et al. (1980) and Milgrom and Murataliev (1989)).

In contrast, a 5-s exposure of $MF_1$-ATPase to 1 mM $Mg^{2+}$ results in 85–90% inhibition of the initial velocity as measured in the presence of azide (Figure 7C), but the enzyme shows a linear rate of MgATP hydrolysis in the absence of azide. The steady-state rate in the absence of azide is equal to the steady-state activity of the control enzyme (Figure 7A). The results of Figure 7 show that binding of $Mg^{2+}$ that occurs in a few seconds gives an enzyme that is inactive if azide is present but that readily regains activity in the presence of MgATP and no azide added. Slow isomerization of this complex (Milgrom & Murataliev, 1989; Murataliev et al., 1991) results in an inactive enzyme which regains activity in the presence of MgATP only slowly (Figure 7B).

An 85–90% inhibition of the enzyme by a 5-s exposure to 1 mM $Mg^{2+}$ (Figure 7, compare panels A and C, controls) requires that at least three half-times of the reaction are completed in that time. The maximal half-time of the reaction can thus be calculated to be about 1.7 s. This corresponds to a $k_{on} = 0.8 \times 10^3$ $M^{-1}$ $s^{-1}$ for inhibitory $Mg^{2+}$ binding by the enzyme with ADP present at a catalytic site and shows that there is no step slower than 0.4 $s^{-1}$ (24 $min^{-1}$) in the inactivation process. The latter value is several times faster than the maximal rate of inactivation measured in Figures 5 and 6 (10 $min^{-1}$) indicating that the rate-limiting step of $MF_1$-ATPase inactivation precedes the step of $Mg^{2+}$ binding. This means that the slow inactivation of the enzyme during ATP hydrolysis results from either a slow formation of or a low steady-state concentration of the $MF_1$-ATPase with inhibitory ADP bound at a catalytic site.

## DISCUSSION

The data of Figures 1–3 show that during the onset of $Mg^{2+}$ inhibition the $MF_1$-ATPase binds MgATP at a noncatalytic site. Exposure of the enzyme to $PP_i$ prevents both binding of noncatalytic AdN and enzyme inactivation during ATP hydrolysis. The MgATP binding at a noncatalytic site accelerates $Mg^{2+}$- and ADP-dependent inactivation of the enzyme in the course of ATP hydrolysis and occurs independently from the binding of the MgATP as a substrate. The requirement of MgATP binding at a noncatalytic site for $MF_1$-ATPase inactivation demonstrates another influence of the noncatalytic site nucleotides on the catalytic properties of the $F_1$-ATPases (Xue & Boyer, 1989; Guerrero et al., 1990a; Milgrom et al., 1990, 1991; Murataliev & Boyer, 1992).

In support of our conclusion that nucleotide binding at noncatalytic sites affects an ADP- and $Mg^{2+}$-induced inactivation of the $MF_1$-ATPase. R. L. Cross and Y. M. Milgrom (personal communications) found that prior binding of GTP at a noncatalytic site of $MF_1$-ATPase resulted in a slower rate of the enzyme inactivation by azide during ATP hydrolysis. Azide is a poor inhibitor of the GTPase activity of mitochondrial $F_1$-ATPase. Filling of the noncatalytic sites of $MF_1$-ATPase with ATP increased the sensitivity of the GTPase activity of the enzyme to azide.

A $K_m$ of 125 $\mu$M for the initial ATPase rate (Figure 1) shows a good reproducibility in different laboratories (Mitchell & Moyle, 1972; Vasilyeva et al., 1980; Vulfson et al., 1986) when the initial velocity without prior exposure of the enzyme to $Mg^{2+}$ is measured. On the other hand, the $K_{ATP}$ value of 25 $\mu$M (Figures 1 and 4) for half-maximal inactivation rate is 4–5 times lower than was found earlier (Vasilyeva et al., 1982b; Vulfson et al., 1986b). The inactivation rate during ATP hydrolysis in the present work was also somewhat higher than was measured earlier (Vasilyeva et al., 1982b; Vulfson et al., 1984; Drobinskaya et al., 1985; Murataliev, unpublished observations). The differences in the $K_{ATP}$ value for the half-maximal inactivation rate may reflect some variability of the properties of noncatalytic sites of $MF_1$-ATPase preparations. Such variability in ATP affinity for noncatalytic sites would not change fundamental catalytic properties. The properties of our $MF_1$-ATPase preparation were fortunate in that they allowed discernment of noncatalytic site binding for the modulating ATP.

It should be noted that there are some analogies between $F_1$-ATPase inactivation by a natural protein inhibitor and by ADP and $Mg^{2+}$ binding. $MF_1$-ATPase inactivation by the inhibitor protein results in entrapment of noncatalytic site nucleotides (Di Pietro et al., 1988). Half-saturation of the rate of $MF_1$-ATPase inactivation by the inhibitor protein occurs in the range of $10^{-5}$ M at pH 7.0 (Panchenko & Vinogradov, 1986, 1989; Milgrom, 1989), which is not far from $K_{ATP}$ = 25 $\mu$M for ADP- and $Mg^{2+}$-dependent inactivation (Figures 1 and 4). The binding of ATP at a site different from the catalytic one was suggested to be necessary for rapid inactivation of $F_1$-ATPase by the inhibitor protein (Panchenko & Vinogradov, 1986, 1989).

It is of interest to note that the inhibitory ADP but not the ion $Mg^{2+}$ arises from MgATP. The formation of inactive complex during MgATPase assay cannot be prevented by decreasing the medium ADP concentration by increasing the concentration of pyruvate kinase (Vasilyeva et al., 1982a; Vulfson et al., 1984; Guerrero et al., 1990). This shows that inhibitory ADP arises at a catalytic site of $MF_1$-ATPase in the course of multisite ATP hydrolysis (Vasilyeva et al., 1982b; Vulfson et al., 1984). On the other hand, the $Mg^{2+}$ needed for inactivation seems to bind from a medium rather than arrive with MgATP. The binding of inhibitory $Mg^{2+}$, MgATP at a catalytic site (as substrate), and MgATP at a noncatalytic site to accelerate $MF_1$-ATPase inactivation appear to be independent processes. Whether the binding of inhibitory $Mg^{2+}$ occurs at the catalytic site where inhibitory ADP is bound or at another site remains uncertain [see Guerrero et al. (1990b)].

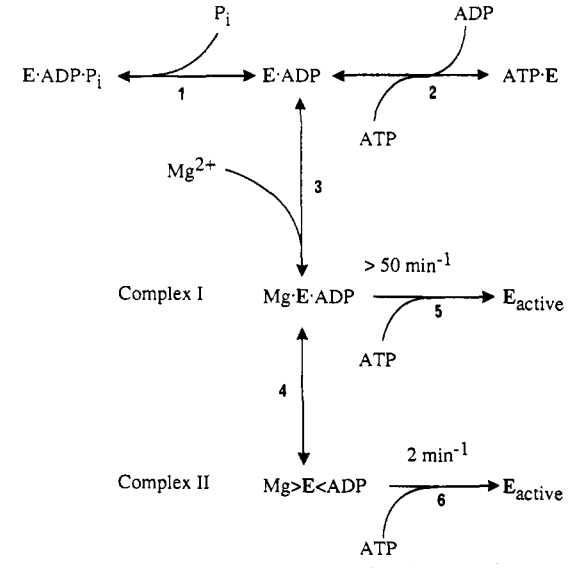
*What Step Limits the Inactivation*? The inhibitory $Mg^{2+}$ binding with $MF_1$-ATPase is known to involve at least two steps: the initial $Mg^{2+}$ binding gives an enzyme–ADP–$Mg^{2+}$ which isomerizes slowly into a second, more stable complex (Milgrom & Murataliev, 1989; Bulygin & Vinogradov, 1991; Murataliev et al., 1991). An uncertainty remained as to whether or not the first complex is active in the case of mitochondrial enzyme. Bulygin and Vinogradov (1991) have concluded that the binding of $Mg^{2+}$ resulted in an active $MF_1$-ATPase that subsequently slowly isomerized to give an inactive enzyme. In contrast, results with $CF_1$ showed that the first complex is inactive but regains an activity after removal of medium $Mg^{2+}$ more readily than the second one (Milgrom & Murataliev, 1989; Murataliev et al., 1991).

The results of Figure 7 show that the $Mg^{2+}$-dependent inactivation of the enzyme containing inhibitory ADP at a catalytic site occurs with $t_{1/2}$ about 1.7 s in the presence of 1 mM $Mg^{2+}$. There is no step slower than 24 $min^{-1}$ leading to the inactivation of $MF_1$-ATPase by $Mg^{2+}$ binding when enzyme already has ADP at a catalytic site. The inactivation in the course of ATP hydrolysis occurs considerably slower even in the presence of saturating $Mg^{2+}$ and ATP ($k_{inact}$ = 10 $min^{-1}$ at infinite $Mg^{2+}$ and ATP; Figures 5 and 6). Neither $Mg^{2+}$ binding nor any subsequent isomerization step, therefore, limits $MF_1$-ATPase inactivation in the course of ATP hydrolysis. The rate-limiting step of $MF_1$-ATPase inactivation during ATP hydrolysis with excess $Mg^{2+}$ present precedes the binding of $Mg^{2+}$. Likely, this is the rate of formation of enzyme with ADP bound at a catalytic site without bound $P_i$. This complex can then rapidly bind $Mg^{2+}$ to inactivate the $MF_1$-ATPase. The binding of the noncatalytic ATP could accelerate the inactivation by promotion of $P_i$ release from the catalytic site to give the E·ADP species required for inactivation.

The data of Figure 7 show another important point. A 5-s exposure of $MF_1$-ATPase to 1 mM $Mg^{2+}$ (Figure 7C) results in rapid formation of the enzyme–ADP–$Mg^{2+}$ complex and 85–90% inactivation as measured in the presence of azide. If azide is omitted, the enzyme shows a linear rate of ATP hydrolysis which is equal to the steady-state activity of the control enzyme. This indicates that the inactive enzyme–ADP–$Mg^{2+}$ complex formed during short-time exposure to $Mg^{2+}$ can regain activity in the presence of MgATP relatively rapidly—within 2–3 s. A long-term exposure of $MF_1$-ATPase to $Mg^{2+}$ results in inactive enzyme with maximal reactivation rate $k_{act}$ about 2 $min^{-1}$ in the presence of saturating ATP ($K_{ATP}$ is about 150–200 $\mu$M, data not shown; see also Vasilyeva et al. (1980) and Milgrom and Murataliev (1989)).

*Effect of the Inactive Enzyme Formation on the Kinetic Properties*. Scheme I illustrates the interaction of the $MF_1$-ATPase with $Mg^{2+}$ to form the inactive enzyme complexes. E·ADP is the $MF_1$-ATPase containing bound ADP at a catalytic site without $P_i$. The E·ADP arises after removal of the loosely bound nucleotides on the Sephadex centrifuge column equilibrated with a $Mg^{2+}$-free buffer or during ATP hydrolysis (steps 1 and 2) as a result of $P_i$ release prior to ADP (step 1). Complex I represents inactive $MF_1$-ATPase after a short time exposure to $Mg^{2+}$ (step 3). This complex rapidly regains activity in the presence of MgATP (step 5), and this activation is prevented by azide (Figure 7C). Prolonged incubation of the complex I (step 4) results in the formation of complex II that has a higher affinity for both ADP (Milgrom & Murataliev, 1989) and $Mg^{2+}$ (Bulygin & Vinogradov, 1991; Murataliev et al., 1991) and regains activity in the presence of MgATP (step 6) with maximal $k_{act}$ of 2 $min^{-1}$ (Figure 7B).

Scheme I: Kinetic Model for $Mg^{2+}$- and ADP-Induced Inactivation of $F_1$-ATPase[a]




[a] The catalysis proceeds through steps 1 and 2. Complex I and complex II are two inhibited states of $F_1$-ATPase.

During ATP hydrolysis by $MF_1$-ATPase, a low steady-state concentration of E·ADP (see Scheme I) arises because sometimes $P_i$ dissociation precedes ADP release (step 1). Free $Mg^{2+}$ binds with the E·ADP complex present and inhibits activity of $MF_1$-ATPase by producing the inactive complex I (step 3). The latter can either isomerize (step 4) into more stable inactive complex II (Milgrom & Murataliev, 1989; Murataliev et al., 1991) or be rapidly reactivated by binding of MgATP (step 5).

The scheme explains why increasing MgATP concentrations result in less inhibition of the ATPase activity at steady state (very little inhibition occurs at 5 mM MgATP (Vulfson et al., 1986b)). High MgATP concentrations decreases the inactivation because (a) MgATP binds faster than $P_i$ release occurs and prevents formation of the E·ADP complex; (b) MgATP rapidly reactivates (step 5) the inactive complex I (Figure 7C), preventing the isomerization reaction (step 4) leading to the more stable complex II; and (c) MgATP competes effectively with $Mg^{2+}$ for the binding with E·ADP (step 2). For these reasons, the increasing of the ATP concentration decreases the extent of inactivation, as shown by Vulfson et al. (1986b). This protecting effect of high MgATP results in an increase of the steady-state activity of the enzyme as MgATP increased and an appearance of an apparent $K_m$ for MgATP hydrolysis in millimolar range. The initial activity of $MF_1$-ATPase at millimolar ATP changes only slightly if any with increasing ATP.

MgATP binding at a noncatalytic site of $MF_1$-ATPase with $K_{ATP}$ = 25 $\mu$M increases the rate of enzyme inactivation (Figures 1 and 4). The rate of ATP-dependent reactivation, on the other hand, does not increase considerably at this ATP range (Milgrom & Murataliev, 1989). The resulting steady-state activity of $MF_1$-ATPase becomes more inhibited compared to initial velocity as the MgATP concentration is increased. This can result in the appearance of an apparent $K_m$ for MgATP in the range 20–50 $\mu$M. The variability of $MF_1$-ATPase preparations in noncatalytic nucleotide binding discussed above could cause the discrepancy in the number of $K_m$s and their values obtained in different laboratories.

The fact that the extent of $MF_1$-ATPase inactivation at steady state varies at different ATP concentrations (Vulfson et al., 1986b) sets some requirements for the kinetic studies

of $MF_1$-ATPase. The mass balance equation for the steady-state conditions includes two enzyme species $E_t = E{\cdot}S + E_{free}$. In the case of $MF_1$-ATPase, the fraction of an inactive E·ADP·Mg complex can reach 70–75% at some concentrations of MgATP (Vulfson et al., 1986b), and the inactive enzyme needs to be included in the mass balance equation. Unfortunately, this makes the reaction velocity equation for steady-state conditions complicated and difficult to handle and measurements of an initial reaction velocity for kinetic analysis of $MF_1$·ATPase become preferable. Activating anions such as sulfite and bicarbonate helps abolish the apparent negative cooperativity (Ebel & Lardy, 1975) by retarding the formation of the inactive $MF_1$-ATPase during ATP hydrolysis (Vasilyeva et al., 1982b) and reducing the interference of the inactive complex formation in steady-state kinetic studies.

The $MF_1$-ATPase is known to fill one catalytic site with an MgATP at a concentration well below micromolar (Grubmeyer et al., 1982; Milgrom & Murataliev, 1987; Cunningham & Cross, 1988). The presence of only one $K_m$ above a micromolar ATP concentration means that a maximal rate of ATP hydrolysis can be attained with only two catalytic sites filled (Milgrom & Murataliev, 1986) or, less likely, that a second and third catalytic sites have nearly the same $K_m$ value.

A similar behavior was shown in ATP synthesis by chloroplast and bacterial synthases. One MgADP binds with a $K_m$ less than micromolar, and nearly maximal velocity can be attained with the saturation of the second catalytic site with a single $K_m$ of about 30–50 μM (Stroop & Boyer, 1985; Perez & Ferguson, 1990; Zhu & Boyer, 1992). The interpretation favored from these and other results is that during catalysis all three catalytic sites participate in an equivalent manner. One site contains very tightly bound ATP that can interconvert to bound ADP and $P_i$. At another site, ADP binding is favored and is necessary for rapid ATP synthesis. ATP binding is favored at a different catalytic site and necessary for rapid hydrolysis of ATP. In either ATP synthesis or hydrolysis reactions, all three catalytic sites sequentially pass through three conformations.

*Comparison of the Mitochondrial and Chloroplast Enzymes.* Recently, we found with $CF_1$-ATPase that the complex I (see Scheme I) regained activity after $Mg^{2+}$ removal several times faster than the complex II formed after prolonged incubation (Murataliev et al., 1991). MgATP, however, did not reactivate complex I rapidly and almost the same extent of inhibition was observed if the assay was performed in either the presence or absence of azide. This difference in reactivation by MgATP of the inactive complex I (step 5, Scheme I) could be the reason why the chloroplast $F_1$-ATPase is more sensitive to $Mg^{2+}$ inhibition than the mitochondrial enzyme.

The modulation of the hydrolytic activity of $CF_1$-ATPase by nucleotide binding at noncatalytic sites has been shown (Xue & Boyer, 1989; Guerrero et al., 1990a; Milgrom et al., 1990, 1991). More recent studies show that this modulation of the $CF_1$-ATPase activity by noncatalytic nucleotides occurs through the promotion of the release of inhibitory ADP from a catalytic site (Murataliev & Boyer, 1992). With $CF_1$-ATPase, a noncatalytic ATP binding accelerates reactivation of the inhibited enzyme. The opposite effect of noncatalytic nucleotides on the chloroplast and mitochondrial $F_1$-ATPases could result from differences in the rate of inhibited complex formation and reactivation. With mitochondrial $F_1$-ATPase, promotion of $P_i$ release by ATP binding at a noncatalytic site increases the steady-state concentration of the E·ADP complex required for $Mg^{2+}$-induced inhibition. With the chloroplast enzyme, the inactive enzyme–ADP–$Mg^{2+}$ forms so readily that no activity is observed at low ATP concentrations until reactivation of the inactive complex is promoted by activating anions and/or ATP binding at noncatalytic sites (Milgrom et al., 1990, 1991; Murataliev & Boyer, 1992). Also, it is possible that with two enzymes different noncatalytic sites are involved.

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