Active/Inactive State Transitions of the Chloroplast F1 ATPase Are Induced by a Slow Binding and Release of Mg2+

RELATIONSHIP TO CATALYSIS AND CONTROL OF F1 ATPases*

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Mg2+ is known to be a potent inhibitor of F1 ATPases from various sources. Such inhibition requires the presence of a tightly bound ADP at a catalytic site. Results with the spinach chloroplast F1 ATPase (CF1) show that the time delays of up to 1 min or more in the induction or the relief of the inhibition are best explained by a slow binding and slow release of Mg2+ rather than by slow enzyme conformational changes. CF1 is known to have multiple Mg2+ binding sites with $K_d$ values in the micromolar range. The inhibitory Mg2+ and ADP can bind independently to CF1. When Mg2+ and ATP are added to the uninhibited enzyme, a relatively fast rate of hydrolysis attained soon after the addition is followed by a much slower steady-state rate. The inhibited steady-state rate results from a slowly attained equilibrium of binding of medium Mg2+. The $K_d$ for the binding of the inhibitory Mg2+ is in the range of 1–8 mM, in the presence or absence of added ATP, as based on the extent of rate inhibition induced by Mg2+. Assessments from 18O exchange experiments show that the binding of Mg2+ is accompanied by a relatively rapid change to an enzyme form that is incapable of hydrolyzing MgATP. When ATP is added to the Mg2+- and ADP-inhibited enzyme, the resulting reactivation can be explained by MgATP binding to an alternate catalytic site which results in a displacement of the tightly bound ADP after a slow release of Mg2+. Both an increase in temperature (to 50°C) and the presence of activating anions such as bicarbonate or sulfate reduce the extent of the Mg2+ inhibition markedly. The activating anions may bind to CF1 in place of P, near the ADP. Whether the inhibitory Mg2+ binds at catalytic or noncatalytic nucleotide binding sites or at another location is not known. The Mg2+- and ADP-induced inhibition appears to be a general property of F1 ATPases, which show considerable differences in affinity for ADP, Mg2+, and P. These differences may reflect physiological control functions.

It has been established that both Mg2+ and ADP can cause strong inhibitory effects on the activity of the separated and membrane-bound F1 ATPase1 from various sources (1–20). However, the factors underlying the Mg2+-induced inhibition and its relationship to the ADP-induced inhibition have not been elucidated clearly. Evidence suggests that both medium Mg2+ and an ADP bound at a catalytic site without accompanying P (16, 18) are required for inhibition (7, 9, 11, 16, 17, 20). Thus, it seems likely that only one type of inhibitory effect is involved.

The inhibition of MgATP hydrolysis or the reactivation of the inhibited enzyme is unusual in that under conditions used frequently, the inhibition or reactivation requires many seconds or even some minutes to develop fully. For example, with ATP-heat-activated CF1, an initial rapid phase of MgATP hydrolysis during the first 30 s may be followed by a decrease of activity to a considerably slower but constant steady-state rate (20). When CF1 is exposed to both Mg2+ and ADP or only to Mg2+, if a tightly bound ADP is present at a catalytic site, an inhibited enzyme form is obtained (11, 18). Upon addition of ATP the enzyme is slowly reactivated, and a maximum steady-state rate is attained in about 2 min.

The transitions between inhibited and active states have generally been regarded as resulting from slow conformational changes following binding of Mg2+ and ADP. However, any conformational changes accompanying binding could be relatively rapid, and the slow transitions could result from a slow binding and release of Mg2+. To distinguish between these two possibilities and to obtain a better understanding of the role of Mg2+, we have explored several parameters of the Mg2+-induced inhibition, including experiments on its time and its Mg2+ concentration dependence. Other experiments using 18O exchange were designed to determine whether the Mg2+- and ADP-induced inhibition could be best explained by a progressive increase in inhibition of all enzyme molecules as the Mg2+ concentration is increased, or if the inhibition, as suggested (2, 15, 18), results from the interconversion of active and essentially inactive enzyme forms. We also report another approach for ascertaining whether tightly bound ADP at a catalytic site (17, 18, 20) is necessary for the Mg2+-induced inhibition and report on the effects of activating anions and temperature in relieving the inhibition. Overall, the results presented add some important new insights and confirm and extend some previous suggestions for the nature of the inhibition induced by both ADP and Mg2+.

EXPERIMENTAL PROCEDURES

Preparation of CF1 CF1, prepared as described previously (21), was heat activated (22) in the presence of either ATP, ADP, or GDP

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\(^1\) The abbreviations used are: F1, ATPase, separated ATPase from the ATP synthase; CF1, the F1 ATPase from spinach chloroplasts; MF1, F1 ATPase from beef heart mitochondria; P, partition coefficient; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
as described. CF₁, activated in the presence of the different nucleotides, will be referred to as either ATP-, ADP-, or GDP-heat-activated enzyme, respectively.

Assays — ATPase assays were performed at pH 8 in 50 mM Tricine buffer, except for those accompanying ³⁰O exchange experiments. Assays were run at room temperature if not otherwise indicated. Some of the experiments included an ATP-regenerating system consisting of 1 mM phosphoenolpyruvate and 100 µg/ml pyruvate kinase as indicated in the figure legends.

For ³⁰O exchange experiments ATP hydrolysis was performed in 50 mM glycyglycine, 100 mM KCl, pH 8. The Mg(γ⁻³⁰O)ATP concentration was maintained constant at 10 µM by using 0.5 mM γ⁻³⁰O phosphoenolpyruvate (23) and 100 µg/ml pyruvate kinase. CF₁ (30–60 µg/ml) was incubated for sufficient time, 30–90 min, to allow for the production of 100–200 nmol of P₂, under the different conditions tested. Samples were quenched by the addition of an equal volume of ice-cold chloroform and processed for analysis as described (24).

Protein determinations were performed routinely with the Lowry method (25) using defatted bovine serum albumin as a standard. Conversion factors were based on an A₂₇₇ at 1 mg/ml = 0.483 (26) for CF₁, and a molecular mass of 400 kDa (27). P₂ production was monitored by a catalyzed P₂ assay (28). Rates of MgATP hydrolysis in the presence of high P₂, backgrounds were determined with a coupling assay that monitors NADH oxidation at 340 nm (29).

Conversion factors were based on an A₂₇₇ at 1 mg/ml = 0.483 (26) for Mg²⁺ binding, chemical relaxation parameters were calculated with a derivative-free nonlinear regression program from BMDP Statistical Software, Inc., Los Angeles, CA (34) provided by Dr. Larry Faller.

Appraisals of ³⁰O distributions in the P₂ samples were made as described previously (35) with use of a computer program designed by Dr. Terry J. Reedy (36).

RESULTS

An Increase in the Free Mg²⁺ Concentration Results in a Decrease of Both an Initial Rapid ATPase Activity and the Steady-state Rate — Fig. 1 shows ATPase activity measurements of ATP-heat-activated CF₁ in the presence of different concentrations of free Mg²⁺, with the MgATP concentration maintained at 2 mM as described under “Experimental Procedures.” For example, with CF₁, at a low free Mg²⁺ concentration (10 µM) an initial lag (about 1 s long), as reported earlier (3,37), is followed by a burst of activity and a transition to a slower steady-state rate (20). At this concentration of free Mg²⁺ the burst lasts about 60 s before a linear inhibited rate is achieved. As the free Mg²⁺ concentration is increased further the extent and the rate of the initial burst, the steady-state rate, and the time before the onset of the linear rate are all decreased. At the highest concentrations of free Mg²⁺ tested (450 µM), the burst is barely apparent, and a strongly inhibited steady-state rate is observed. If the free Mg²⁺ concentration is decreased below 10 µM by increasing further the total ATP concentration (>12 mM), activity is inhibited because of competition of free ATP for binding at the catalytic sites (3).

After the steady-state rate is reached, ATP hydrolysis continues linearly for many min in the presence of a pyruvate kinase trap to prevent the accumulation of ADP, which at higher concentrations (>70 µM) causes a weak competitive inhibition (38). The slower rates observed at the higher Mg²⁺ concentrations do not involve nonspecific electrostatic effects since CaATP hydrolysis does not exhibit a similar transition to an inhibited steady-state rate, and the slower steady-state rates do not result when the free Ca²⁺ concentration is increased (20). The strong activity dependence on the free Mg²⁺ concentration is consistent with a binding of Mg²⁺ to an inhibitory site on CF₁. The final linear rate thus would reflect the attainment of an equilibrium between enzyme with bound Mg²⁺ at the inhibitory site and that without bound Mg²⁺. The slow transition to the inhibited rate at low Mg²⁺ concentrations suggests that Mg²⁺ binding might be relatively slow, a possibility supported further by other data in this paper.

If the initial high rate of activity is assumed to correspond to 100% active CF₁, then the steady-state rates can give an approximation of the percent of inactive enzyme at equilibrium in the presence of the different concentrations of free Mg²⁺. Estimates of Kd values for the inhibitory Mg²⁺ binding when the Mg²⁺ concentration was increased as reported in Fig. 1 were 1.1, 2.0, 3.6, 1.8, 5.6, 7.3, and 6.3 µM, respectively, corresponding to an average of 4.0 ± 2.5 µM.

The Kd, k₅₀, and k₉ₐt rate constants were also derived from the data of Fig. 1 and other similar unreported data using a derivative-free nonlinear regression statistical software package as described under “Experimental Procedures” by fitting the observed data with the data predicted by a model in which the inhibition might result from one inhibitory Mg²⁺ binding to CF₁. Analysis of the rate of approach to a linear steady state resulted in an estimate for the Kd of 8.4 ± 6.0 µM and k₅₀ and k₉ₐt rate constants of 4.5 ± 3 × 10⁻⁵ M⁻¹ s⁻¹ and 0.03 ± 0.02 s⁻¹, respectively.

Only One Catalytic Pathway Is Found with an Increased Inhibition of the Steady-state Rate by Increasing Mg²⁺ Concentrations — The effect of increasing the Mg²⁺ concentration could result from a progressive change in the reaction rate constants governing the catalytic pathway or from a conversion of active enzyme with one catalytic pathway to an inactive enzyme form. For the former, a progressive shift in characteristics of the reaction pathway would result. For the latter, only one catalytic pathway should be operative at all Mg²⁺ concentrations.

Measurement of the distribution of [³⁰O]P₂ isotopomers formed during the hydrolysis of [γ⁻³⁰O]ATP provides a sensitive probe of the reaction pathway for F₁ ATPases. When ATP is hydrolyzed, the bound P₂ formed at the catalytic site can exchange oxygens with water before its release. The probability of the exchange occurring prior to release is expressed as the partition coefficient, P₂. As the ATP concentration is lowered, the P₂ value increases from near 0 (little

Fig. 1 MgATPase activity in the presence of increasing concentrations of free Mg²⁺. Assays were performed at 22 °C and pH 8 in 50 mM Tricine in the presence of an ATP-regenerating system (1 mM phosphoenolpyruvate, 100 µg/ml pyruvate kinase). The MgATP concentration was maintained constant at 2 mM as described under “Experimental Procedures.” The CF₁ concentration was 60 µg/ml. The free Mg²⁺ concentrations used were: 10 µM (O), 20 µM (●), 35 µM (■), 55 µM (○), 100 µM (△), 220 µM (□), and 460 µM (▲).
Absence of Medium ATP or ADP—To state rate is inhibited by increasing the free Mg²⁺ concentration. Assays were performed at 22 °C and pH 8 in 50 mM buffer (0.5 mM phosphoenolpyruvate, 100 mM KCl, and a pyruvate kinase-regenerating system. Fig. 2 compares the degree of inhibition of the steady-state rate and the changes in Pₐ values as the free Mg²⁺ concentration is increased. The measurements were somewhat difficult to perform since at the low concentration of substrate used, Pₐ values are very sensitive to MgATP concentration. In most ¹⁸O experiments, the Mg²⁺ concentration is in excess to maintain the MgATP concentration constant. However, in these experiments both Mg²⁺ and ATP concentrations were varied to allow experiments to be performed in the presence of both excess and very low values of Mg²⁺. What is noteworthy is that in all trials only one major Pₐ value was observed for each condition tested. The average Pₐ value obtained was 0.56.

The Mg²⁺ Inhibition Can Be Induced and Reversed in the Absence of Medium ATP or ADP—To determine whether MgATP hydrolysis might be a requirement to observe the inhibition, as has been noted for the action of inhibitory proteins from various F, ATPases (39), the following experiments were performed. ATP-heat-activated CF₁ that contains an ADP at one of the three noncatalytic sites and adenine nucleotides at two of the three noncatalytic sites (40) was used. The enzyme was exposed to different concentrations of Mg²⁺ for different periods of time prior to catalysis. Fig. 3 shows the effect on ATP hydrolysis of prior exposure to 250 μM Mg²⁺ for different periods of time. A control without the prior exposure to Mg²⁺ shows the usual kinetics when assayed with 5 mM ATP and 2 mM Mg²⁺, which provides a low free Mg²⁺ concentration (20 μM). A 1-min exposure to 250 μM Mg²⁺ is enough to induce a pronounced lag that recovers slowly (about 1 min) before the same steady-state rate is observed as for the control. An increase in the time of exposure to Mg²⁺ to 5 min prior to assay increased the duration of the lag. Additional exposure for another 5 min gave no further increase. Therefore, it appears that at 250 μM Mg²⁺ the binding site has been essentially saturated within 5 min. The results show that Mg²⁺ does not require either the presence of medium ATP or ADP or the occurrence of catalysis to be able to bind to ATP-heat-activated CF₁ and to effect the changes required for the transition to the inactive enzyme.

Fig. 4 shows the effect of time of exposure to increasing Mg²⁺ concentrations on the subsequent rate of ATP hydrol-
ysis during the burst phase as compared with a control without prior exposure to Mg\(^{2+}\). The activity during the burst phase was approximated from the slope of the time course between 0 and 20 s of catalysis in the presence of 2 mM Mg\(_{\text{ATP}}\). Between 0 and 20 s most of the Mg\(^{2+}\)-inhibited enzyme has not had sufficient time to recover any significant activity, and the control has not yet reached its slower equilibrium rate. Therefore, the observed 0–20-s rate gives an approximation of the amount of inhibited enzyme present at the time of Mg\(^{2+}\) addition. Other experiments were performed to determine the Mg\(^{2+}\) concentration dependence of the inhibition after a 10-min exposure to Mg\(^{2+}\) (data not shown). The inhibition pattern as the Mg\(^{2+}\) concentration is increased shows a typical hyperbolic dependence.

One important conclusion from the data of Fig. 4 is that upon exposure to 250 \(\mu\)M Mg\(^{2+}\) the decrease in activity is rapid, resulting in more than a 90% inhibition at the first assay point (30 s). With 1 mM Mg\(^{2+}\) no activity was detected after 30 s of exposure. Increasing the Mg\(^{2+}\) concentration thus speeds up the transition to the inhibited enzyme. These results show that the Mg\(^{2+}\)-induced inhibition does not result from stabilization of a slowly formed inactive enzyme conformation. The binding of Mg\(^{2+}\) causes or allows the transition to an inactive enzyme form within a few seconds or less. Relatively rapid conformational changes could accompany or follow the Mg\(^{2+}\) binding. A second important point from the data in Fig. 4 is that as the Mg\(^{2+}\) concentration is lowered, the Mg\(^{2+}\) binding appears to be approaching an equilibrium with time. The results may be explained by a relatively slow reversible binding of Mg\(^{2+}\) with a \(K_d\) of less than 10 \(\mu\)M.

To assess further the equilibrium of Mg\(^{2+}\) binding, an experiment was conducted to find if the same final equilibrium position was approached from both the fully active and the fully inactive enzyme forms. Fully active CF\(_{1}\) and fully inactive CF\(_{1}\) were allowed to reach an equilibrium between active and inactive forms in the presence of 5 \(\mu\)M Mg\(^{2+}\). For the data presented in Fig. 5 either CF\(_{1}\) (30 \(\mu\)g/ml) was exposed to 5 \(\mu\)M Mg\(^{2+}\) for various time intervals prior to 2 mM Mg\(_{\text{ATP}}\) hydrolysis, or CF\(_{1}\) (300 \(\mu\)g/ml) was exposed to 50 \(\mu\)M Mg\(^{2+}\) for 20 min. This allowed nearly complete inactivation of the enzyme. The sample was then diluted to a final Mg\(^{2+}\) concentration of 5 \(\mu\)M and a final enzyme concentration of 30 \(\mu\)g/ml. The time course of inactivation in the former case and the time course of reactivation in the latter case were monitored as described previously. In both instances close to the same final equilibrium between active and inactive enzyme forms (ratio 60/40) was achieved, indicating a \(K_d\) of about 3 \(\mu\)M.

An Additional Assessment of the Requirement of Enzyme-bound ADP for the Mg\(^{2+}\)-induced Inhibition—The hydrolysis of MgGTP does not show the pronounced Mg\(^{2+}\)-induced inhibition as does Mg\(_{\text{ATP}}\) hydrolysis (20). Therefore, it was of interest to compare the degree of the Mg\(^{2+}\)-induced inhibition observed upon exposure of Mg\(^{2+}\) to either ADP-heat-activated CF\(_{1}\) or GDP-heat-activated CF\(_{1}\). Heat activation with ADP results in a tightly bound ADP at a catalytic site and an ADP incorporated into one of the three noncatalytic sites. Heat activation in the presence of GDP prevents the ADP incorporation and likely results in the incorporation of a GDP into a catalytic site (40). The inhibition observed at two different Mg\(^{2+}\) concentrations (25 \(\mu\)M and 1 mM) was compared with either ADP-heat-activated CF\(_{1}\) or GDP-heat-activated CF\(_{1}\). As shown in Fig. 6, when the tight binding of ADP at a catalytic site is prevented, the extent of Mg\(^{2+}\)-induced inhibition is reduced drastically. If 2 mM ADP is added to GDP-heat-activated CF\(_{1}\), at any time during the Mg\(^{2+}\) incubation, the same degree of activity inhibition is attained as for the ADP-heat-activated enzyme by the time of the first time point (10 s). Therefore, ADP binding occurs quickly (<10 s) in comparison with the binding of Mg\(^{2+}\), which takes about 15 min to reach equilibrium at a concentration of 5 \(\mu\)M. The ADP binding to CF\(_{1}\) could be inducing a conformational change that in itself does not inhibit enzyme activity but may be necessary to allow the transition to the inhibited form induced by Mg\(^{2+}\). Also the results show that Mg\(^{2+}\) is still able to bind to the enzyme in the absence of the bound ADP at a catalytic site. An inhibition is observed, however, only after the ADP has also bound to the enzyme.

The presence of bicarbonate or an increase in the temperature of the incubation decreases the inhibition of the steady-state rate—Either an increase in temperature (to 50 °C) or the presence of bicarbonate (60 mM) reduces drastically the Mg\(^{2+}\)-induced inhibition of the steady-state rate. Both temperature and bicarbonate exert their maximal activation when

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**Fig. 5.** Mg\(^{2+}\) binding to CF\(_{1}\) is reversible and at 5 \(\mu\)M reaches an equilibrium within 15 min. CF\(_{1}\) (30 \(\mu\)g/ml) was exposed to 5 \(\mu\)M Mg\(^{2+}\) for different lengths of time prior to activity assay (\(\square\)). CF\(_{1}\) (300 \(\mu\)g/ml) was exposed to 50 \(\mu\)M Mg\(^{2+}\) for 10 min prior to a 10-fold dilution to 30 \(\mu\)g/ml CF\(_{1}\) and 5 \(\mu\)M Mg\(^{2+}\). The time course of reactivation was followed after the dilution step (\(\square\)). Assays and the method to determine the percent activity remaining after the different Mg\(^{2+}\) incubation conditions were performed as in Fig. 4.

**Fig. 6.** GDP-heat-activated CF\(_{1}\) does not show the same degree of Mg\(^{2+}\)-induced inhibition as does ADP-heat-activated CF\(_{1}\). GDP heat-activated or ADP heat-activated CF\(_{1}\) (1 mg/ml) was exposed to different concentrations of Mg\(^{2+}\) for different periods of time. Assay conditions were as in Fig. 4. Conditions used were: GDP-heat-activated CF\(_{1}\) with either 25 \(\mu\)M Mg\(^{2+}\) (\(\square\)) or 1 mM Mg\(^{2+}\) (\(\triangle\)), and ADP-heat-activated CF\(_{1}\) with either 25 \(\mu\)M Mg\(^{2+}\) (\(\square\)) or 1 mM Mg\(^{2+}\) (\(\triangle\)).
CF, is hydrolyzing ATP in the presence of excess Mg\(^{2+}\). That bicarbonate increases the catalytic activity of CF, hydrolyzing MgATP in the presence of excess Mg\(^{2+}\) was first reported by Nelson et al. (41). Fig. 7 shows the effect of increasing the temperature under conditions in which there is a 1 mM excess of either Mg\(^{2+}\) or ATP, and the substrate concentration is maintained within the range of 30–40 \(\mu\)M MgATP. At higher temperatures the Mg\(^{2+}\)-induced inhibition is reduced drastically. The fractional inhibition caused by 1 mM excess Mg\(^{2+}\) decreases from approximately 80% at room temperature to 5% at 62 °C.

For the experiments of Fig. 7, the estimated MgATP concentration was 30 \(\mu\)M at room temperature. The concentration of MgATP increased slightly as the temperature was increased. A 1.4-fold increase in the \(K_a\) for MgATP formation results from the increase in temperature from 25 to 37 °C (42). Even if the \(K_a\) values doubled in going from 25 to 50 °C, the change in MgATP concentration would only be from about 30 \(\mu\)M to about 37 \(\mu\)M.

Other Consequences of Bicarbonate Activation—A possibility that warranted further appraisal was whether bicarbonate decreased the affinity of ADP for its inhibitory site. To determine whether ADP binding to the enzyme might be affected by bicarbonate, the degree of inhibition of MgGTP hydrolysis caused by the addition of 2 \(\mu\)M ADP was compared in the presence or absence of bicarbonate. Addition of 2 \(\mu\)M ADP during MgGTP hydrolysis results in a 50% inhibition of the steady-state rate. The extent of the inhibition was nearly the same (5% less) in the presence of 60 mM bicarbonate (data not shown). Thus, the activation caused by bicarbonate does not result from a decreased binding of ADP to CF, during MgGTP hydrolysis. Similarly, when CF, was exposed to 50 \(\mu\)M Mg\(^{2+}\) in the presence or absence of 60 mM bicarbonate prior to dilution and testing of MgATP hydrolysis, no change was observed in either the time course or the final degree of the inhibition (data not shown). Therefore, Mg\(^{2+}\) binding and release and any resultant enzyme conformational changes that occur in the absence of catalysis are not affected by the presence of bicarbonate.

In other experiments the effect of Pi on the bicarbonate activation was assessed (Fig. 8). Experiments were performed in the presence of 3 mM Mg\(^{2+}\) and 3 mM ATP to have a high degree of the Mg\(^{2+}\)-induced inhibition, and activity was monitored by the disappearance of NADH using a coupling assay (29). As the Pi concentration is increased from 0 to 80 mM in the absence of 60 mM bicarbonate, a small degree of activation (1.5 \(\times\)) of MgATP hydrolysis is observed. On the other hand, the extent of bicarbonate activation decreases by about 3-fold as the Pi concentration is increased. This is consistent with Pi binding to the same site as the bicarbonate. However, the binding of Pi, is much less effective than the binding of bicarbonate for activation of MgATP hydrolysis. Experiments performed with 30 mM sulfite, another activating anion, exhibited a similar inhibition of the sulfite activation in the presence of Pi. Bicarbonate and other activating anions do not appear to bind to the site to which MgATP binds since increasing the MgATP concentration from 0.5 to 3 mM while maintaining the free Mg\(^{2+}\) constant (1 mM) did not change the extent of anion activation or the inhibition by Pi of the bicarbonate activation (data not shown).

**DISCUSSION**

The Time Delays for Inhibition and Reactivation of CF, MgATP Hydrolysis Can Be Explained by the Slow Binding and Slow Release of Inhibitory Mg\(^{2+}\)—The results show that the time course of the inhibition of MgATP hydrolysis observed with ATP-heat-activated CF, is strongly dependent on the free Mg\(^{2+}\) concentration. The free Mg\(^{2+}\) concentration affects both the length of the initial burst of activity and the degree of inhibition attained during the subsequent linear steady-state rate. The Mg\(^{2+}\) causes inhibition by binding to an enzyme with a tightly bound ADP at a catalytic site. The Mg\(^{2+}\) binding promotes a transition to an enzyme form that is incapable of hydrolyzing MgATP. The slow transitions from an active to an inactive enzyme form when MgATP hydrolysis is initiated or from an inactive to an active enzyme form when the enzyme is exposed to Mg\(^{2+}\) prior to catalysis is due to the slow binding or slow release of Mg\(^{2+}\) instead of slow conformational changes associated with a fast binding and release of Mg\(^{2+}\). The steady-state rate achieved at a given concentration of free Mg\(^{2+}\) reflects the balance between Mg\(^{2+}\) binding and release and the level of tightly bound ADP at a catalytic site. Once Mg\(^{2+}\) dissociates, the binding of MgATP...
at an alternate catalytic site will promote ADP release.

The results as given in Figs. 1, 4, and 5 clearly suffice to show that the combination of the inhibitory Mg\(^{2+}\) with CF\(_1\) is unusually slow. They suffice to give only a reasonable approxi-
mation of the values for \(K_0\) and \(k_{on}\) and \(k_{off}\) rate constants, principally because it is difficult to obtain accurate measure-
ment of the uninhibited velocity. This is taken as the maxi-
imum velocity observed during the burst phase and is not a
precise value. If the rate uninhibited by Mg\(^{2+}\) were higher
than our estimated maximum, the values of \(K_0\) would be
lower. The various estimates of \(K_0\) reported under “Results”
fail in the range of 1–8 \(\mu\)M. Low free Mg\(^{2+}\) concentra-
tions thus suffice for maximal inhibition. The data do provide
convincing evidence that the binding of the inhibitory Mg\(^{2+}\)
is unusually slow (4.5 \(\times\) 10\(^{-3}\) M\(^{-1}\) s\(^{-1}\)). In contrast, based on
the rapid inhibition by 2 \(\mu\)M ADP of the GTP-heat-activated
enzyme already exposed to Mg\(^{2+}\) as shown in Fig. 5, the \(k_{on}\)
for ADP is of the order of 1 \(\times\) 10\(^{6}\) M\(^{-1}\) s\(^{-1}\) or more than 200
times more rapid than the rate of Mg\(^{2+}\) binding. The slow
Mg\(^{2+}\) binding could result if the Mg\(^{2+}\) must cross some type
of barrier, e.g. conformational or charge, to reach the binding
site.

In a previous investigation, Hochman et al. (3) obtained a
\(K_0\) of 20 \(\mu\)M for the Mg\(^{2+}\)-induced inhibition observed during
MgATP hydrolysis for CF\(_1\). This is not far from our value.
Malyan (12) obtained a \(K_0\) of 56 \(\mu\)M for the Mg\(^{2+}\) inhibition
during CaATP hydrolysis. The differences in \(K_0\) may result
from differences in the preparation of the enzyme, such as
loading of the noncatalytic sites, or may reflect differences in
enzyme conformation of the static enzyme and an enzyme
undergoing either MgATP or CaATP hydrolysis.

**Only Fully Active or Essentially Inactive Enzyme Is Present as the Mg\(^{2+}\) Concentration Is Increased**—Previous research led
to the suggestion that the inhibition by Mg\(^{2+}\) resulted
from the conversion of an active to an inactive enzyme state
(2, 7). Our results using the \(^{18}O\) exchange methodology vali-
date this previous suggestion. Hydrolysis of a low concentra-
tion of \([\gamma-\text{\(^{18}\)O}]\)ATP shows the existence of only one catalyt-
ically active enzyme form, with kinetic characteristics that
result in a single \(P_1\) value of 0.56 at free Mg\(^{2+}\) concentrations
varying from below 1 \(\mu\)M to 1 \(\mu\)M. No gradual change in the
\(P_1\) value was observed as Mg\(^{2+}\) concentrations were increased,
ruling out the possibility that the Mg\(^{2+}\) affects all CF\(_1\) mole-
cules similarly at a given Mg\(^{2+}\) concentration. Such equal
effect on all CF\(_1\) molecules could result if the association and
dissociation of Mg\(^{2+}\) to one or more sites to cause inhibition
were more rapid than the time for catalytic turnover.
The finding of only one \(P_1\) value means that the Mg- and ADP-
inhibited enzyme did not have any detectable catalytic activ-
ity.

**Additional Evaluation of the Requirement for Both Tightly Bound ADP and Mg\(^{2+}\) to Observe the Inhibition**—As shown in
Figs. 3 and 4, ATP- or ADP-heat-activated CF\(_1\) needs to
be exposed only to Mg\(^{2+}\) in the absence of added medium
ADP or ATP to induce the inhibition. Heat activation with
adenine nucleotides results in the tight binding of an ADP
into a catalytic site (40). Prior exposure to Mg\(^{2+}\) has been
noted to inhibit MgATP hydrolysis by rat liver mitochondrial
particles (2) and by MF\(_1\) (6). In these studies the endogenous
nucleotide content was not determined, and the stringent
requirement for a tightly bound ADP at a catalytic site was
not reported. Other experiments with either MF\(_1\) or CF\(_1\) have
detected a strict requirement for a tightly bound ADP to induce
the inhibition of MgATP hydrolysis (15, 17, 20).

Similarly, light activation of the membrane-bound CF\(_1\) ATP-
ase has been correlated with the release of a tightly bound
ADP (43, 44). The deactivation that occurs once the thylakoid
membranes are placed in the dark is accelerated by the
presence of medium ADP (45, 46) and is prevented if medium
ADP is removed (1, 47). The presence of medium Mg\(^{2+}\) is
necessary for the deactivation process (47, 48).

The results given in Fig. 6 confirm the mutual requirement
for both Mg\(^{2+}\) and ADP to induce the inhibition and show
the strong Mg\(^{2+}\) concentration dependence on the time re-
quired to induce the inhibition. GDP-heat-activated CF\(_1\) does
not contain the tightly bound ADP at the catalytic site (40).
GDP may be more loosely bound to the site if bound, may
be unable to induce the conformational changes required for
Mg\(^{2+}\) to induce the inhibitory enzyme conformation. The
presence of GDP in the medium decreases the Mg\(^{2+}\) inhibition
of the light-activated membrane-bound CF\(_1\), ATPase (46). The
low level of inhibition during MgATP hydrolysis may result
with the catalytic site empty or loaded with GDP or may be
due to ADP contamination of the GDP or of the enzyme. The
rapid inhibition by ADP of the GDP-heat-activated enzyme
that has been exposed to Mg\(^{2+}\) confirms that Mg\(^{2+}\) binds to
the enzyme lacking the tightly bound ADP but is not capable
by itself of inducing the change to the inactive enzyme form.
Only when ADP is bound at a catalytic site does the transition
to the inhibited state occur.

**Location of the Inhibitory Mg\(^{2+}\) Binding Site**—Where the
inhibitory Mg\(^{2+}\) binds is not known, and because up to six
Mg\(^{2+}\) binding sites have been reported for CF\(_1\), (49) the loca-
tion is difficult to assess. Early studies to characterize the
cation binding sites were performed by following Mn\(^{2+}\)
induction to CF\(_1\) by EPR (50, 51). Mn\(^{2+}\), like Mg\(^{2+}\), induces the
inhibition of ATP hydrolysis and has an even higher affinity
than Mg\(^{2+}\). The EPR studies indicated the presence of one
tight Mn\(^{2+}\) binding site and five loose cation binding sites in
the absence of exogenous nucleotide. In the presence of ade-
nine nucleotide the cation binding pattern shifted to two tight
cation binding sites and four loose binding sites. However,
later studies with CF\(_1\) from the same laboratory (49, 52)
showed three cation binding sites with positive cooperativity
and with a high affinity for Mg\(^{2+}\) plus three noninteracting
cation binding sites with a lower affinity. The three high
affinity Mg\(^{2+}\) binding sites were suggested (52) to have higher
\(k_{on}\) and \(k_{off}\) rates than those reported in our studies. The
slower binding and release of the inhibitory Mg\(^{2+}\) may not
have been detected.

Differences observed in the cation binding characteristics
have been suggested to reflect differences in the method of
preparation and storage of the enzyme (49). Our enzyme
preparation was more like that used in the earlier cation
binding studies (50), particularly in that the enzyme was
stored as an ammonium sulfate precipitate instead of being
stored in glycerol at −80 °C (49).

All sites with high affinity for added Mn\(^{2+}\), and thus likely
for added Mg\(^{2+}\), can also bind nucleotides (50, 53). Reports
from Carmelli’s laboratory (50–52) favor a catalytic site loca-
tion for high affinity Mg\(^{2+}\) binding sites, a view supported by
Haddy et al. (53). However, the possibility that the inhibitory
Mg\(^{2+}\) binds to a noncatalytic site needs consideration. Non-
catalytic site nucleotides on F\(_1\) ATPases are not readily re-
placed during ATP hydrolysis (54). One noncatalytic site on
CF\(_1\) binds ATP tightly only in the presence of Mg\(^{2+}\) (40), but
filling this site does not inhibit catalysis. The function of
noncatalytic binding sites is obscure although filling of the
sites with ATP promotes GTP hydrolysis (40), and the sites
may function similarly in ATP hydrolysis.

If the inhibitory Mg\(^{2+}\) binds to a catalytic site then the
question arises as to whether the inhibitory Mg\(^{2+}\) is bound at

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the same site as the inhibitory ADP. A possibility raised by Haddy et al. (53) is that with ADP already present at one catalytic site, the inhibitory Mg\(^{2+}\) binds at a second or third catalytic site in preference to MgATP binding and thus inhibits catalysis. This possibility is eliminated by the characteristics of the Mg\(^{2+}\) inhibition reported here. The $K_c$ for the Mg\(^{2+}\) is sufficiently high and the time of Mg\(^{2+}\) release sufficiently rapid so that over minutes of exposure to excess MgATP the inhibition should be nearly completely reversed. This is not the case. The enzyme remains strongly inhibited when the MgATP to Mg\(^{2+}\) ratio is well over 1,000/1. The inhibitory Mg\(^{2+}\); if it binds at a catalytic site, must combine to a catalytic site to which MgATP cannot bind, namely the site already occupied by ADP.

Combination of the inhibitory Mg\(^{2+}\) and ADP at the same site would mean that binding of only one Mg\(^{2+}\)/enzyme is necessary for inhibition. As mentioned briefly under "Results," the inhibition induced by Mg\(^{2+}\) shows a typical hyperbolic dependence, which is in harmony with the need for tight binding of only one Mg\(^{2+}\)/enzyme to induce inhibition. The evaluation of the $K_c$ estimated from the data of Fig. 1 gave similar values over the wide range of free Mg\(^{2+}\) concentrations tested if binding of a single Mg\(^{2+}\) was considered necessary for the inhibition but markedly increasing values of $K_c$ if the combination of two Mg\(^{2+}\)/enzyme were required for the inhibition. Such results strongly favor a requirement of binding of only one Mg\(^{2+}\)/enzyme for inhibition. Although only one bound Mg\(^{2+}\)/enzyme may induce the inhibition, from previous Mg\(^{2+}\) binding studies mentioned above (49-52) it appears likely that other Mg\(^{2+}\) binding sites are also filled in the l-10 μM range.

On the Nature of the Mg\(^{2+}\)-induced Inhibition—A reasonable possibility is that the inhibitory Mg\(^{2+}\) and ADP bind at the same catalytic site. If so, hydrolysis of MgATP should provide bound ADP, Mg\(^{2+}\), and P, at the potential inhibitory site. An explanation is then needed as to why the onset of the inhibition is slow and why the extent of the inhibition depends on the medium Mg\(^{2+}\) concentration if bound ADP and Mg\(^{2+}\) could already be present. A hypothesis that might account for these and other related observations is given in the following paragraphs.

The inhibited linear steady-state rate is much lower in comparison with the initial burst of activity (Fig. 1). Thus, during steady-state catalysis nearly all of the enzyme could have inhibitory ADP and Mg\(^{2+}\) at a catalytic site. When tightly bound ADP, P, and Mg\(^{2+}\) arise at the site, most of the time P, could dissociate before ADP does, accompanied or preceded by dissociation of the Mg\(^{2+}\), which was originally bound to ATP. After this event medium Mg\(^{2+}\) could rebind to enzyme with ADP remaining at a catalytic site in the absence of P. It would be this enzyme form that cannot hydrolyze MgATP. These suggestions are in accord with observations from others that P, binding under certain conditions is able to prevent the Mg\(^{2+}\)-induced inhibition (7, 18, 19). As long as P, is tightly bound, the Mg\(^{2+}\) inhibition is reduced considerably. Binding of MgATP at a second catalytic site promotes the release or loosens binding of P, and probably Mg\(^{2+}\), as is evidenced by the ability of relatively low concentrations of MgATP to inhibit sharply the reversals of bound ATP formation at the catalytic site (23).

That the dissociation of inhibitory Mg\(^{2+}\) is necessary for subsequent promotion of the release of the tightly bound ADP by ATP for reactivation is supported by the data presented in Figs. 3 and 5. The time required to reach a steady-state rate of MgATP hydrolysis, after exposure of CF, to 250 μM-free Mg\(^{2+}\) for 10 min, as shown in Fig. 3, is only about 2-fold less than the time that is required to dissociate Mg\(^{2+}\), from CF, in the dilution experiment of Fig. 5. The relatively small time difference could result from a slight promotion of Mg\(^{2+}\) release by the binding of MgATP at a second site under the experimental conditions of Fig. 5.

We suggest that when P, ATPases have ADP tightly bound at a catalytic site in the absence of P, or an activating anion and have the inhibitory Mg\(^{2+}\) bound, the catalytic cooperativity essential for catalysis is blocked. Because an increase in the ATP concentration with an excess of medium Mg\(^{2+}\) present will not prevent or overcome the inhibition, it is probable that the binding of MgATP to additional catalytic sites is not prevented but that the conformational changes that cause release of ADP from the tight site are prevented or rendered ineffective. Without bound Mg\(^{2+}\) present, or if activating anions are bound, the requisite changes occur more readily. This interpretation is in accord with the observation that at low substrate concentrations at which cooperativity does not occur, bicarbonate loses its ability to activate (55).

Location of the Activating Anion Binding Site—Our data support the suggestions (19, 56) that activating anions and inhibitory anions combine in place of P, at the site with inhibitory ADP present. This explains the ability of P, to decrease the activation by bicarbonate (Fig. 8) and sulfate, P, protects MF, against azide inhibition (56). Earlier, Moyle and Mitchell (2) showed antagonistic effects of azide and activating anions with MF,. Neither the degree of bicarbonate activation nor the inhibition of bicarbonate activation which results from P, addition is affected by MgATP concentration at a constant free Mg\(^{2+}\) concentration. Therefore, the activating anions or P, is not competing for MgATP binding sites.

The concentration of P, required to inhibit activation by other anions is considerably less than that found necessary by Feldman and Sigman (57) for the formation of bound ATP from the tightly bound ATP at a catalytic site of CF,. We suggest that the release of tightly bound P, formed by ATP hydrolysis is accompanied by a conformational change such that P, can only bind loosely. Tight P, binding competent for bound ATP synthesis by the isolated CF, may be induced only by very high concentrations of P,.

How the activating anions promote reactivation is puzzling. We noted that bicarbonate did not prevent the inhibition induced by exposure to Mg\(^{2+}\) prior to the onset of MgATP hydrolysis. Reports in the literature on the effects of activating anions on binding affinities of MgATP, ADP, and Mg\(^{2+}\) are contradictory and include as examples both an increase (50) and a decrease in the affinity for Mg\(^{2+}\) (5). Neither an increase nor the inhibition of bicarbonate activation nor the inhibition of bicarbonate activation which results from P, addition is affected by MgATP concentration at a constant free Mg\(^{2+}\) concentration. Therefore, the activating anions or P, is not competing for MgATP binding sites.

Differences in the Mg\(^{2+}\) and ADP Inhibition Observed for F, ATPases from Various Sources—The Mg\(^{2+}\) and ADP inhibition appears to be a general phenomenon for all F, type
ATPases. However, the characteristics of the inhibitions vary widely (41, 61, 62). The strong retention of catalytic site ADP and the relatively tight binding of Mg\(^{2+}\) by CF\(_1\) make this enzyme, either membrane bound or free, particularly susceptible to the inhibition. At the other extreme, the addition of azide is necessary to elicit prominent Mg\(^{2+}\) inhibition with the membrane-bound enzyme from Lactobacillus casei (62). MFI appears to have a higher affinity than CF\(_1\) for P\(_i\), at the catalytic site. This could explain why the induction of the enzyme, either membrane bound or free, particularly susceptible to the inhibition, such as azide. It appears that nature has fine tuned ATP synthase to differences in cellular needs by changes in the affinities for Mg\(^{2+}\), ADP, and P\(_i\).

These differences in inhibitory patterns may be related to control functions. With chloroplasts, prevention of ATP hydrolysis in the dark may conserve ATP, as has often been suggested. Such control could also be useful under some conditions for mitochondria and Escherichia coli, but for an anaerobe such as L. casei unhindered cleavage of ATP to produce a protomotive force is essential. In this case the Mg\(^{2+}\) inhibition is prominent only in the presence of reagents that promote the inhibition, such as azide. It appears that nature has fine tuned ATP synthase to differences in cellular needs by changes in the affinities for Mg\(^{2+}\), ADP, and P\(_i\).

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