Influence of Divalent Cations on Nucleotide Exchange and ATPase Activity of Chloroplast Coupling Factor 1†

Jeanne G. Digel,‡ Nakisha D. Moore, and Richard E. McCarty*

The Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

Received August 21, 1998

ABSTRACT: The ATPase activity of the catalytic part of ATP synthases is inhibited by free Mg^{2+}, even though MgATP is the substrate. Here we show that the inhibition of the MgATPase activity of chloroplast coupling factor 1 deficient in its ϵ subunit (CF_1-ϵ) by Mg^{2+} is complex. The hydrolysis of MgATP by CF_1-ϵ that contains tightly bound ADP, but no bound Mg^{2+}, is initially rapid and decreases within about 1 min to a steady-state rate. The bound MgADP content of CF_1-ϵ was varied. The initial fast phase of MgATP hydrolysis is eliminated when the molar ratio of MgADP to CF_1-ϵ approaches 2. Loosely bound Mg^{2+} also affects the initial kinetics of the enzyme that contains bound MgADP. At molar ratios of bound MgADP to enzyme in excess of 1, the initial ATPase activity was low and reached the steady state after about 30 s. Free Mg^{2+} in the assay mix also inhibited steady-state ATP hydrolysis by all forms of the enzyme. The results are consistent with a model in which two Mg^{2+} bind cooperatively, probably to the dissociable nucleotide-binding sites on CF_1-ϵ. Thus, four different nucleotide-binding sites may be involved in the inhibition of the MgATPase activity of CF_1-ϵ. Three of these sites are potentially catalytic, and the fourth may be regulatory. The exchange of bound trinitrophenyl-ADP induced by the addition of MgATP or CaATP was found to be fast enough for the site to be involved in catalysis.

The chloroplast ATP synthase, CF_1-CF_o,[1] synthesizes ATP from ADP and P_i driven by the electrochemical proton gradient across the thylakoid membrane. CF_o is integral to the thylakoid membrane, whereas CF_1, the catalytic portion of the complex, is soluble when released from the membrane. CF_1 in solution hydrolyzes rather than synthesizes ATP (*1*).

CF_1 contains the six nucleotide-binding sites of CF_1-CF_o. Of these, four are tight binding sites for adenosine di- or triphosphates, where "tight" means that bound nucleotide is not readily removed by gel filtration or dialysis. Two of the sites, N2 and N5, are noncatalytic and will only bind ATP or AMP−PNP tightly, requiring Mg^{2+} for tight binding (*2*). The remaining tight sites are N1 and N4. Isolated CF_1 has approximately 1.5 mol of ADP/mol of enzyme distributed between these two sites (*3*). Although dissociation of ADP bound to either N1 or N4 is slow in the absence of nucleotide in the medium, when medium nucleotide is present they may undergo exchange. Bound ADP is released relatively rapidly as medium nucleotide binds elsewhere (*2*). The exchange properties of N1 and N4 are quite different. TNP-ADP in N1 exchanges readily for medium ADP, AMP−PNP, and ATP (*4*). This site can also be emptied of bound nucleotide by incubation of CF_1 with sulfite or with high concentrations of P_i (*3*). N4 exchanges much more slowly than N1 (*5*).

Tightly bound MgADP is a strong inhibitor of the ATPase activity of F_1ATPases in solution (*6*). Drobinskaya et al. (*6*) showed using nucleotide-depleted mitochondrial F_1 (MF_1) that MgADP rather than ADP alone or Mg^{2+} alone caused the observed inhibition, and concluded that the inhibitory MgADP was at a catalytic site. Milgrom and Murataliev (*7*) determined that there are two tight binding sites for ADP on MF_1, one of which had very high affinity (K_d = 4−5 nM) that they assigned as the inhibitory MgADP site and the other with a $K_d \cong 200$ nM that they concluded is a catalytic site. As MgATP is hydrolyzed by MF_1, the rate of hydrolysis declines to a steady-state value. Murataliev (*8*) determined that the MgATP concentration for the half-maximal rate of inactivation was 4−5 times lower than the K_m for ATP hydrolysis and concluded that both a catalytic and noncatalytic binding site were involved in inhibition.

Free Mg^{2+} in the reaction mixture inhibits steady-state ATPase activity (*8*, *9*). Guerrero et al. (*9*) noted that the rate of the onset of MgATPase activity inhibition was slow relative to enzyme turnover and concluded that inhibition was the result of slow binding of free Mg^{2+} to the catalytic site containing tightly bound ADP. However, as MgATP is the substrate for ATP hydrolysis, it seems that each turnover must result in MgADP at the catalytic site unless Mg^{2+} can dissociate together with P_i.

The rate of the initial exchange of ADP-bound N1 in heat-activated CF_1 was found to be much slower than the steady-state rate of catalysis, leading to the conclusion that the N1 site could not be catalytic even though it was capable of hydrolyzing ATP (*4*). Further investigation suggested that

† This work was supported by Grant MCB 94-05713 from the National Science Foundation. N.D.M. was the recipient of a Howard Hughes Medical Institute Summer Research Fellowship in 1996.

* To whom correspondence should be addressed. Tel: 410-516-5356. Fax: 410-516-5213.

‡ Present address: Medical Biotechnology Center, The University of Maryland, 725 W. Lombard St., Baltimore MD, 21201.

[1] Abbreviations: CF_1-CF_o, the chloroplast ATP synthase; CF_1, chloroplast coupling factor 1; MF_1, mitochondrial coupling factor 1; TNP-ADP, 2′(3′)-*O*-trinitrophenyl-ADP; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside.

10.1021/bi982027p CCC: $15.00
Published on Web 11/14/1998

only the first turnover of exchange was slow; during steady-state CaATPase activity, exchange was found to be fast enough to be part of catalysis. Also, the initial CaATPase activity was found to be slower than the steady-state rate, leading to the suggestion that the first release of bound ADP was part of an activation of CF_1 and that N1 was in fact a catalytic site (*10*).

The question of whether exchange from N1 was fast enough to be part of the catalytic mechanism was revisited with studies of sulfite-stimulated MgATPase activity (*11*, *12*) of CF_1 bound to thylakoids with opposite conclusions being reached. Du and Boyer (*12*) found a lag in the onset of MgATPase activity that corresponded to the time that it took to exchange much of the tightly bound exchangeable ADP, whereas Larsen et al. in a similar study(*11*) saw no such lag.

By selectively loading the tight binding sites of CF_1 we have shown that binding of MgADP to both N1 and N4 contributes to ATPase inhibition, with the maximum inhibition from tight binding of MgADP obtained when both sites contain tightly bound MgADP. In addition, free Mg^{2+} in the assay mixture further inhibits the rate of steady-state ATPase activity. Analysis of the inhibition suggests that two cooperatively interacting sites for free Mg^{2+} are involved. These sites are likely to be the dissociable sites, N3 and N6.

The initial variations in the rate of MgATP hydrolysis by CF_1 with time are shown to depend strongly on the occupancy of the nucleotide-binding sites, as well as on the content of the reaction mixture. Comparison of the rate of exchange of nucleotide in N1 with the time course of ATPase activity under the same circumstances demonstrates that the loss of tightly bound nucleotide from N1 is kinetically consonant with the activation of CF_1 and that, in the absence of tightly bound ADP, the enzyme is already active.

EXPERIMENTAL PROCEDURES

CF_1 was prepared from market spinach by the procedure described in Shapiro and McCarty (*2*), with modifications described in refs *13* and *14*. CF_1-ϵ was prepared according to the method of Richter et al. (*15*), with modifications described in Soteropoulos et al. (*16*). CF_1-ϵ was stored at 4 °C as a precipitate in 50 mM Tris-HCl (pH 8.0), 1 mM ATP, and 50% saturated ammonium sulfate with 5 mM EDTA to remove bound Mg^{2+}. CF_1-ϵ depleted of its endogenous nucleotide (CF_1-ϵ-NT) was prepared as in Digel et al. (*5*), except that 25 mM Na_2SO_3 was added during the incubation with alkaline phosphatase. The presence of the Na_2SO_3 facilitated the removal of bound ADP without affecting the ATPase activity. Buffer solutions were passed through a column of Chelex 100 resin to remove residual Mg^{2+} and were stored in plastic. the CF_1-ϵ concentration was determined by the Lowry method (*17*).

Steady-state MgATPase activities were in some cases measured using a coupled enzyme assay as described in ref *5*. The variation of the ATPase activity of CF_1-ϵ and CF_1-ϵ-NT over time was also monitored by measuring the amount of P_i produced over different time intervals following the addition of CF_1-ϵ or CF_1-ϵ-NT to a solution of $MgCl_2$ or $CaCl_2$ and ATP in 50 mM Tris-HCl (pH 8.0). P_i produced was determined by the sensitive malachite green assay (*18*) with modifications (*19*). ATPase activity as a function of time was also monitored using a continuous spectrophotometric assay (*20*), in which P_i production is measured as a shift in the absorbance at 360 nm of 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) when it is phosphorylitically cleaved by purine-nucleoside phosphorylase. MESG was synthesized using the procedure in ref *20*, except that the silicic acid column was not used. Nucleoside phosphorylase was from Sigma (St. Louis, MO).

To avoid the error inherent in numerical differentiation, we fit the curves from the continuous spectrophotometric P_i assay directly using nonlinear least-squares analysis and the activities calculated from derivatives of the fits. It is the calculated activities that appear in figures in which activity is plotted as a function of time. All of the equations used were determined empirically and are given under Results.

The bound nucleotide content of CF_1 was measured by ion-pairing high-pressure liquid chromatography, using the procedure of Moal et al. (*21*) with samples prepared as described (*5*). The relationship between the integrated peak area and the nucleotide quantity was determined for each experiment using nucleotide standards of known concentration. Bound Mg^{2+} was measured using atomic absorption spectroscopy (*3*).

CF_1-ϵ and CF_1-ϵ-NT at 10−40 mg/mL were loaded with TNP-ADP by incubation in 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 0.5 mM TNP-ADP for 30−50 min at room temperature. Excess and loosely bound TNP-ADP were removed by passage of the enzyme through two or three consecutive Sephadex G-50 columns equilibrated with 5 mM Tris-HCl (pH 8.0). The extent of TNP-ADP loading was determined by the absorbance of tightly bound TNP-ADP at 418 nm using an extinction coefficient of $2.51 \times 10^4\ M^{-1}\ cm^{-1}$ (*22*) and blanked against CF_1-ϵ at the same concentration that had not been loaded with TNP-ADP. The concentration of free Mg^{2+} in a mixture with ATP was calculated using a program by Grunwald and Lemasters (*23*).

Binding of TNP-ADP as well as exchange of tightly bound TNP-ADP with nucleotide in solution was observed by measuring TNP-ADP fluorescence. The fluorescence of TNP-ADP increases linearly with the amount bound to CF_1-ϵ (*12*). Fluorescence measurements were made using an OLIS-modified SLM/Aminco SPF-500 spectrofluorometer. The excitation wavelength was 418 nm and the emission wavelength, 560 nm. Exchange was initiated by stopped-flow mixing. All ATPase assays and fluorometric exchange measurements were carried out at 25 °C.

RESULTS

To monitor changes with time of MgATPase activity of CF_1-ϵ, we used an assay in which ADP formation was coupled to NADH oxidation. By using this assay, we observed a lag in the initial rate of ADP production by MgATPase activity (*5*). Direct addition of a relatively large amount of ADP to the reaction mix resulted in rapid initial NADH oxidation, suggesting that the lag observed in the presence of CF_1-ϵ was attributable to an activation of the activity of the CF_1-ϵ. The experiment was repeated using direct measurement of the P_i produced by the malachite green colorimetric assay. Upon addition of Mg^{2+}-depleted CF_1-ϵ to a reaction mix containing 1 mM $MgCl_2$ and 5 mM ATP, the MgATPase activity is initially rapid and decreases over

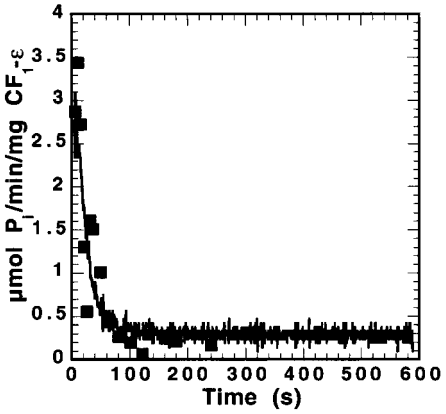

FIGURE 1: Activity as a function of time as determined by two different assay methods. Spectrophotometric assay with 5 mM ATP, 2 mM $MgCl_2$, 200 μM MESG, 5 units nucleoside phosphorylase, and 20 μg of CF_1-ϵ in 50 mM Tris-HCl (pH 8.0), 1 mL total volume (solid line). (squares) Colorimetric malachite green P_i assay of aliquots of a stirred mixture containing 5 mM ATP, 2 mM $MgCl_2$, 50 μg/mL CF_1-ϵ in 50 mM Tris-HCl (pH 8), 50 mM NaCl (squares). Both reactions were initiated by addition of CF_1-ϵ. Note that ATPase activity is plotted as a function of time.

about 1 min to the steady-state activity observed with the coupled enzyme assay (Figure 1). Thus the lag in MgATPase activity of CF_1-ϵ reported previously (*5*) is very likely an artifact caused by the coupling reactions, possibly the need to accumulate a reservoir of ADP to drive the regeneration of ADP to ATP.

For continuous monitoring of P_i production, a spectrophotometric assay requiring nucleoside phosphorylase and MESG was used. Although there is no regeneration of ADP to ATP using this assay, it has the advantages that nucleoside phosphorylase does not require Mg^{2+} and is not sensitive to Mg^{2+} or Ca^{2+} at the concentrations used in the MgATPase and CaATPase reaction mixes. Also, it requires only one enzyme. The time course of P_i production by CF_1-ϵ in the presence of 1 mM $MgCl_2$ and 5 mM ATP determined by the continuous spectrophotometric assay was similar to that obtained by malachite green assay (Figure 1). By both assays ATPase activity was rapid at first and declined within about 100 s to a steady-state value about 7% of that in the initial stage of the reaction.The first 5 s or more of each spectrophotometric assay are lost to mixing. Where presented, data for initial activities ($t = 0$) are extrapolated from nonlinear least-squares analysis. The extrapolated initial activities were 20% or less higher than those directly observed in the assay. In some cases, the initial activities were less than the steady-state activities.

Samples of CF_1-ϵ that contained varying amounts of bound ADP were produced by incubating nucleotide-depleted CF_1-ϵ (0.14 mol of ADP/mol of CF_1-ϵ) with increasing substoichiometric amounts of ADP in a buffer of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA for 1 h before passage of the enzyme solutions through two Sephadex G-50 centrifuge columns equilibrated with 50 mM Tris-HCl (pH 8.0). A fraction of each sample was then incubated with 1 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0) before passage through two more centrifuge columns. This protocol has been shown to produce samples with only as much bound Mg^{2+} as ADP (*3*). Neither the initial rate of MgATP hydrolysis nor the steady-state rate was affected significantly by the content of tightly bound ADP itself (data not shown). However, the

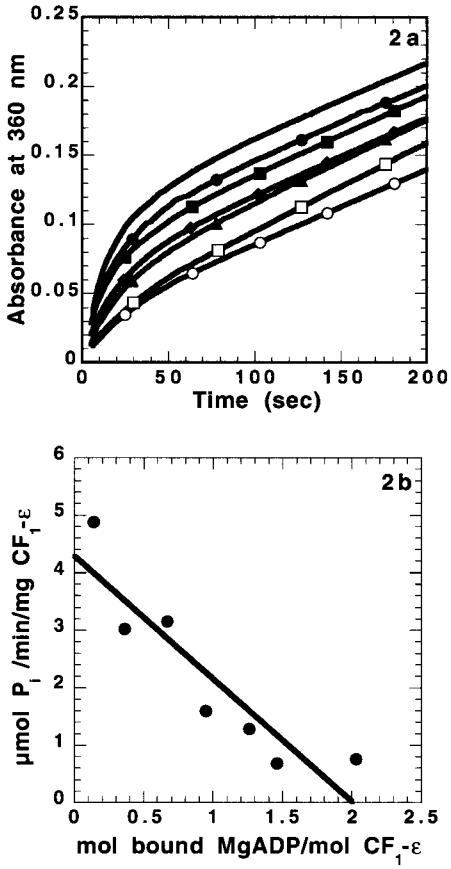

FIGURE 2: (a) Assay of P_i production for CF_1-ϵ with different bound MgADP content. Assays contained 5 mM ATP, 2 mM $MgCl_2$, 200 μM MESG, 5 units of nucleoside phosphorylase, and 20 μg of CF_1-ϵ in 50 mM Tris-HCl (pH 8), 1 mL total volume. Samples of CF_1-ϵ were loaded with tightly bound MgADP (mol/mol of CF_1-ϵ) as follows: 0.14 (solid line), 0.36 (closed circles), 0.67 (closed squares), 0.95 (diamonds), 1.26 (triangles), 1.46 (open circles), and 2.03 (open squares). Data points were taken 1/s. Symbols are to distinguish between data sets. Absorbance of the reaction mixture at 360 nm prior to the addition of the enzyme was subtracted. (b) Difference between the initial and steady-state MgATPase activities of CF_1-ϵ as a function of bound MgADP content. The initial and steady-state activities were calculated from fits to the data shown in part (a). The difference between the initial and steady-state activities is plotted against the bound MgADP content.

rate at which the activity approaches the steady state is slowed by the presence of tightly bound ADP. The half-time for decay to the steady-state activity was 8 s in a sample that contained 0.21 mol of ADP/mol of CF_1 and 14 s in a sample that had 1 mol of ADP/ mol of CF_1.

Dramatically different results were obtained with the enzyme that contained tightly bound MgADP. As the amount of tightly bound MgADP increases, the initial activity, assayed in the presence of 5 mM ATP and 2 mM Mg^{2+}, decreases (Figure 2a). Note that the data in Figure 2a are presented as the absorbance of the assay mixtures at 360 nm, the wavelength at which the difference in absorbance by the MESG and its phosphorylytically cleaved product is maximal. The change in absorbance at this wavelength is proportional to the P_i concentration. The initial activity, calculated from fitting the data shown in Figure 2a to either bi- (0.14 and 0.36 mol of ADP/mol of CF_1) or mono- (all other samples) exponential decays, approaches the steady-state activity as the ratio of ADP to CF_1-ϵ approaches 2 (Figure 2b). Thus, the loss in activity of the enzyme that

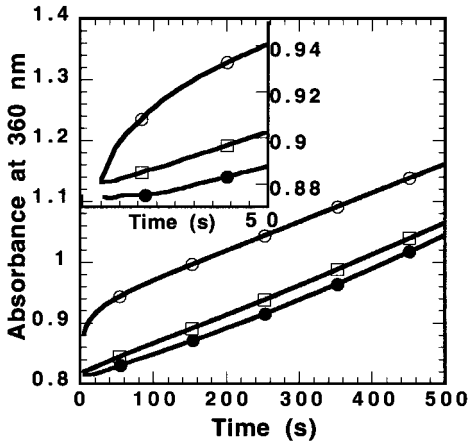

FIGURE 3: The effect of Mg^{2+} pretreatment on the activity of CF_1-ϵ containing tightly bound MgADP. Assay conditions were as described for Figure 2a, except that samples were incubated with 1 mM $MgCl_2$ for 8 min prior to initiation of activity. Aliquots of the samples were added directly to the assay mixtures. Only three of the runs are shown. The MgADP contents (mol/mol of CF_1) were: 0.14 (open circles), 0.95 (open squares), and 2.03 (closed circles). The inset shows the first 50 s of the reaction. The lowermost curve in the inset was offset for clarity. Data points were taken at 1 s intervals; the symbols are used to identify the curves.

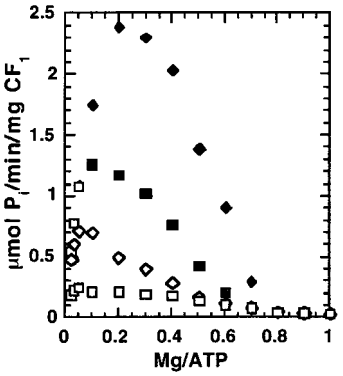

FIGURE 4: Activity of CF_1-ϵ with different [Mg^{2+}]. Assays contained 5 mM ATP, varying [Mg^{2+}], 200 μM MESG, 5 units of nucleoside phosphorylase, and 40 μg of CF_1-ϵ in 50 mM Tris-HCl (pH 8.0), 1 mL total volume. At each Mg^{2+} concentration activity was measured for 600 s. Activities were calculated from fits of the data. The activities at 10 s (solid diamonds), 30 s (filled squares), 60 s (open diamonds), and 600 s (open squares) are shown.

does not contain tightly bound Mg^{2+} is likely the result of the binding of Mg^{2+} from the assay mixture to bound ADP as well as the filling of empty sites with MgADP.

The incubation of the samples that contained varying amounts of tightly bound MgADP with free Mg^{2+} prior to assay also had dramatic effects on the MgATPase activity. In these samples, excess and loosely bound Mg^{2+} were not removed prior to assay. Pretreatment of nucleotide-depleted CF_1-ϵ with Mg^{2+} does not affect the initial or final MgATPase activity. Incubation of the enzyme samples that contained bound MgADP with Mg^{2+} further inhibits ATPase activity (Figure 3). The rapid, initial phase of the reaction is eliminated by the pretreatment when the stoichiometry of bound Mg ADP to CF_1 approaches 1 on a molar basis. In the sample that contained 0.95 mol of ADP/mol of CF_1, the steady-state rate of ATPase was evident throughout the time course of the reaction. The amount of inhibition increases with the amount of bound MgADP. As the MgADP content approaches 2 mol of MgADP/mol of CF_1-ϵ, the initial ATPase activity was lower than the steady-state rate assayed in the presence of 1 mM $MgCl_2$ and 5 mM ATP (Figure 3). This increase in activity with time is not seen in the absence of Mg^{2+} pretreatment (Figure 2a). Thus, the loose binding of Mg^{2+} to one or more sites potentiates the inhibition by bound MgADP. The lag in the actvity of the enzyme that contained 2 mol of MgADP/mol of CF_1 may indicate that Mg^{2+} binding to a site other than the two tight MgADP sites is involved. Activation in these samples probably results from removal of the loosely bound Mg^{2+} by chelation to ATP in the assay mixture.

The tight, noncatalytic MgATP sites as well as the tight MgADP sites were filled by overnight incubation of the enzyme in 50 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, and 5 mM ATP. Excess nucleotide and Mg^{2+} were removed by passage of the preparation through two consecutive Sephadex G-50 centrifuge columns. The sample was incubated with Mg^{2+} prior to assay. An increase in MgATPase activity with time similar to that detected in CF_1 that contained just tightly bound MgADP was observed (data not shown). Thus, the increase in activity of the MgADP-loaded enzyme cannot be the result of the binding of MgATP in the reaction mixture to the noncatalytic MgATP sites.

Varying the ratio of Mg^{2+} to ATP in the reaction mix also affects the change with time of the ATPase activity of CF_1-ϵ. The CF_1 preparation used in this experiment contained tightly bound ADP but no bound Mg^{2+}. At concentration ratios of Mg^{2+} to ATP of 0.02−0.1 there is a rapid increase of activity upon initiation, followed by a decrease that probably reflects the binding of Mg^{2+} together with ADP (Figure 4). At Mg^{2+}/ATP ratios from 0.2 to 0.7, activity starts at its maximum value and becomes inhibited. At concentrations of Mg^{2+}/ATP ratios greater than 0.8, the activity starts at the steady-state rate. The observed activation at low [Mg^{2+}] is likely the same activation observed by Milgrom et al. (*24*) and shown to correlate to binding of noncatalytic MgATP. The activation becomes too fast to see in the presence of greater than 0.96 mM MgATP in the medium.

In the hopes of determining whether noncatalytic MgATP binding was responsible for the observed activation, samples were prepared with and without prior loading of the noncatalytic MgATP sites. CF_1-ϵ was incubated overnight at room temperature in a solution of 50 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, and 5 mM ATP which results in the filling of N2 and N5 with MgATP, and N1 and N4 with MgADP. As a control, an aliquot of the same CF_1-ϵ was incubated for 30 min in 5 mM ADP. Excess nucleotide was removed by two consecutive Sephadex G-50 centrifuge columns. After removal of excess ADP, $MgCl_2$ was added to the ADP-loaded sample at a 2:1 molar excess of Mg^{2+} to CF_1-ϵ. This results in a sample in which N1 and N4 are filled with tightly bound MgADP. The activity of both samples was measured as a function of time using the nucleoside phosphorylase assay with 20 μg of CF_1-ϵ, 5 mM ATP, and 0.1 mM $MgCl_2$.

Neither sample showed the activation phase observed in samples that had not been preloaded with either MgADP or MgATP. In both cases the activity was dominated by the inhibition by the two tightly bound MgADP and had the same constant ATPase rate (data not shown). If the activation of the ATPase activity of Mg^{2+}-depleted CF_1-ϵ at relatively low

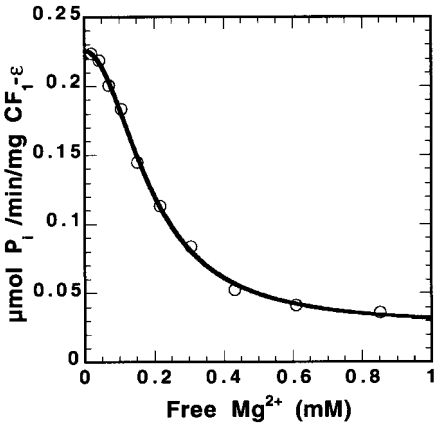

FIGURE 5: Effect of free Mg^{2+} on steady-State MgATPase. Steady-state ATPase versus free Mg^{2+} concentration of the reaction mixture. Data are from Figure 4. Results of the fit are $k = 0.033 \pm 0.008$ mM^2, $n = 2.06 \pm 0.14$, $V_{\Delta max} = 0.200 \pm 0.006$, and $V_{min} = 0.025 \pm 0.004$.

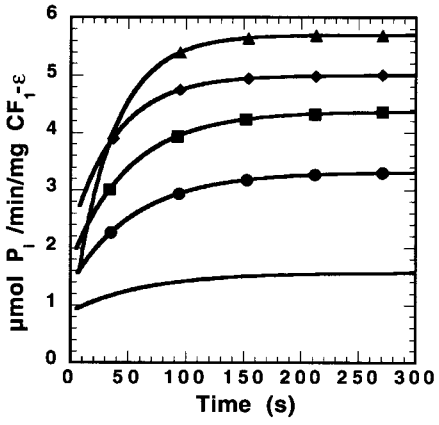

FIGURE 6: Activity of CF_1-ϵ with different [Ca^{2+}]. Assays contained 5 mM ATP, varying [Ca^{2+}], 200 μM MESG, 5 units of nucleoside phosphorylase, and 4.0 μg of CF_1-ϵ in 50 mM Tris-HCl (pH 8.0), 1 mL total volume. Ca^{2+} concentrations were as follows: 1.0 mM (solid line), 2.0 mM (circles), 3.0 mM (squares), 4.0 mM (diamonds), and 5.0 mM(triangles). Activities were calculated from fits.

MgATP concentrations is the result of noncatalytic MgATP binding, then this effect is completely overcome by the inhibition from tightly bound MgADP.

The steady-state rate also changes as the Mg/ATP ratio is varied. Plotting the steady-state rate versus the calculated concentration of free Mg^{2+} for data in the range 0.1−1 Mg^{2+}/ATP with 5 mM ATP (Figure 5) yields a sigmoidal curve which was fit to the equation

$$V = V_{\Delta max}(1 - ([Mg^{2+}]^n)/(k + [Mg^{2+}]^n)) + V_{min}$$

where V ≡ steady-state activity, $V_{\Delta max} \equiv V_{max} - V_{min}$, and [$Mg^{2+}$] is the concentration of free Mg^{2+} in the reaction mix. Nonlinear least-squares fitting gives $k = 0.033 \pm 0.008$ mM^2, $n = 2.1 \pm 0.1$, $V_{\Delta max} = 0.200 \pm 0.006$ μmol of P_i/min^{-1} (mg of CF_1-ϵ)$^{-1}$, and $V_{min} = 0.025 \pm 0.004$. These data are consistent with free Mg^{2+} inhibiting by binding cooperatively to at least two sites.

If site N1 is a catalytic site, the ADP initially in this site must come off before the enzyme is active. Loading the N1 site of CF_1-ϵ with TNP-ADP causes a drop in the initial ATPase activity at 5 mM ATP and 1 mM $MgCl_2$ from 2.4 to 0.9 μmol of P_i min^{-1} (mg CF_1-ϵ)$^{-1}$, where there was 1.49 mol of ADP in 1 mol of the initial CF_1-ϵ, and 0.66 mol of ADP and 1.1 mol of TNP-ADP after loading. TNP-ADP binds more tightly to N1 than ADP, and the loss of activity may be due to the increased difficulty of emptying the N1 site. Both the TNP-ADP loaded and the unloaded sample reach the same steady-state rate. The $T_{1/2}$ to reach steady-state activity is 30 s and the $T_{1/2}$ for complete exchange is 23 s, so the enzyme is essentially free of TNP-ADP when it has reached steady state.

In contrast to that of TNP-ADP, the release of ADP bound to N1 does not appear, on the surface, to result in activation of CF_1-ϵ. Within the initial time resolution of the assay, the enzyme with ADP bound to N1 and sufficient MgATP as substrate starts in its most active state and the activity decreases rapidly. However, there is a sufficient fraction of the enzyme that has site N1 empty to account for this apparent discrepancy. The activity is first observed at about 5 s after hand mixing of the contents of the cuvette. For the case TNP-ADP exchange observed using stopped-flow fluorescence in the presence of 2 mM $MgCl_2$ and 5 mM ATP in a typical measurement, 25% of bound TNP-ADP is released in the first 5 s. The exchange of ADP is probably faster than that of TNP-ADP. When MgADP inhibition of the MgATPase activity of CF_1-ϵ is obviated by the presence of oxyanions in the reaction mixture, the rates of ATP hydrolysis under these conditions range from 10 to 20 μmol min^{-1} mg^{-1}. Thus, there is easily enough of the enzyme free of nucleotide at site N1 to account for the relatively low initial activity of about 0.8 μmol of P_i min^{-1} (mg CF_1-ϵ)$^{-1}$ observed under the same conditions.

Lags in the initial activity of CF_1 were previously investigated using CaATPase activity (*10*) or the MgATPase activity in the presence of sulfite (*11*, *12*). Steady-state CaATPase and sulfite-MgATPase activities are much higher than that of MgATPase in the absence of oxyanions. In the presence of Ca^{2+}, initial ATPase activities of CF_1-ϵ that does not contain bound Mg^{2+} are in the range of those of MgATPase. In contrast to MgATPase, the rate of CaATPase increases with time rather than decreases. This increase in activity follows a single-exponential decay. The steady-state CaATPase rates are 10−20 times that of the MgATPase for a divalent cation to ATP ratio of at least 0.4 (Figure 6). As for MgATPase, the continuous spectrophotometric P_i assays of CaATPase activities were found to give results similar to those of the malachite green P_i assay.

Comparison of the CaATPase activity with exchange from N1 was done with TNP-ADP-loaded samples. The presence of bound TNP-ADP lowers the initial activity, as it did for MgATPase, and increases the time it takes to the reach steady state without affecting the steady-state rate (Table 1). As for MgATPase, the enzyme is essentially free of TNP-ADP at the steady state. Again the initial exchange rate is much slower than the initial activity. In this case the N1 site is emptied faster than the rate at which the activity increases, as shown by comparison of the half-time of TNP-ADP release and that of the increase in CaATPase activity with time.

When CF_1-ϵ is depleted of its endogenous ADP there is no longer a large difference between the initial and final CaATPase activities. Instead the initial activity of CF_1-ϵ with 0.17 mol of ADP/mol of CF_1-ϵ starts at 80% of the final

Table 1: CaATPase Activity Compared with Exchange Rates[a]

	activity			exchange	
	A_{min} (μmol min^{-1} mg^{-1})	$T_{1/2}$ (s)	A_{max} (μmol min^{-1} mg^{-1})	V_{max} (nmol min^{-1} mg^{-1})	$T_{1/2}$ (s)
2 mM Ca,B	1.3	45	2.6		
2 mM Ca, L	0.5	108	2.1	7.8	43
5 mM Ca, B	1.6	33	5.1		
5 mM Ca, L	0.9	110	4.9	11.1	24

[a] Activity values were calculated from the nonlinear least-squares fit of a single-exponential decay to spectrophotometric activity assays that contained 5 mM ATP, 2 or 5 mM $CaCl_2$, as indicated, 200 μM MESG, 5 units of nucleoside phosphorylase, and 2 μg of CF_1-ϵ in 50 mM Tris-HCl (pH 8.0), 1 mL total volume. Exchange values were calculated from stopped-flow fluorometric data fit to the sum of three decaying exponentials. A_{min} is the initial, lowest rate of CaATPase, and A_{max} is the final steady-state rate presented with the half-time for the activity to rise to the steady state. V_o is the initial, fastest rate of TNP-ADP exchange, presented with the half time for total exchange. Sample B is CF_1-ϵ prepared with its endogenous bound ADP. Sample L is from the same batch of CF_1-ϵ, loaded with 1.0 mol of TNP-ADP/mol of CF_1-ϵ.

steady-state rate. Adding back ADP to the sample prior to initiating the activity restores the activation phase seen in samples containing endogenous ADP (Figure 7a). The lower steady-state activity of the ADP-treated sample may be attributed to Mg^{2+} contamination of the ADP.

Pretreatment of the nucleotide-depleted sample with Mg^{2+} does not restore the activation phase but does cause a drop of 45% in the steady-state CaATPase activity. In this case the reaction mix contains 44 μM $MgCl_2$ carried over from the pretreatment. When a CF_1-ϵ sample containing endogenous ADP is added to a CaATPase reaction mixture containing 44 μM $MgCl_2$, there are two distinct effects (Figure 7b). First, the rate at which the enzyme is activated increases. Initial exchange rates are faster when Mg^{2+} is present in the reaction mix (*3*), providing further evidence that activation of the ATPase activity is coupled to exchange from N1. Second, at longer times the CaATPase activity becomes increasingly more inhibited as MgADP binds to the enzyme. The steady state was not reached at the end of the 600 s assay. Thus, as in the case of the MgATPase data with nucleotide-depleted CF_1-ϵ, MgADP inhibition occurs much faster when the N1 and N4 sites are initially empty.

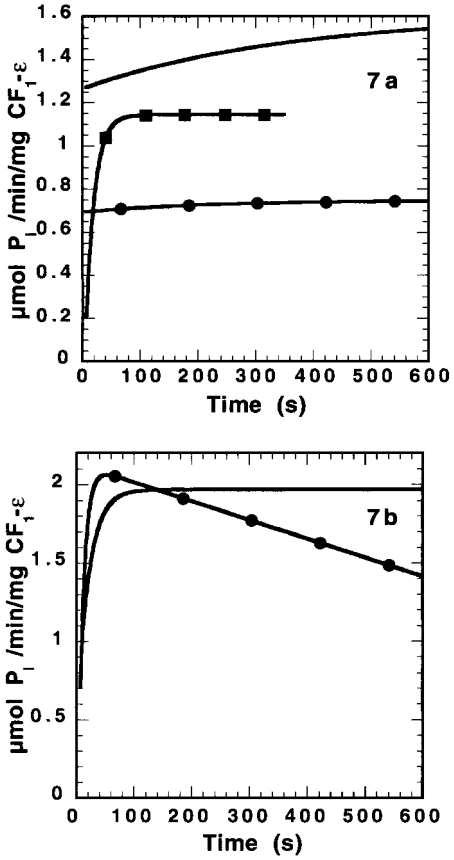

FIGURE 7: The effect of tightly bound ADP on CaATPase activity. Assays contained 5 mM ATP, 2 mM Ca^{2+}, 200 μM MESG, 5 units of nucleoside phosphorylase, and 4.0 μg of CF_1-ϵ in 50 mM Tris-HCl (pH 8.0), 1 mL total volume. (a) Nucleotide-depleted CF_1-ϵ with 0.24 mol of nucleotide bound/mol of CF_1-ϵ (solid line). Nucleotide-depleted CF_1-ϵ pretreated with 1 mM $MgCl_2$ for 11.5 min prior to assay; reaction mix contained an additional 44 μM $MgCl_2$ carried over from the incubation (circles). Nucleotide-depleted CF_1-ϵ pretreated with 1 mM ADP for 5 min prior to assay; reaction mix contained an additional 44 μM $MgCl_2$ (squares). (b) CF_1-ϵ with 1.49 mol of bound ADP/mol of CF_1-ϵ (solid line). Same sample, reaction mix contained an additional 44 μM $MgCl_2$ (circles). Activities were calculated from fits.

DISCUSSION

The interactions of CF_1 with MgADP and MgATP result in complex effects on the MgATPase activity of the enzyme. All six nucleotide-binding sites likely affect MgATPase activity. Binding of MgATP to the tight, noncatalytic sites (N2 and N5) stimulates the initial ATPase activity, as suggested by Milgrom et al. (*24*). This activation may be observed when CF_1 that does not contain bound MgATP or bound MgADP is added to assay mixtures that contain low concentrations of MgATP. At concentrations of MgATP of 1 mM or higher, the activation is complete within 5 s.

The binding of MgADP to the tight ADP sites (N1 and N4) inhibits MgATPase activity. This inhibition overrides the activation by the binding of MgATP to the noncatalytic sites. Although others have suggested that a single site is involved in MgADP inhibition (see, for example, ref *6*), our data show that the tight binding of more than one MgADP per CF_1 is necessary to abolish the initial, fast phase of ATPase activity. The rate at which MgATPase activity is lost depends on the initial ADP content of the enzyme. The filling of one tight ADP site actually slows the decay of activity. It seems likely that MgADP binds more rapidly to an empty site than does Mg to ADP that already occupies the site. The higher-affinity ADP site is probably N4.

Even when each of the tight nucleotide-binding sites is filled with either MgADP or MgATP, CF_1 will hydrolyze MgATP at a steady-state rate of about 200 nmol min^{-1} mg^{-1} at 25 °C in 5 mM ATP and 2 mM Mg^{2+}. Increasing the free Mg^{2+} concentration in the assay mixture inhibits activity further. The curve relating steady-state activity to free Mg^{2+} concentration suggests that two sites interact cooperatively. Since incubation of the nucleotide-depleted enzyme with Mg^{2+} does not affect ATPase activity, it seems likely that the inhibition by Mg^{2+} is mediated by its interactions with nucleotides and CF_1. The two interacting sites could be the dissociable sites N3 and N6. The binding of MgADP to these sites could fix the enzyme in a less active form, perhaps by retarding the release of product MgADP.

Comparison of the MgATPase activity of the enzyme with the rate at which TNP-ADP exchanges from site N1 in CF_1-ϵ shows that the initial release of bound TNP-ADP is much slower than the rate of catalytic turnover, even when compared to the very slow steady-state rate. The discrepancy can be explained if there is an active fraction without bound TNP-ADP that is responsible for the observed activity. When CF_1-ϵ is added to the reaction mixture the active fraction is reduced by binding of the MgADP being formed from ATP hydrolysis.

Extrapolation of Figure 2b suggests that the steady-state MgATPase is achieved with CF_1-ϵ that has both N1 and N4 filled with MgADP. Murataliev (*8*) suggested that CF_1-ϵ with two tightly bound MgADP had both an active and an inactive form, and that free Mg^{2+} inhibited by shifting the balance between the two forms. Thus, binding of Mg^{2+} to N3 and N6 may be fixing the MgADP-loaded enzyme in its inactive form. Or it may be that at the steady state there is still some fraction of the enzyme which does not have MgADP in both N1 and N4. In this case it seems possible that free Mg^{2+} assists in filling the remaining empty sites with MgADP.

Where a substantial fraction of the enzyme must be active, such as for CaATPase, the activity increases as TNP-ADP is released from N1 (Table 1). Though it was not possible to observe the initial exchange of bound ADP for medium nucleotide, the CaATPase of CF_1-ϵ with ADP rather than TNP-ADP in N1 also had an activation phase (Figure 6). In the absence of bound ADP there was no lag in the onset of ATPase activity, demonstrating that bound ADP is responsible for the low initial activity in CaATPase of samples containing endogenous ADP.

Our results shed new light on the discrepancy in the results of Larsen et al. (*11*) and Du and Boyer (*12*) in comparing activities with nucleotide exchange rates. The samples of Du and Boyer (*12*) were incubated in 5 mM $MgCl_2$ without added nucleotide. ATP and sulfite were added together to initiate the ATPase reaction. A substantial decrease in free Mg^{2+} would result, and this decrease may have been responsible for some of the observed increase in ATPase activity. We showed (Figure 3) that the incubation of CF_1-ϵ that contains more than one mole of tightly bound MgADP with Mg^{2+} induces a lag in the onset of MgATPase activity. ATP in the assay mixture, which is present in excess of Mg^{2+}, likely slowly removes some of the bound Mg^{2+} resulting in activation. In both papers the CF_1 studied was bound to thylakoids and there was a significant difference in how the samples were loaded with [^{3}H]ADP. Larsen et al. illuminated the thylakoids during labeling which causes the release of one tightly bound ADP (*28*). Du and Boyer labeled their sample in the dark. It is possible that the ADP which is released during illumination is actually in site N4 and that Du and Boyer measured exchange from a site different than that studied by Larsen et al.

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BI982027P