Two Tight Binding Sites for ADP and Their Interactions during Nucleotide Exchange in Chloroplast Coupling Factor 1†

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ABSTRACT: Chloroplast coupling factor 1 (CF_1) deficient in its ϵ subunit was loaded with 2′(3′)-*O*-trinitrophenyl-ADP (TNP-ADP), and the release of tightly bound TNP-ADP was followed as a decrease in fluorescence. TNP-ADP could be exchanged for medium ADP, ATP, MgADP, and MgATP. The preferred substrate for exchange was MgADP, particularly in the presence of P_i. One nucleotide binding site contained ADP which was not displaced during TNP-ADP loading. When Mg^{2+} was bound at this site, complete exchange of bound TNP-ADP for medium nucleotide was prevented. This tightly bound MgADP was removed by incubation of the enzyme with EDTA. Tightly bound TNP-ADP was removed by high concentrations of sulfite, sulfate, or P_i in the absence of medium nucleotide and free Mg^{2+}, regardless of the bound Mg^{2+} content of the enzyme. Sulfite and P_i, in the presence of medium nucleotide and Mg^{2+}, enabled complete exchange of tightly bound TNP-ADP. The combination of Mg^{2+} and sulfite, or Mg^{2+} and P_i, caused exchange of tightly bound ADP from two different sites. These results suggest that both sites exchange when the enzyme is fully active, and that at least three sites are likely to participate in catalysis.

The synthesis of ATP in chloroplasts is catalyzed by ATP synthase (CF_1-CF_o),[1] and is driven by an electrochemical proton gradient across the thylakoid membrane. CF_1 contains the nucleotide binding sites of the complex, and, released from the membrane, it catalyzes ATP hydrolysis rather than synthesis (McCarty, 1992). ATP hydrolysis by CF_1 is often studied to provide insight into ATP synthesis.

CF_1 is believed to contain six binding sites for adenine nucleotides (Girault et al., 1988), not all of which participate directly in catalysis. In a proposed model (Boyer, 1989), at least two catalytic sites alternate properties. One of these sites is highly dissociable, while bound nucleotide is not removed from the other site by passage through centrifuged gel filtration columns, or by dialysis. As an operational definition, the dissociable site is termed "loose", and the other is termed "tight". The binding of nucleotide to a loose site causes that site to become a tight binding site, while a second site which had contained tightly bound nucleotide becomes dissociable. Catalysis occurs while the substrate is tightly bound. This mechanism is referred to as the binding change model.

Shapiro and McCarty (1990) showed that the alternating of tight and loose sites was induced by nucleotide binding, and would occur even in the absence of enzymatic activity. This switching of binding properties could be observed for ATP and the nonhydrolyzable ATP analog adenylyl β,γ-imidodiphosphate (AMP-PNP) only in the presence of added Mg^{2+}. After the removal of free nucleotide from the medium, the enzyme gradually relaxed back to its original state with the tightly bound nucleotide located at the same site in each enzyme.

Four tight binding sites on CF_1 have been characterized (Shapiro et al., 1991). Two of these sites only bind ATP when loading is done in a buffer containing 1 mM $MgCl_2$. These two sites did not fill in the presence of 2 mM EDTA. ATP bound to these sites was not released or hydrolyzed, even after extensive turnover of the enzyme. These sites were emptied by the precipitation of the enzyme with ammonium sulfate in the presence of EDTA. The other two sites will bind ADP or ATP tightly, and they do not require Mg^{2+} to fill. One of these two sites has been previously characterized as catalytic (Bruist & Hammes, 1981). Evidence has been presented suggesting that the second Mg^{2+}-independent tight binding site is also catalytic (Shapiro et al., 1991). These two sites will exchange their contents for medium nucleotide with one nucleotide from the medium binding for each nucleotide released.

The exchange of tightly bound nucleotide with medium nucleotide in beef heart mitochondrial F_1 was examined by Nalin and Cross (1982). F_1 was loaded with a single tightly bound [^{3}H]AMP-PNP, which did not dissociate from F_1 until nucleotide was added to the medium. Exchange was observed for ADP, ATP, and AMP-PNP, and found to be biphasic in all cases. The exchange of bound [^{3}H]AMP-PNP for medium ADP was enhanced by the addition of P_i. Pyruvate kinase and phosphoenolpyruvate were added to prevent the accumulation of ADP, and as the concentration of the ATP regeneration system increased, the exchange rate in the presence of medium ATP decreased, leading to the conclusion that only ADP caused exchange in F_1. All of the buffers used in these experiments contained Mg^{2+}.

Prior exposure of CF_1 loaded with ADP to Mg^{2+} was shown to inhibit the exchange of tightly bound ADP for medium nucleotide (Feldman & Boyer, 1985). Inhibition was gradually relieved when EDTA was added to the sample.

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[1] Abbreviations: CF_1-CF_o, chloroplast ATP synthase; CF_1, chloroplast coupling factor 1; AMP-PNP, adenylyl β,γ-imidodiphosphate; TNP-ADP, 2′(3′)-*O*-trinitrophenyl-ADP; HPLC, high-pressure liquid chromatography; AA, atomic absorption.

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Free Mg^{2+} added simultaneously with the ATPase reaction medium likewise inhibited the exchange of tightly bound ADP. The presence of P_i in the activity assay medium overcame the inhibition caused by prior incubation of the enzyme with Mg^{2+}. The slow exchange induced by Mg^{2+} correlated with a slow onset of ATPase activity (Feldman & Boyer, 1985). The presence of 100 mM P_i in solution prevented inhibition of both exchange and activity by Mg^{2+}. The ADP site studied was determined to be catalytic, with the exchange of tightly bound ADP from this site being the first step in the binding change mechanism.

Komatsu-Tokaki (1984) determined that millimolar concentrations of P_i enhanced the release of tightly bound ADP from thylakoid bound CF_1 in the dark, even in the absence of medium nucleotide. Released ADP was associated with that site on CF_1 which is emptied upon illumination of the thylakoids. Sulfite has also been shown to overcome Mg^{2+}-induced inhibition of both the ATPase activity of thylakoid-bound CF_1 and the exchange of tightly bound ADP from thylakoid-bound CF_1 (Larson et al., 1989; Du & Boyer, 1990).

2′(3′)-*O*-Trinitrophenyl-ADP (TNP-ADP) is a fluorescent variant of ADP. TNP-ADP and TNP-ATP were used by Grubmeyer and Penefsky in 1981 to study nucleotide binding to mitochondrial F_1, and TNP-ATP was shown to be a substrate for F_1 hydrolysis (Grubmeyer & Penefsky, 1981). TNP-ADP has also been used to study nucleotide binding in CF_1 (Musier & Hammes, 1988; Mills & Richter 1991). The fluorescence of TNP-ADP is strongly enhanced upon binding to CF_1. The relationship between the TNP-ADP fluorescence and the fraction of TNP-ADP in a solution with CF_1 that is bound to the enzyme has been shown to be linear (Soteropoulos et al., 1994).

This change in fluorescence intensity provides a convenient method for observing either the binding of ADP to CF_1 or the release of tightly bound ADP from CF_1. The binding affinity of CF_1 for TNP-ADP is much greater (Murataliev & Boyer, 1994) than the affinity for ADP. Therefore, rate constants obtained for exchange of tightly bound TNP-ADP may not be directly comparable to those for tightly bound ADP. However, this method remains useful for examining the rates of exchange under different conditions.

In this work, the ability to continuously monitor TNP-ADP binding has been used in combination with precise measurements of the bound nucleotide content of CF_1 by high-pressure liquid chromatography, and with measurements of the bound Mg^{2+} content by atomic absorption spectroscopy. This has made it possible to examine the effects of Mg^{2+}, sulfite, and phosphate on the exchange of bound nucleotide from CF_1, and to clarify some of the interactions between different sites of tight nucleotide binding on the enzyme.

MATERIALS AND METHODS

CF_1 was prepared from market spinach using the procedure described by Shapiro and McCarty (1990), with modifications described by Soteropoulos et al. (1994). An additional modification was the replacement of the Zetaprep 100 QAE-cellulose disk (CUNO, Inc.) with two Productiv QM Pre-Pack column cartridges of 90 mm diameter and 50 mm depth. The column was equilibrated with a buffer of 20 mM HEPES, 1 mM EDTA, and 0.5 mM ATP, pH 7.3 (HEA buffer). After protein loading, the column was washed with a 2 L linear gradient of 0−70 mM $(NH_4)_2SO_4$ in HEA buffer. CF_1 was eluted from the column with HEA + 250 mM $(NH_4)_2SO_4$. Contaminating ribulosebisphosphate carboxylase/oxygenase was removed by immunoaffinity chromatography as described by Soteropoulos et al. (1992). CF_1-ϵ was prepared according to the method of Richter et al. (1984), with modifications described in Soteropoulos et al. (1992).

CF_1-ϵ was desalted by passage through two consecutive 3 mL Sephadex G-50 centrifuge columns. Samples were prepared in a buffer of 50 mM Tris-HCl, pH 8.0, 50 mM NaCl (TN buffer) which was passed through a column of Chelex 100 resin to remove residual Mg^{2+}. ADP and ATP stock solutions were prepared at pH 7.0 and also passed through Chelex. Solutions of NaH_2PO_4, Na_2SO_3, and Na_2SO_4 were used at pH 6−7. After desalting, CF_1-ϵ was stored in plastic, as was the chelated buffer to prevent Mg^{2+} contamination.

For exchange studies, CF_1-ϵ was loaded with TNP-ADP. Desalted CF_1-ϵ at concentrations between 20 and 50 mg/mL in TN buffer was incubated with 500 μM TNP-ADP for about 40 min. Excess and loosely bound TNP-ADP were removed by passage of the enzyme through two consecutive Sephadex G-50 centrifuge columns as described above. In some instances, the TN buffer contained 2−10 mM EDTA as specified in the description of the individual experiment. The extent of TNP-ADP loading was determined by the absorbance of tightly bound TNP-ADP at 418 nm where $\epsilon = 2.51 \times 10^4$ mol^{-1} cm^{-1} (Cerione & Hammes, 1982). Samples were measured against a blank of CF_1-ϵ at the same concentration which had not been loaded with TNP-ADP. The concentration of CF_1-ϵ was determined by a modified Lowry method (Lowry et al., 1951).

Exchange of tightly bound TNP-ADP was followed by measuring the TNP-ADP fluorescence. The fluorescence of TNP-ADP has been shown to increase linearly with the amount that is tightly bound to CF_1-ϵ (Soteropoulos et al., 1994). 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 the addition of free nucleotide into the sample medium.

The content of tightly bound ADP or ATP in samples of CF_1-ϵ was determined by ion-pairing high-pressure liquid chromatography (HPLC) by the procedure of Moal et al. (1989). Samples were prepared as described in Soteropoulos et al. (1994). A Beckman 342 gradient liquid chromatograph with either an Ultrasphere ODS 4.6 mm × 15 cm reversed-phase column or a Chromega chromegabond mc18/5 4.6 mm × 10 cm reversed-phase column was used.

Atomic absorption (AA) was performed with a Perkin-Elmer Model 4000 flame AA spectrometer. Protein samples were prepared by addition of an equal volume of 2 N nitric acid (trace metal grade from Sigma) to an enzyme preparation of 4-6 mg/mL. Samples were centrifuged for 30 min in a microcentrifuge and the supernatants collected. Magnesium standards and nucleotide solutions for AA were prepared in 1 N nitric acid.

Table 1: Removal of ADP and Mg^{2+} by EDTA[a]

	mol of Mg^{2+}/ mol of CF_1-ϵ	mol of nucleotide/mol of CF_1-ϵ			
		ADP	ATP	TNP-ADP	total
sample prior to TNP-ADP loading	0.43	1.26	0.12	0	1.37
TNP-ADP-loaded sample after EDTA treatment for					
15 min	0.42	0.81	0.18	0.83	1.82
30 min	0.41	0.79	0.17	0.86	1.82
60 min	0.36	0.75	0.14	0.86	1.75
120 min	0.28	0.66	0.12	0.80	1.58
180 min	0.31	0.71	0.11	0.86	1.68
240 min	0.24	0.65	0.11	0.81	1.57

[a] CF_1-ϵ loaded with TNP-ADP was incubated with TN buffer + 5 mM EDTA. Samples were then passed through two consecutive Sephadex G-50 centrifuge columns equilibrated with TN buffer.

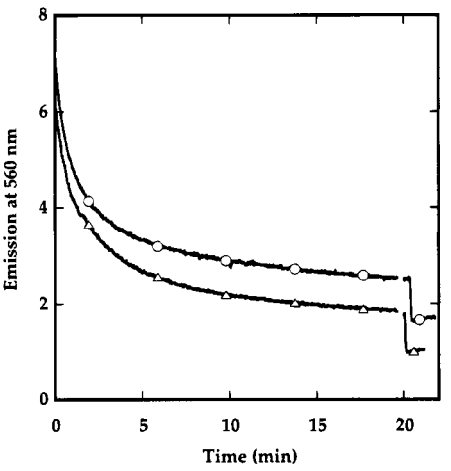

FIGURE 1: Exchange of tightly bound TNP-ADP for medium nucleotide. (○) ADP or (△) ATP was added to CF_1-ϵ which has 1.15 mol of tightly bound TNP-ADP/mol of CF_1-ϵ in TN buffer. The final concentration of CF_1-ϵ was 0.5 μM, and the final nucleotide concentration was 5 mM for both scans. At the end of each scan, SDS was added to 0.5%. The greater fluorescence of the ADP trace is the result of a fluorescent contaminant in ADP. Data points were taken every 2 s. The symbols mark only a few of the data points and identify the different scans.

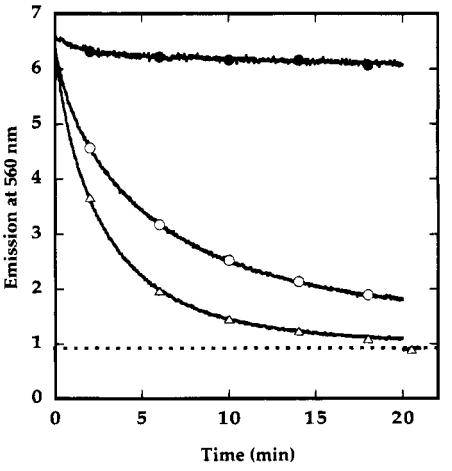

FIGURE 2: Effect of EDTA upon exchange. (○) ATP was added to 5 mM in 0.5 μM CF_1-ϵ which had 0.75 mol of tightly bound TNP-ADP/mol of CF_1-ϵ in TN buffer, or (△) the same batch of TNP-ADP-loaded CF_1-ϵ which had been incubating in a buffer of TN + 5 mM EDTA for 3.5 h. (●) Base line scan. The dotted line at the bottom of the graph indicates the base line obtained by the addition of SDS to 0.5%.

RESULTS

CF_1 used in these studies was depleted of its ϵ subunit which allows for more rapid exchange (Soteropoulos et al., 1994). As prepared, CF_1-ϵ contains between 1.2 and 1.6 mol of tightly bound ADP. Loading CF_1-ϵ with TNP-ADP in TN buffer produces an enzyme which has close to 1 mol of TNP-ADP and 1 mol of ADP tightly bound per mole of enzyme (Table 1). Tightly bound TNP-ADP dissociated slowly, with a half-time on the order of 1 day. Upon addition of ADP or ATP to the medium, the tightly bound TNP-ADP exchanged with the medium nucleotide. This exchange was easily monitored as a decrease in fluorescence intensity of TNP-ADP as it was released into solution (Figure 1).

The fluorescence decrease was found to be biphasic in all cases, and fit the equation:

$$F(t) = F_1 \exp(-k_1 t) + F_2 \exp(-k_2 t) + F_{min} \quad (1)$$

where F = the sample fluorescence at time t and F_{min} is the minimum fluorescence which would be obtained at $t = \infty$. The resulting fits have correlation coefficients R^2 of 0.999 in the case of both scans presented in Figure 1, and fits of other scans were similarly accurate. The residuals show a slight systematic fluctuation that is about 2% of the total ΔF, particularly in the first 3 min. While this may be evidence that the process is more complicated, it could easily be the result of a finite mixing time and a small nonlinear photobleaching effect which proved too inconsistent to subtract accurately. A typical example of a sample base line can be seen in Figure 2. From the data in Figure 1, initial velocities of TNP-ADP exchange for ADP and ATP were 0.78 and 0.76 μM TNP-ADP/min, respectively, as calculated from fitting the data to eq 1.

Unlike observations made in beef heart mitochondrial F_1 (Nalin & Cross, 1982), the exchange of tightly bound TNP-ADP for medium ATP cannot be attributed to contaminating ADP. The ATP stock solution contained a 1−2% ADP contamination; however, both the rate and the extent of exchange are noticeably less for this concentration of medium ADP. As an additional test, exchange was observed for medium AMP-PNP which had been purified by HPLC, and was free of ADP. Exchange for medium AMP-PNP was slightly slower than that for ATP or ADP and consisted of two phases with rates and extents similar to those of exchange for ADP and ATP (data not shown).

The exchange of TNP-ADP for medium ADP or ATP was not complete. At the end of the fluorescence scan SDS was added at $^1/_{20}$th of the total volume to a final concentration of 0.5%. This treatment results in the release of all bound nucleotide from the enzyme. Upon addition of SDS, an additional drop in fluorescence was seen (Figure 1). In both cases, the difference between the calculated F_{min} and the base

line obtained by SDS addition indicates that approximately 15% of the total bound TNP-ADP was not exchanged.

TNP-ADP-loaded CF_1-ϵ was incubated in 5 mM EDTA, which has been shown to reverse inhibition by Mg^{2+} (Feldman & Boyer, 1985). After an incubation of several hours, exchange was initiated by the addition of ATP (Figure 2). The calculated F_{min} for ATP exchange in the EDTA-treated sample was 1.06 V, as opposed to 1.60 V obtained from the same preparation of TNP-ADP-labeled CF_1-ϵ in the absence of EDTA. Addition of 0.5% SDS brought the fluorescence to 0.92 V in both cases. EDTA incubation also enhanced the extent of exchange of bound TNP-ADP for medium ADP.

The Mg^{2+} content of TNP-ADP-loaded CF_1-ϵ incubated with EDTA was measured by AA (Table 1). After 4 h of incubation in EDTA, the enzyme lost 0.18 mol of Mg^{2+}/mol of CF_1-ϵ. The EDTA-induced removal of Mg^{2+} occurred slowly, and the loss was approximately linear with a rate of 0.78 ng of Mg^{2+}/min. EDTA incubation also resulted in the loss of 0.16 mol of tightly bound ADP/mol of CF_1-ϵ, nearly identical to the loss of bound Mg^{2+}. The amount of tightly bound TNP-ADP did not decrease. Incubation of the enzyme in EDTA results in the removal of MgADP, which increases the extent of TNP-ADP exchange. The inhibition of complete TNP-ADP exchange shown in Figures 1 and 2 is the result of Mg^{2+} that is at a different site than that to which TNP-ADP is bound. In overcoming this inhibition, EDTA removes ADP as well as Mg^{2+}. The Mg^{2+} content of several different preparations of CF_1-ϵ was measured and found to vary from 0.3 to 0.6 mol of Mg^{2+}/mol of CF_1-ϵ.

In addition to Mg^{2+} present in CF_1-ϵ , Mg^{2+} contaminates ADP and ATP stock solutions. The ATP stock was found to have 340 ppm of Mg^{2+} by mole fraction. The ADP came from different lots, one of which had 3970 ppm of Mg^{2+} and the other, 570 ppm. In the case of the ADP with the higher Mg^{2+} content, elevated F_{min} values were observed. Solutions of ADP and ATP were passed through a 1.5 cm diameter column of 3 g dry weight of Chelex 100 resin. Although Mg^{2+} was not removed by the Chelex column, nucleotide which had received this treatment gave faster exchange rates. All nucleotide stocks used were Chelex-treated except for the TNP-ADP used to load the enzyme.

CF_1-ϵ loaded with TNP-ADP was incubated with substoichiometric amounts of Mg^{2+} for 40 min before exchange was initiated by the addition of 5 mM ATP (Figure 3). As the amount of Mg^{2+} was increased from 25 nM to 500 nM with 500 nM of CF_1-ϵ, V_0, the rate of exchange at $t = 0$, decreased from 1.9 to 1.1 V/min and F_{min} increased from 1.7 to 2.4 V. Inhibition was observed for even the lowest amounts of Mg^{2+} used. Preincubation of the prepared sample with Mg^{2+} was required for strong effects of low concentrations of Mg^{2+} on ATP exchange. Measurements made with Mg^{2+} concentrations above 0.5 μM show that the inhibition continues to increase. The same effect was also observed with exchange for ADP.

No change was observed in the relative extents of the fast and slow phases, although the total extent of exchange decreased with increasing Mg^{2+} concentration (Figure 2). Nor were the relative extents of the slow and fast phases changed by the removal of MgADP during the EDTA incubation presented in Table 1. Thus, the fact that exchange consists of a fast and a slow phase is not the result of Mg^{2+}

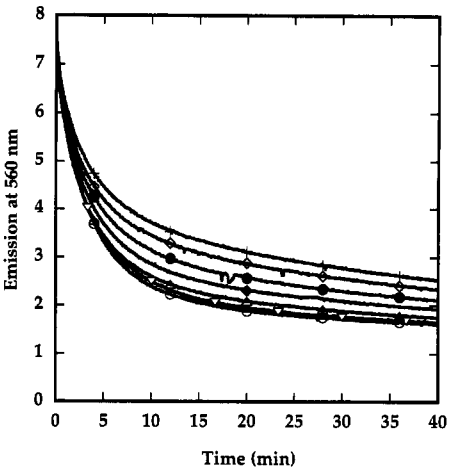

FIGURE 3: Effect of the addition of Mg2+ on exchange. 5 mM ATP was added to 0.5 μM CF_1-ϵ with 1.07 mol of tightly bound TNP-ADP/mol of CF_1-ϵ in TN buffer. The enzyme was loaded with TNP-ADP in a buffer of TN + 10 mM EDTA and returned to TN buffer by passage through two consecutive Sephadex G-50 centrifuge columns. (○) CF_1-ϵ prepared in TN buffer; (▽) CF_1-ϵ incubated for 40 min in TN + 25 nM $MgCl_2$; (△) TN + 100 nM $MgCl_2$; (♦) TN + 200 nM $MgCl_2$; (●) TN + 300 nM $MgCl_2$; (◇) TN + 400 nM $MgCl_2$; (+) TN + 500 nM $MgCl_2$.

Table 2: Exchange of TNP-ADP for MgADP and MgATP[a]

	5 mM ADP		4.75 mM ATP	
exchange initiated with nucleotide plus	V_0 (V min^{-1})	extent (V)	V_0 (V min^{-1})	extent (V)
nucleotide alone	3.77	4.85	1.47	4.76
200 μM Mg^{2+}	5.79	4.09	2.11	4.30
500 μM Mg^{2+}	7.57	3.70	1.95	4.12
200 μM Mg^{2+} added prior to ADP addition	0.28	2.41		

[a] Exchange was initiated by a mixture of ADP or ATP, and Mg^{2+} added to 0.5 μM CF_1-ϵ with 1.03 mol of tightly bound TNP-ADP/mol of CF_1-ϵ (ADP samples) or 0.75 mol of tightly bound TNP-ADP/mol of CF_1-ϵ (ATP samples) in TN buffer.

bound to the enzyme, or of the presence of tightly bound ADP in a site different from that of the bound TNP-ADP.

If TNP-ADP were to bind to more than one type of binding site, this might account for the biphasic kinetics. To examine this possibility, CF_1-ϵ was loaded with TNP-ADP from 0.21 to 0.76 mol of TNP-ADP/mol of CF_1-ϵ. Neither the rate constants nor the relative extents of the two phases were dependent on the labeling stoichiometry, making it unlikely that the two phases result from exchange from two different sites.

In the case of exchange for ADP, an additional effect of Mg^{2+} was observed. If Mg^{2+} was added to the ADP stock and the resulting mixture used to initiate exchange, the rate of exchange increased mainly by an increase of constant k of eq 1 for the faster of the two phases (Table 2). In the absence of added Mg^{2+}, V_0 was half of what it was when 500 μM Mg^{2+} was added together with the ADP stock. Values for F_{min} also increased as the concentration of free Mg^{2+} increased along with the concentration of MgADP. Similar measurements were made with Mg^{2+} and ATP (Table 2). In the case of ATP, the effect of adding Mg^{2+} combined with nucleotide was less, and increases in the rate constants were masked by the decreasing extent of the exchange. The presence of a small amount of ADP in the ATP stock may account for the slight increases seen.

Table 3: Effect of Combined Mg^{2+} and P_i on Exchange of Tightly Bound CF_1-ϵ[a]

	ADP				ATP		
	F_{min} (V)	extent (V)	V_0 (V min^{-1})		F_{min} (V)	extent (V)	V_0 (V min^{-1})
ADP only	2.60	5.26	4.52	ATP only	1.32	5.64	3.71
P_i, in EDTA[b]	2.43	5.55	4.95	P_i	1.48	5.23	3.66
P_i	1.90	5.46	14.14	P_i, 23.6 μM Mg^{2+}	1.67	5.25	6.46
P_i, 23.6 μM Mg^{2+}	2.02	5.83	25.21	P_i, 94.4 μM Mg^{2+}	1.70	5.01	8.56
P_i, 94.4 μM Mg^{2+}	1.98	4.77	32.08	P_i, 472 μM Mg^{2+}	1.62	5.17	9.80
P_i, 944 μM Mg^{2+}	2.09	4.20	53.56	P_i, 944 μM Mg^{2+}	1.43	5.07	9.98
SDS base line[c]	1.86			SDS base line[c]	0.98		

[a] CF_1-ϵ was loaded with 0.93 mol of tightly bound TNP-ADP/mol of CF_1-ϵ in a buffer of TN + 10 mM EDTA and then passed through two consecutive Sephadex G-50 centrifuge columns equilibrated with TN. [CF_1-ϵ] was 0.47 μM. Exchange was initiated with a mixture of 4.7 mM ADP or ATP with 4.7 mM NaH_2PO_4 and $MgCl_2$. [b] CF_1-ϵ was incubated in a buffer of TN + 10 mM EDTA for 13 min before ADP addition. [c] Indicates the signal obtained by the addition of SDS to 0.5%.

Exchange rates were further accelerated by the addition of P_i. In the case of exchange for ADP, 5 mM P_i had a dramatic effect on the rate in a manner dependent on the presence of Mg^{2+} (Table 3). Because the ADP stock was not free of Mg^{2+}, the effect of P_i on exchange was tested first in the presence of EDTA. When EDTA was present, P_i increased the total extent of exchange. EDTA treatment alone causes a similar increase in extent but requires a much longer incubation time than 13 min. Without EDTA, 5 mM ADP which contained 3970 ppm or 20 μM Mg^{2+} was sufficient to produce an initial velocity, V_0, of 14.14 V min^{-1}, which compares to the value of 4.52 V min^{-1} obtained in the absence of added P_i. As the Mg^{2+} content increased, the rate of exchange continued to increase, but the value of F_{min} did not increase as before. In the presence of P_i, all of the tightly bound TNP-ADP was exchanged over the range of Mg^{2+} concentrations measured, as determined by SDS addition. Fits of the extremely fast rates obtained by addition of ADP, P_i, and $MgCl_2$ are less accurate. This results in the apparently smaller extents, and most likely an underestimate of V_0. That exchange was complete for these samples can be seen by the F_{min} values.

A similar, but less pronounced, increase of the exchange rate for medium ATP was observed in the presence of P_i and Mg^{2+} (Table 3). In contrast, F_{min} values did not noticeably decrease, remaining above the base line of 0.98 V obtained by the addition of SDS. The presence of the approximately 20 μM MgADP present in the ADP stock was sufficient to produce an initial rate V_0 of 14.14 V/min corresponding to 2.1 μmol of TNP-ADP exchanged/min, whereas the fastest V_0 measured in the ATP runs was 9.98 V/min or 1.5 μmol of TNP-ADP exchanged/min for the case of 5 mM ATP, 1 mM $MgCl_2$, and 5 mM P_i. Thus, the rate increase can be accounted for by ADP contamination of the ATP.

Sulfite is commonly used to stimulate the Mg^{2+}-ATPase activity of CF_1, overcoming the inhibition of activity that is caused by the presence of MgADP bound to the enzyme (Larson et al., 1989; Du & Boyer, 1990). Sulfite, P_i, or sulfate was added along with ADP, ATP, or with a combination of $MgCl_2$ and nucleotide to initiate exchange (Table 4). When added together with ATP, the only effect of sulfite, P_i, or sulfate on TNP-ADP exchange was a slight quenching of the fluorescence of the bound TNP-ADP and/or possibly a small inhibition of the exchange rate. The observed quenching of the fluorescence accounts for the smaller extents seen in the presence of sulfite or P_i. When sulfite or phosphate was added together with Mg^{2+} and ATP, V_0 was faster than in the absence of Mg^{2+}, and much faster than the case of exchange for ATP in the presence of Mg^{2+} alone. Also, the Mg^{2+}-induced inhibition of complete exchange of the tightly bound TNP-ADP was overcome. In contrast, the inhibition produced by the addition of Mg^{2+} and sulfate was greater than the inhibition produced by Mg^{2+} alone.

Table 4: Effects of Sulfite, P_i, and Sulfate on Exchange

	5 mM ADP		4.5 mM ATP	
nucleotide added with	V_0 (V min^{-1})	extent (V)	V_0 (V min^{-1})	extent (V)
nucleotide only[a]	6.88	4.86	2.28	6.01
2 mM Mg^{2+} [a]	1.41	5.48	0.73	6.08
100 mM Na_2SO_3[a]	2.75	4.44	1.16	4.29
100 mM Na_2SO_3, 2 mM Mg^{2+} [a]	2.10	5.09	2.47	4.73
75 mM NaH_2PO_4[a]	8.66	4.44	1.24	4.52
75 mM NaH_2PO_4, 2 mM Mg^{2+} [a]	7.85	4.72	2.00	4.74
	2.86 mM ADP		4.5 mM ATP	
nucleotide added with	V_0 (V min^{-1})	extent (V)	V_0 (V min^{-1})	extent (V)
nucleotide only	3.63	3.66	2.06	5.05
2 mM Mg^{2+} [b]	1.70	4.55	0.53	5.30
100 mM Na_2SO_4[b]	2.10	4.16	0.99	4.05
100 mM Na_2SO_4, 2 mM Mg^{2+} [b]	0.18	2.15	0.60	5.78

[a] Exchange was initiated by addition of nucleotide in mixtures of either Na_2SO_3, NaH_2PO_4, or Na_2SO_4, with or without $MgCl_2$, to CF_1-ϵ with 1.09 mol of tightly bound TNP-ADP/mol of CF_1-ϵ in TN buffer for a final concentration of 0.31 μM CF_1-ϵ. [b] CF_1-ϵ loaded with 0.90 mol of tightly bound TNP-ADP/mol of CF_1-ϵ in TN buffer, at a final concentration of 0.50 μM.

Similar results were obtained for exchange with ADP (Table 4), except in the case of P_i. At 75 mM P_i, the same effect on ADP-induced exchange was seen as in Table 3 where 5 mM P_i was used. At the elevated P_i levels, the exchange rates were considerably slower than at 5 mM P_i. This may be due to increased Na^+ inhibition which is present at 125 mM due to the added NaH_2PO_4, or simply to ionic strength effects. The addition of 2 mM Mg^{2+} with 75 mM P_i and ADP has a slight inhibitory effect, unlike the rate increase seen with ATP in the presence of P_i. Exchange for ADP in the presence of sulfite is also slightly inhibited by Mg^{2+}. Exchange of bound TNP-ADP for either medium ADP or ATP in the presence of Mg^{2+} was accelerated by the presence of sulfite or P_i, and complete exchange of tightly bound TNP-ADP for medium ADP or ATP was observed in the presence of sulfite or P_i and 2 mM Mg^{2+}. Thus, sulfite

Table 5: Removal of ADP by Sulfite and P_i[a]

	mol of ADP/ mol of CF_1-ϵ	$MgCl_2$ present at 2 mM with sulfite and Pi	mol of ADP/ mol of CF_1-ϵ
control	1.33	control	1.26
ADP-loaded sample	1.94	ADP-loaded sample	1.93
4 min, 100 mM P_i	1.17	5 min, 100 mM P_i	1.74
11 min, 100 mM P_i	1.10		
26 min, 100 mM P_i	1.08	20 min, 100 mM P_i	1.62
4 min, 100 mM sulfite	1.09	5 min, 100 mM sulfite	1.69
26 min, 100 mM sulfite	1.02	20 min, 100 mM sulfite	1.77

[a] CF_1-ϵ in TN buffer was incubated with 10 mM ADP for 1 h and then passed through two consecutive Sephadex G-50 centrifuge columns equilibrated with TN. Na_2SO_3 or NaH_2PO_4 was added with or without $MgCl_2$. At the indicated times, the sulfite or P_i was removed along with loose nucleotide and Mg^{2+} by two consecutive Sephadex G-50 centrifuge columns.

Table 6: Removal of MgADP by Sulfite[a]

	mol of Mg/ mol of CF_1-ϵ	mol of nucleotide/ mol of CF_1-ϵ			
		AMP	ADP	ATP	total
ADP loaded	0.10	0.03	2.42		2.45
MgADP loaded	2.87	0.39	2.11	0.76	3.25
ADP loaded + sulfite	0.15		1.45		1.45
MgADP loaded + sulfite	1.89	0.03	1.67	0.59	2.29

[a] CF_1-ϵ stored for 4 days as a precipitate in 50% $(NH_4)_2SO_4$ and 5 mM EDTA was spun down and desalted into either TN buffer or TN + 10 mM EDTA. CF_1-ϵ in EDTA buffer was incubated with 5 mM ADP for 80 min, and CF_1-ϵ in buffer without EDTA was incubated in 2 mM $MgCl_2$ and 5 mM ADP for the same time. Excess ADP and Mg^{2+} were removed by two consecutive Sephadex G-50 centrifuge columns. Samples were then incubated for 20 min in 100 mM Na_2SO_3 and again passed through two consecutive Sephadex G-50 centrifuge columns.

or P_i is capable of overcoming the inhibition of exchange rate and extent caused by MgADP tightly bound to CF_1-ϵ, and sulfate is not.

The addition of high concentrations of sulfite, P_i, or sulfate without the addition of any nucleotide resulted in the release of tightly bound TNP-ADP, which was observed as a drop of about 50% in the fluorescence intensity. This effect was strongly inhibited by Mg^{2+}. CF_1-ϵ was loaded with ADP to slightly less than 2 mol of ADP/mol of CF_1-ϵ (Table 5). The addition of sulfite or P_i reduced the ADP content to just over 1 mol of ADP/mol of CF_1-ϵ. The addition of Mg^{2+} and sulfite or Mg^{2+} and P_i resulted in a much smaller decrease to about 1.6 mol of ADP/mol of CF_1-ϵ.

A similar experiment was performed with CF_1-ϵ loaded with ADP in the presence of Mg^{2+} or EDTA (Table 6). Sulfite removed 1 mol of nucleotide/mol of CF_1-ϵ from both CF_1-ϵ loaded with ADP and CF_1-ϵ loaded with Mg^{2+} and ADP. CF_1-ϵ loaded with Mg^{2+} and ADP also lost 1 mol of Mg^{2+}/mol of CF_1-ϵ, indicating that sulfite is capable of removing tightly bound MgADP. Thus, the Mg^{2+}-induced inhibition of the sulfite-stimulated release of tightly bound ADP does not arise from CF_1-ϵ-bound MgADP. The significant amount of AMP in the sample of CF_1-ϵ loaded with ADP in the presence of Mg^{2+} is probably a result of a contaminating adenylate kinase activity. AMP will not remain bound to CF_1-ϵ after its passage through Sephadex G-50 centrifuge columns. The adenylate kinase activity was likely manifest after the nucleotides were released from CF_1-ϵ by the comparatively mild treatment of precipitation of CF_1-ϵ by methanol in the presence of NaCl. Adenylate kinase activity, which is totally dependent on Mg^{2+}, is likely responsible for the production of AMP from the ADP and for some of the ATP. It is apparent that sulfite must inhibit the adenylate kinase activity.

Table 7: Effect of Mg^{2+} on Exchange of Tightly Bound TNP-ADP for ATP in the Presence of Sulfite[a]

	V_0 (V min^{-1})	F_{min} (V)	extent (V)
ATP	2.74	0.89	5.44
ATP, sulfite	2.15	0.96	4.77
ATP, 25 μM Mg^{2+}, sulfite	2.65	1.20	4.72
ATP, 500 μM Mg^{2+}, sulfite	2.43	1.06	4.95
ATP, 2 mM Mg^{2+}, sulfite	2.98	0.66	5.16
ATP, 5.3 mM Mg^{2+}, sulfite	3.85	0.99	4.51

[a] Exchange was initiated with a combination of ATP, Mg^{2+}, and sulfite to CF_1-ϵ containing 0.84 mol of tightly bound TNP-ADP/mol of CF_1-ϵ. Final concentrations were 0.5 μM CF_1-ϵ, 5 mM ATP, and 100 mM Na_2SO_3.

In order to ensure that the adenylate kinase activity did not influence exchange measurements, ATP production was measured under conditions under which exchange was observed. ATP production was determined by measuring the reduction of $NADP^+$ by the absorbance change at 340 nm ($\epsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}), using a coupled assay of 5 mM ADP, 10 units mL^{-1} hexokinase, 10 units mL^{-1} glucose-6-phosphate dehydrogenase, 0.5 mM $NADP^+$, 25 mM glucose, and 0.5 μM CF_1-ϵ in TN buffer. The concentration of ATP was found to increase by 0.25 μM min^{-1} as a result of the adenylate kinase activity. Even during the longest exchange measurements, the amount of ATP produced in this fashion was less than the amount of ATP already present in the ADP stock.

The ability of sulfate to remove tightly bound MgADP was also tested. Sulfate was more effective than sulfite in removing MgADP, although it does not stimulate exchange of bound TNP-ADP for medium nucleotide in the presence of Mg^{2+}. Therefore, the ability of sulfite or P_i to stimulate exchange under these conditions is not directly related to the release of tightly bound TNP-ADP observed in the absence of medium nucleotide.

At low ratios of Mg^{2+} to ATP the rate and extent of exchange in the presence of sulfite are not altered by Mg^{2+} addition (Table 7). Only when the Mg^{2+} concentration becomes 40% of the ATP concentration does the exchange rate start to increase, and the rates continue to increase as the Mg^{2+} concentration is increased.

Sulfite with Mg^{2+}, or P_i with or without Mg^{2+}, all of which enable complete exchange of bound TNP-ADP, might affect a second tight ADP binding site, the one which contains inhibitory MgADP and which did not load with TNP-ADP in the absence of Mg^{2+}. Sulfite, P_i, or sulfate removed at most one of two tightly bound ADP, and is seen in Table 7 to cause release from the site that is easily loaded with TNP-ADP. If P_i, or sulfite with Mg^{2+}, in the presence of medium nucleotide causes exchange of both the site from which MgADP inhibits and that which is TNP-ADP loaded, then it should be possible to load both sites with TNP-ADP under these conditions.

Loading of CF_1-ϵ was tried first in the presence of 2 mM Mg^{2+}, and P_i or sulfite (Table 8). This resulted in the

Table 8: TNP-ADP Loading of CF_1-ϵ in the Presence of P_i and Sulfite

	mol of ADP/ mol of CF_1-ϵ	mol of TNP-ADP/ mol of CF_1-ϵ	ADP + TNP-ADP
TNP-ADP loaded in TN[a]	0.60	1.06	1.66
TNP-ADP loaded in P_i, Mg^{2+} [a]	0.81	1.46	2.27
TNP-ADP loaded in sulfite, Mg^{2+} [a]	0.73	1.85	2.58
sample prior to loading[b]	1.68	0	1.68
ADP-loaded sample[b]	1.96	0	1.96
TNP-ADP-loaded sample[b]	0.66	0.9	1.56
TNP-ADP loaded in P_i[b]	0.88	1.08	1.96
TNP-ADP loaded in P_i, Mg^{2+} [b]	0.74	1.36	2.10

[a] CF_1-ϵ was loaded with TNP-ADP in TN buffer, TN buffer + 100 mM NaH_2PO_4 + 2 mM $MgCl_2$, and TN buffer + 100 mM Na_2SO_3 + 2 mM $MgCl_2$. [b] CF_1-ϵ was loaded with ADP in TN buffer, and then with TNP-ADP in the following buffers: TN, TN + 5 mM NaH_2PO_4, TN + 5 mM NaH_2PO_4 + 100 μM $MgCl_2$.

incorporation of 1.85 mol of TNP-ADP/mol of enzyme in the presence of sulfite. In the presence of 2 mM Mg^{2+}, however, a third site on CF_1-ϵ becomes tight binding for ADP and in addition to the TNP-ADP the sample also contained 0.73 tightly bound ADP.

To determine where the excess TNP-ADP was binding, the experiment was repeated in the presence of P_i with either no added Mg^{2+} or 100 μM Mg^{2+} in TN buffer (Table 8). The complete exchange of tightly bound TNP-ADP occurred under both conditions (Table 3). In the presence of 100 μM Mg^{2+} and P_i, 1.36 mol of TNP-ADP was bound, and 1.22 of the original 1.96 mol of tightly bound ADP was displaced in the process. Under these conditions, at least two sites which bind ADP in the absence of Mg^{2+} exchange. With low Mg^{2+} concentrations, one of these two sites appears to exchange much more quickly than the other. At higher Mg^{2+} concentrations, commonly used for sulfite-stimulated Mg^{2+}-ATPase assays, the exchange rate of the slower site may increase to match that of the site whose exchange was studied directly by TNP-ADP loading. Some evidence for faster exchange of the second site in the presence of 2 mM Mg^{2+} is provided by the experiment presented in Table 8.

DISCUSSION

At least three interacting sites are required to explain the observations reported in this paper. The first is the dissociable or "loose" site. It is the binding of medium nucleotide to this site which initiates exchange (Shapiro & McCarty, 1990). In addition, there is a second site which binds TNP-ADP readily, exchanging previously bound ADP for TNP-ADP. The third site is one which retains bound ADP in the presence of medium TNP-ADP unless in the presence of P_i, or a combination of sulfite and Mg^{2+}.

Unlike previous studies of the exchange of tightly bound ADP in CF_1 (Shapiro & McCarty, 1990), and MF_1 (Nalin & Cross, 1982), it is clear that TNP-ADP that is tightly bound to CF_1 (in the "second" site) will exchange for both medium ADP and ATP, and that this process does not require a divalent cation (Figure 1). The observed exchange rate was similar for medium ADP, ATP, and MgATP, and faster for MgADP. Despite the fact that exchange was studied in solubilized CF_1, the preferred substrate for exchange was clearly MgADP + P_i (Table 3), which has been shown to be the substrate for ATP synthesis in the membrane-bound enzyme (Zhou & Boyer, 1992).

While MgATP is believed to be the preferred substrate for ATPase activity of CF_1 (Hochman & Carmelli, 1981), exchange for medium ATP was only stimulated by Mg^{2+} in the presence of an activating anion, and only when the concentration of Mg^{2+} approached that of the ATP. While not precluding MgATP as the substrate for hydrolysis, this result does support the suggestion by Berger et al. (1994) that free Mg^{2+} activates hydrolysis in solubilized CF_1.

The presence of tightly bound MgADP (at the "third" site) is sufficient to prevent exchange of tightly bound TNP-ADP for medium nucleotide. Guerrero et al. (1990) determined that it was the formation of a tightly bound MgADP that inhibited the Mg^{2+}-ATPase activity of CF_1, and that the Mg^{2+} binding or release was slow. They most likely observed MgADP formation at the "third" site. The release of Mg^{2+} (and ADP) from this site by EDTA was slow, taking hours to complete.

The ability of P_i to remove tightly bound ADP has been demonstrated (Komatsu-Takaki, 1984), and it was determined that ADP released by P_i was in the same site as ADP released upon illumination of CF_1-CF_o in thylakoids. Sulfite, P_i, and sulfate all cause release of ADP from the site which readily binds TNP-ADP. The amount of ADP released was never observed to be more than 1 of 2 mol of ADP/mol of CF_1 suggesting that only one site may be emptied. This is not the site which released ADP in the presence of EDTA. Incubation in EDTA did not affect the amount of bound TNP-ADP (Table 1).

The release of tightly bound ADP (or TNP-ADP) by sulfite, P_i, or sulfate was strongly inhibited by the addition of 2 mM Mg^{2+}. This inhibition was not the result of formation of tightly bound MgADP. Both sulfite and sulfate are capable of removing tightly bound MgADP in an enzyme where the amount of tightly bound nucleotide is only slightly less than the amount of bound Mg^{2+}. Rather than inhibiting release, free Mg^{2+} may accelerate the rebinding of the released ADP or TNP-ADP. Hisabori and Mochizuki (1993) reported that in CF_1 with only 1 mol of tightly bound ADP/mol of CF_1 that Mg^{2+} accelerates the rate of ADP binding.

The release/exchange of tightly bound ADP in the presence of Mg^{2+}, sulfite, and added nucleotide has been observed before (Du & Boyer, 1990; Larson et al., 1989), as has the ability of added sulfite in the presence of Mg^{2+} and ATP to stimulate ATPase activity of CF_1. It seems reasonable that the release of tightly bound ADP, or MgADP, is the cause of this stimulation. However, sulfate, which is even more effective than sulfite at releasing ADP, does not stimulate either exchange or hydrolysis in the presence of Mg^{2+} and nucleotide.

Mg^{2+} and sulfite (or P_i) do enable exchange of tightly bound ADP for TNP-ADP at the "third" site, although evidence suggests that the rate of exchange at this site is less than that of the "second" site. We suggest that both of these tight binding sites must exchange when CF_1 is fully active as an ATPase, as in the sulfite-stimulated Mg^{2+}-ATPase assay, or as an ATP synthase when bound to thylakoids in the presence of Mg^{2+} and P_i. At least two of these sites interact in a cooperative manner as proposed previously (Boyer, 1989; Shapiro & McCarty, 1990). Whether

the third site fulfills a solely regulatory function or is also involved in catalysis has yet to be determined.

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