Chloroplast ATP synthase, CF$_1$CF$_0$, couples the phosphorylation of ADP to ATP on electron transfer at the expense of the electrochemical proton gradient across the thylakoid membrane. The catalytic portion of the complex, CF$_1$, contains the nucleotide binding sites. In solution, CF$_1$ catalyzes a low rate of ATP hydrolysis, but removal of the inhibitory e subunit increases the activity to a high level (1).

CF$_1$ likely contains six nucleotide binding sites (2), not all of which are catalytic (2). These binding sites have been partially characterized (3, 4). Two sites are dissociable. There are two noncatalytic sites that are tight binding only for ATP and only that of exchange of bound nucleotide for medium nucleotide. The nucleotide depleted enzyme binds one additional mol of TNP-ADP/mol of CF$_1$, indicating that there is a tightly bound site that does not exchange readily with medium nucleotide.

It is MgADP in this nonexchanging site, not the easily exchanging ADP site, that is responsible for the MgADP-induced inhibition of the ATPase activity. The rate of exchange of tightly bound ADP from CF$_1$ matches the rate at which the Mg$^{2+}$/ATPase activity of CF$_1$ is activated but is not itself responsible for the activation.
alcaline phosphatase activity and must be removed from solution by passage through two consecutive 3-ml Sephadex G-50 columns equilibrated with 50 mM Tris-HCl, pH 8.0, 50 mM NaCl (TN buffer). This and all subsequent buffers were passed through a column of Chelex 100 resin to remove residual Mg2+ and were stored in plastic.

CF1-NT concentration was determined by the Lowry method (12) or by absorbance, using an extinction coefficient of ε = 0.483 cm3/mg of CF1 at 277 nm (3). CF1-NT (15 mg) was diluted to 5 mg/ml with TN buffer, and 280 units of StrataGene calf intestinal alkaline phosphatase (catalog number 600015) were added. Samples were incubated 18–20 h at room temperature. Alkaline phosphatase was removed from the CF1-NT by DEAE-cellulose chromatography at room temperature. Alkaline phosphatase was eluted from the column (0.1-ml column volume) of CF1-NT with 15 ml of 50 mM Tris-HCl, 50 mM NaCl, followed by elution with 45 ml of 50 mM Tris-HCl, 100 mM NaCl. Nucleotide-depleted CF1-NT was eluted by 5 ml of 50 mM Tris-HCl, 400 mM NaCl and was collected in 0.5-ml fractions.

The CF1-NT fractions were assayed for alkaline phosphatase activity by following p-nitrophenyl phosphate hydrolysis at 410 nm at 25 °C. The fractions that contained most of the CF1-NT were then combined and desalted by two consecutive Sephadex G-50 columns equilibrated with TN buffer. The phosphatase activity of the CF1-NT-NT was 1 nmol min−1 mg CF1-NT or less.

ATPase activity of CF1-NT in the presence of Ca2+ was assayed by the incubation of 10 µg of CF1-NT for 3–5 min at 37 °C in 50 mM Tris-HCl, 5 mM ATP, and 5 mM CaCl2. The amount of P, produced was determined colorimetrically (13). ATPase activity of CF1-NT in the presence of Mg2+ was measured with a coupled enzyme assay at 25 °C. CF1-NT (50 µg) was added to a mixture of 1 ml total volume containing 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM MgCl2, 1 mM phosphoenolpyruvate, 5 mM ATP, 20 units each of Sigma lactate dehydrogenase (catalog number L-1254), Sigma pyruvate kinase (catalog number P-9136), and 0.25 mM NADH. The decrease in NADH concentration was measured spectrophotometrically at 340 nm.

The cold stability of CF1-NT was monitored by loss of ATPase activity upon incubation at 0 °C in the manner of Hightower and McCarty (14). CF1-NT was incubated at 15 µg/ml in Chelex 100-treated 50 mM Tris-HCl, pH 8.0, at 0 and 25 °C. At various times, 200-µl aliquots were taken for the assay of Ca2+-ATPase activity for 3 min at 25 °C. The concentration of P, produced was determined by the sensitive malachite green assay (15) with modifications described elsewhere (14).

The analysis of nucleotide bound to CF1 was performed with ion-pairing high pressure liquid chromatography (HPLC). Bound nucleotides were released from CF1 by methanol precipitation (16). The relationship between the integrated peak area and the nucleotide quantity was determined by HPLC (Fig. 1) over 11 trials to be 0.094 ± 0.04 mol/mmol of CF1-NT. The nucleotide-depleted enzyme was stored in 50 mM Tris-HCl, 20% glycerol at −80°C. Overnight storage in 20% glycerol proved most conducive to retaining Ca2+-ATPase activity, with storage at 25 °C and −80 °C equally effective. CF1-NT fully retains its Ca2+-ATPase activity for up to 3 weeks in 50 mM Tris-HCl, 20% glycerol at −80 °C.

RESULTS

As prepared, CF1-NT, stored as an (NH4)2SO4 precipitate in the presence of EDTA, contains 1.2–2 mol of tightly bound ADP/mol of CF1. Tightly bound ADP is released into the medium at a slow rate and is re-bound at a fast rate (10). Alkaline phosphatase degrades ADP to AMP and then to adenosine. Neither AMP nor adenosine binds to CF1. In principle, therefore, alkaline phosphatase should promote the removal of bound ADP from CF1, simply by pulling the equilibrium toward the dissociation of bound nucleotide. Although the mechanism of alkaline phosphatase-induced release of bound ADP has not been investigated in detail, results of preliminary experiments suggest that alkaline phosphatase attacks ADP that had dissociated from CF1. Alkaline phosphatase added to the outside of a dialysis bag containing CF1 enhanced ADP removal (data not shown). This method has been routinely effective in producing nucleotide-depleted CF1 with an ADP content determined by HPLC (Fig. 1) over 11 trials to be 0.094 ± 0.04 mol/mmol of CF1-NT compared with an untreated control content of 1.2 ± 0.2 mol of ADP/mmol of CF1-NT. In more recent trials, the CF1-NT used had a tightly bound ADP content in the range of 1.6–1.8 mol of ADP/mmol of CF1-NT and the bound nucleotide content after depletion was correspondingly higher, in the range 0.18–0.32 mol/mmol of CF1-NT.

The nucleotide-depleted enzyme was stored in 50 mM Tris-HCl, pH 8.0, with and without 20% glycerol, with and without 50% saturated ammonium sulfate, at 25 or 4 °C and, for samples without ammonium sulfate, at −80 °C. Overnight storage in 20% glycerol proved most conducive to retaining Ca2+-ATPase activity, with storage at 25 °C and −80 °C equally effective. CF1-NT fully retains its Ca2+-ATPase activity for up to 3 weeks in 50 mM Tris-HCl, 20% glycerol at −80 °C.

Nucleotide depletion appears to have no effect on the steady-state rate of ATP hydrolysis activity of the enzyme. The ATPase activity of CF1-NT was equivalent to that of CF1-NT with endogenous nucleotide (Table I). p-Nitrophenylphosphatase assays revealed no significant residual alkaline phosphatase activity in the CF1-NT-NT. To ensure that the ATPase activity measured was that of CF1-NT and not of residual alkaline phosphatase, Ca2+-ATPase assays were also performed in the presence of tentoxin, a potent inhibitor of the ATPase activity of CF1 but not of alkaline phosphatase. The ATPase activity of CF1-NT was fully inhibited by µM concentrations of tentoxin.
Nucleotide depletion also has no effect on the cold stability of the enzyme. CF<sub>1</sub>-e loses its ATPase activity upon exposure to low temperatures (0 °C), a result of at least a partial dissociation of the complex (18). Nucleotides in the medium protect from the loss of ATPase activity in the cold (18, 19). Nucleotide-depleted CF<sub>1</sub>-e lost its ATPase activity at 37 °C at a rate identical to that for CF<sub>1</sub>-e (Fig. 2). The CF<sub>1</sub>-e used contained 1.8 mol of tightly bound ADP/mol of CF<sub>1</sub>-e, and the nucleotide-depleted enzyme contained 0.20 mol of ADP tightly bound/mol of CF<sub>1</sub>-e.

Measurements were taken of the effect of bound ADP on the loading of the enzyme with TNP-ADP. CF<sub>1</sub>-e-NT containing 0.05 mol of bound nucleotide/mol of CF<sub>1</sub>-e-NT was prepared in TN buffer. Half was loaded with ADP by incubation in 5 mM ADP for 1 h. Excess ADP was removed by passage through two consecutive Sephadex G-50 centrifuge columns. TNP-ADP binding to both the nucleotide-depleted and the ADP-loaded enzyme was measured as the increase in fluorescence of TNP-ADP as it binds to the enzyme in solution (Fig. 3).

When 1 μM TNP-ADP was added to 0.5 μM CF<sub>1</sub>-e-NT essentially all of the TNP-ADP bound to the enzyme resulting in 1.95 mol of bound TNP-ADP/mol of CF<sub>1</sub>-e. The binding was complete within 1 min. The ADP-loaded enzyme in contrast bound 1.1 mol of TNP-ADP/mol of CF<sub>1</sub>-e, at these concentrations, and binding took close to 10 min to complete. When the concentration of TNP-ADP was increased to 4 μM with 0.5 μM CF<sub>1</sub>-e, the nucleotide-depleted enzyme bound 2.5 mol of TNP-ADP/mol of CF<sub>1</sub>-e, and the ADP-loaded enzyme bound 1.6 mol of TNP-ADP/mol of CF<sub>1</sub>-e (Fig. 3A). These data suggest that a nonexchanging or slowly exchanging tight ADP binding site exists, although it is possible that the site may be made to exchange at higher concentrations of TNP-ADP.

TNP-ADP binding was studied using stopped-flow fluorescence in order to resolve the phases of binding to CF<sub>1</sub>-e-NT. In this case, either CF<sub>1</sub>-e-NT containing 0.20 mol of tightly bound ADP/mol of CF<sub>1</sub>-e or CF<sub>1</sub>-e which contained an endogenous 1.81 mol of tightly bound ADP/CF<sub>1</sub>-e were mixed in the stop-flow with TNP-ADP in TN buffer to a final concentration of 0.4 μM CF<sub>1</sub>-e and 1.6 μM TNP-ADP (Fig. 3B). The resulting scans of fluorescence intensity versus time showed two distinct phases and were fit to Equation 1:

\[ F(t) = F_0 + F_1 \exp(-k_1 t) + F_2 \exp(-k_2 t) \]  

(Eq. 1)

where \( F(t) \) is the fluorescence intensity at time \( t \), \( F_1 \) and \( F_2 \) are the extent of the two phases, and \( k_1 \) and \( k_2 \) are their rate constants. \( F_0 \) is the base-line fluorescence of the unlabeled sample with the unbound TNP-ADP.

As can be seen in Table I, in the case of binding to CF<sub>1</sub>-e that contains endogenous nucleotide, there is one rapid phase, the extent of which corresponds well to the filling of the 0.19 mol of empty ADP tightly bound site/mol of CF<sub>1</sub>-e, and a second, much slower phase whose rate constant is a good match for those observed for exchange of tightly bound TNP-ADP (7). In the nucleotide-depleted sample, at these concentrations, a total of 1.4 sites are filled per CF<sub>1</sub>-e, and two rate constants are observed, one equivalent to the fast rate constant from the sample with endogenous ADP, and one 9 times faster. Close to one site filled at the fast rate, with the remaining 0.5 nucleotide binding at the slower rate.

The measurements were repeated, mixing the enzyme solution with a mixture of MgCl<sub>2</sub> and TNP-ADP for a final concentration of 1.6 μM for each. The sample of CF<sub>1</sub>-e with its endogenous nucleotide showed an increase in its slower rate constant. The rate of exchange of bound nucleotide has been shown to be faster for medium MgADP then for ADP alone (7), a further indication that the slow rate constant represents exchange of bound ADP for medium TNP-ADP. There was a drop of about half for both rate constants for the nucleotide-depleted sample showing that binding of MgTNP-ADP to either empty site is slower than binding of TNP-ADP without Mg<sup>2+</sup>.

It has previously been suggested that both of the tight ADP binding sites will exchange for medium ADP in the absence of Mg<sup>2+</sup> (4). In order to test this hypothesis, CF<sub>1</sub>-e-NT containing 0.05 mol of ADP/mol of CF<sub>1</sub>-e was loaded with TNP-ADP. This resulted in CF<sub>1</sub>-e with 1.44 mol of TNP-ADP tightly bound/mol of CF<sub>1</sub>-e. Exchange of the bound TNP-ADP was observed in the presence of 5 mM ADP and 5 mM EDTA for more than 10 min and fit accurately to Equation 2:

\[ F(t) = F_1 \exp(-k_1 t) + F_2 \exp(-k_2 t) + F_{min} \]  

(Eq. 2)

Here \( F_1 \) and \( F_2 \) are the extents of a biphasic decay, and \( k_1 \) and \( k_2 \) are the rate constants, with \( F_{min} = F(t = \infty) \). The extent of exchange was calculated from the fit, and from a base line obtained by addition of 0.1% SDS to remove all bound nucleotides. Of 1.44 tightly bound TNP-ADP, 1.1 would exchange, suggesting that if the second tight ADP site does exchange, it does so very slowly.

Mg<sup>2+</sup> inhibits the ATPase activity of CF<sub>1</sub> and is believed to inhibit through the formation of a tightly bound Mg<sup>2+</sup>-ADP complex (20, 21). In order to determine whether ADP was a requirement of Mg<sup>2+</sup>-induced inhibition, the activity as a function of time of CF<sub>1</sub>-e-NT was observed at 25 °C by the coupled enzyme assay, with and without prior exposure of the enzyme to Mg<sup>2+</sup>. The CF<sub>1</sub>-e-NT contained 0.18 mol of tightly bound nucleotide/mol of CF<sub>1</sub>-e, and the control was CF<sub>1</sub>-e which contained 1.6 mol of tightly bound nucleotide/mol of CF<sub>1</sub>-e. The activity was observed by monitoring the absorbance of NADH.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Specific activity</th>
<th>μmol/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF&lt;sub&gt;1&lt;/sub&gt;-e</td>
<td>14.7 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>CF&lt;sub&gt;1&lt;/sub&gt;-e-NT</td>
<td>16.1 ± 5.2</td>
<td></td>
</tr>
<tr>
<td>CF&lt;sub&gt;1&lt;/sub&gt;-e + tentoxin (1 μM)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CF&lt;sub&gt;1&lt;/sub&gt;-e-NT + tentoxin (1 μM)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
TNP-ADP was added to the cuvette at the time indicated by the addition of 0.1% SDS, which causes release of all TNP-ADP from the enzyme. The activity as a function of time fit to the empirical absorbance with respect to time giving the activity of the enzyme. The rate of change of the absorbance at 340 nm with respect to time, with the rate of change of the absorbance as defined by Equation 1. The first 10 s of a 720-s scan are shown.

Fig. 3. Binding of TNP-ADP to CF$_{1-e}$ and CF$_{1-e}$-NT. A, the addition of 4 μM TNP-ADP to 0.5 μM CF$_{1-e}$-NT + ADP (that was loaded in 5 mM ADP for 1 h followed by passage through two Sephadex G-50 columns) and CF$_{1-e}$-NT in TN buffer at 25 °C was monitored by observing the rise in fluorescence intensity as TNP-ADP binds to the enzyme. The dotted line indicates the fluorescence in the presence of 0.1% SDS, which causes release of all TNP-ADP from the enzyme. TNP-ADP was added to the cuvette at the time indicated by the arrow below the x axis. The addition of SDS to the nucleotide-depleted sample is indicated by a downward arrow. B, the mixture of 1.6 μM TNP-ADP with 0.4 μM CF$_{1-e}$-NT as observed by stopped-flow fluorescence, overlaid by the fit to Equation 1. The first 10 s of a 720-s scan are shown.

at 340 nm with respect to time, with the rate of change of the absorbance with respect to time giving the activity of the enzyme. The activity as a function of time fit to the empirical Equation 3:

\[
\text{Activity (time)} = \Delta A(1 - \exp(-k_2^*t)) - k_2^*t + A_0 \quad \text{(Eq. 3)}
\]

where $\Delta A$ and $k_2^*$ are the extent and the rate constant, respectively, of the initial increase in activity; $k_2$ is the rate constant for a slow inhibition that underlies the first term, and $A_0$ is the activity at $t = 0$, when the enzyme was added to the reaction mixture. In order to avoid the error introduced by numerical differentiation, the absorbance scans were fit to the integral of Equation 3, and the activity as a function of time was calculated from the fit. Excellent fits were obtainable by this method.

As can be seen from the plots of activity versus time (Fig. 4A), incubation of the nucleotide-depleted enzyme with Mg$^{2+}$ prior to the initiation of activity had relatively little effect, particularly in contrast to the enzyme containing its endogenous ADP. More can be learned from the rate constants presented in Table III. There is only slight variation in the rate constant of the initial increase in activity; however, the activity has a lower maximum value in the Mg$^{2+}$-pretreated case, and the rate $k_2^*$ of the underlying inhibition was much less. In contrast, in the sample of CF$_{1-e}$ that retained its endogenous ADP, incubation with Mg$^{2+}$ caused a large decrease in $k_1$. In this case, the sample pretreated with Mg$^{2+}$ showed no competing inhibitory term.

Inhibition of the rate constant $k_1^*$ by Mg$^{2+}$ pretreatment is clearly dependent on the presence of tightly bound ADP. The inhibitory term $k_2^*$ is also affected by Mg$^{2+}$, but the dependence of $k_2$ on ADP is less clear. The lower maximum activity and extremely low $k_2^*$ of the nucleotide-depleted sample when pretreated with Mg$^{2+}$ suggests that this inhibitory process is mostly complete in this case relative to the sample without Mg$^{2+}$ pretreatment. This difference in $k_2^*$ values in the nucleotide-depleted sample may result in some fashion from the remaining 0.18 mol of ADP/mol of enzyme or it may be evidence of an interaction between the enzyme and Mg$^{2+}$, in solution that does not require the presence of bound nucleotide.

It is interesting to note that the activity of CF$_{1-e}$ that retained its endogenous ADP reaches a higher maximum activity then that of the nucleotide-depleted sample before falling, relatively rapidly, to the same level. The sample of CF$_{1-e}$ had been stored as a precipitate in 50% saturated ammonium sulfate with 5 mM EDTA for several days prior to use. This procedure has been shown to produce CF$_{1-e}$ effectively free of bound Mg$^{2+}$ (7). These data suggest that tightly bound ADP actually transiently enhances the activity in the absence of bound Mg$^{2+}$. In order to ensure that the lag in activity described by the rate constant $k_1$ was not the result of the coupling enzymes rather than the CF$_{1-e}$, ADP was added directly to the reaction mixture in the absence of CF$_{1-e}$. No such lag was observed.

CF$_{1-e}$ from the same batch as the preceding experiment was loaded with ADP by incubation for 1 h in 5 mM ADP with an additional 5 mM EDTA to prevent Mg$^{2+}$ binding. Excess and loosely bound ADP were removed by passage through two consecutive Sephadex G-50 centrifuge columns. Both it and the CF$_{1-e}$-NT (0.18 mol of ADP/mol of CF$_{1-e}$) were loaded with TNP-ADP by incubation with a stoichiometric amount of TNP-ADP, with excess and loosely bound TNP-ADP removed by more Sephadex G-50 centrifuge columns. This resulted in a sample with 0.86 mol of TNP-ADP and 0.18 mol of ADP/mol of CF$_{1-e}$, and one with 0.55 mol of TNP-ADP and 1.36 mol of ADP/CF$_{1-e}$. The activities were measured as before with and without Mg$^{2+}$ pretreatment (Table III and Fig. 4B). Despite the presence of tightly bound TNP-ADP, there is remarkably little difference from the previous measurements. The exchange of the tightly bound TNP-ADP for medium nucleotide was observed in both samples, and 90% of the bound TNP-ADP exchanged showing that the TNP-ADP was bound mainly in the easily exchanging ADP tight-binding site. Thus, it must be the second ADP tight-binding site, the one which exchanges slowly if at all, that is responsible for the Mg$^{2+}$-induced delay.

### Table II

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>$k_1^*$</th>
<th>$k_2^*$</th>
<th>$k_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF$_{1-e}$ + TNP-ADP</td>
<td>0.11</td>
<td>0.10</td>
<td>0.41</td>
</tr>
<tr>
<td>CF$_{1-e}$ + Mg$^{2+}$, TNP-ADP</td>
<td>0.18</td>
<td>0.10</td>
<td>0.35</td>
</tr>
<tr>
<td>CF$_{1-e}$-NT + TNP-ADP</td>
<td>0.52</td>
<td>0.11</td>
<td>0.88</td>
</tr>
<tr>
<td>CF$_{1-e}$-NT + Mg$^{2+}$, TNP-ADP</td>
<td>0.36</td>
<td>0.06</td>
<td>1.12</td>
</tr>
</tbody>
</table>
in the onset of ATPase activity. The fact that the easily exchanging site fills fastest is evidence that the fast phase of binding of TNP-ADP to CF$_{1-e}$-NT that was observed represents binding to the easily exchanging site.

When tightly bound ADP was removed from CF$_{1-e}$, preincubation of the enzyme with Mg$^{2+}$ no longer inhibited the rate of ATP hydrolysis. Whether tightly bound ADP had a similar effect on Mg$^{2+}$ inhibition of the exchange of bound nucleotide for medium nucleotide was determined. CF$_{1-e}$-NT and CF$_{1-e}$ were loaded with substoichiometric amounts of TNP-ADP, and the initial rate of exchange ($v_0$) of tightly bound TNP-ADP for medium nucleotide was measured (Table IV). $v_0$ is determined by fitting the TNP-ADP fluorescence to Equation 2 and is described by the apparent first order rate constant for exchange multiplied by the extent of exchange. Exchange under catalytic conditions (both Mg$^{2+}$ and ATP present) was triphasic and fit to a suitably expanded version of Equation 2. For the purpose of comparison $v_0$ values were scaled by the amount TNP-ADP bound to each sample when calculating the ratio of $v_0$ for the two samples.

CF$_{1-e}$ and CF$_{1-e}$-NT were prepared in TN buffer treated with Chelex 100 resin and loaded with TNP-ADP by incubation in TN + 5 mM EDTA + TNP-ADP, where the TNP-ADP concentration was slightly less than the enzyme concentration as determined by absorbance at 277 nm. Thus loaded, the CF$_{1-e}$ contained 1.09 mol of ADP and 0.85 mol of TNP-ADP per mol of CF$_{1-e}$. The nucleotide-depleted enzyme started with 0.16 mol of nucleotide/mol of CF$_{1-e}$-NT and after TNP-ADP loading contained 1.03 mol of TNP-ADP/mol of CF$_{1-e}$. Samples used to study exchange with medium ATP rather than ADP were the same TNP-ADP-loaded samples used for the measurements of activity as a function of time. The fraction of tightly bound TNP-ADP that would exchange was the same for sample with and without endogenous nucleotide when initiated with the same nucleotide mix, typically around 80–90% again showing that TNP-ADP was bound mainly at the same exchangeable site in each sample.

Tightly bound ADP affected exchange rates of bound TNP-ADP only in the presence of Mg$^{2+}$. The variations produced in exchange rates by Mg$^{2+}$, EDTA, sulfite, and P$_i$ are comparable with what has been observed previously, with MgADP causing faster exchange than ADP, and MgADP + P$_i$, or MgATP and sulfite being faster still (7). It is interesting to note that Mg$^{2+}$ incubation reduces the rate of exchange of the nucleotide-depleted samples both for medium ADP and ATP. It was suggested by the activity of Mg$^{2+}$-incubated CF$_{1-e}$-NT that Mg$^{2+}$ is coordinated with the enzyme prior to ADP addition. If so, the Mg$^{2+}$ coordination combined with the very rapid binding of ADP shown in Fig. 3 may result in rapid inhibition of the Mg$^{2+}$-pretreated enzyme in the presence of ADP. However, these samples still contained 0.18 or 0.26 mol of ADP/mol of CF$_{1-e}$ which may be enough by itself to account for the decrease.

The ability to measure both activity and exchange as a function of time provides an opportunity to compare them directly. CF$_{1-e}$ was prepared with 0.9 mol of ADP and 1.1 mol of TNP-ADP/mol of CF$_{1-e}$, and from CF$_{1-e}$-NT a sample was prepared with 0.27 mol of nucleotide with an additional 1.3 mol of TNP-ADP/mol of CF$_{1-e}$. The activity of these samples was monitored using the coupled enzyme assay at 25°C, and the exchange of tightly bound TNP-ADP was observed by fluorescence using the same assay mix and sample volumes and concentrations as the activity assay also done at 25°C. The only exception was that the exchange measurements were done without NADH, the fluorescence of which interferes with the determination of TNP-ADP fluorescence.

The exchange of TNP-ADP under these circumstances had three phases, one of which was faster than that which could be observed in the coupled enzyme assay, where the sample cuvette had to be mixed by inversion prior to measurement. The extent of this rapid phase as a fraction of the total observed exchange corresponds well to the earliest observed activities as a fraction of the maximum activity ($A_0$, and the fast phase extent of Table V). This suggests that there may be a faster, unobserved phase to the increase in the ATPase activity with time and that the actual initial activity of the enzyme is much closer to zero. The maximum rate of exchange observed of the bound TNP-ADP is the initial rate ($v_0$), as only the very first
The effect of tightly bound ADP on the initial rates of exchange of bound TNP-ADP for medium nucleotide

The units of $v_0$ are nmol of TNP-ADP exchanged per min/mg CF$_1$. The samples with prior Mg$^{2+}$ incubation were incubated ~1 h in 5 mM MgCl$_2$ prior to the addition of ADP. The samples with prior EDTA incubation were incubated ~2 h in 5 mM EDTA prior to the addition of ADP.

<table>
<thead>
<tr>
<th>Exchange initiated with</th>
<th>CF$_1$-e-NT $v_0$ (CF$_1$-e-NT)</th>
<th>CF$_1$-e</th>
<th>$v_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM ADP</td>
<td>0.87</td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>5 mM Mg$^{2+}$, 5 mM ADP</td>
<td>4.9</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>5 mM Mg$^{2+}$, 25 mM P$_i$, 5 mM ADP</td>
<td>11.7</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>5 mM ADP (prior Mg$^{2+}$ incubation)</td>
<td>0.54</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>5 mM ADP (prior EDTA incubation)</td>
<td>1.2</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>5 mM ATP</td>
<td>1.54</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>5 mM ATP (prior Mg$^{2+}$ incubation)</td>
<td>1.05</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>5 mM Mg$^{2+}$, 5 mM ATP, 50 mM sulfite</td>
<td>32.96</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>5 mM ATP (prior EDTA incubation)</td>
<td>1.28</td>
<td>1.05</td>
<td></td>
</tr>
</tbody>
</table>

Comparison between exchange and the increase in activity as a function of time

CF$_1$-e-NT and CF$_1$-e refer to the TNP-ADP-loaded enzyme. $A_{\text{max}}$ has units of nmol of P$_i$/min/mg CF$_1$ and $v_0$ units of nmol of TNP-ADP exchanged per min/mg CF$_1$. The half-time of the fast phase of exchange was 4 s for the sample of CF$_1$-e-NT and 11 s for the sample of CF$_1$-e.

<table>
<thead>
<tr>
<th>CF$_1$-e-NT</th>
<th>CF$_1$-e</th>
</tr>
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<tbody>
<tr>
<td>Activity</td>
<td>Exchange</td>
</tr>
<tr>
<td>$A_{\text{max}}$</td>
<td>0.37</td>
</tr>
<tr>
<td>$A_{\text{max}}$</td>
<td>0.09</td>
</tr>
<tr>
<td>$v_0$</td>
<td>330</td>
</tr>
<tr>
<td>$v_0$</td>
<td>11</td>
</tr>
<tr>
<td>$v_0$</td>
<td>2.2</td>
</tr>
</tbody>
</table>

DISCUSSION

Our results show that CF$_1$-e with markedly decreased bound nucleotide may be obtained by treatment of the enzyme with alkaline phosphatase for at least 18 h. This nucleotide-depleted enzyme had rates of Ca$^{2+}$- and Mg$^{2+}$ ATPase activity (Table I and Fig. 3) equivalent to those of CF$_1$-e with endogenous ADP. These findings are consistent with the results obtained from nucleotide-depleted E. coli and mitochondrial F$_1$. The initial presence of tightly bound nucleotide was not required for catalysis in E. coli F$_1$ and mitochondrial F$_1$, exhibited no loss of ATPase activity following depletion of tightly bound nucleotide. However, bound nucleotide appears to stabilize mitochondrial F$_1$, as the presence of 50% glycerol is required throughout the depletion and further procedures to prevent protein denaturation (9). Our depletion procedure as well as all our assays are conducted in the absence of glycerol. Our cold inactivation data (Fig. 2) demonstrated that the absence of bound nucleotide has no significant effect on the structural stability of CF$_1$-e. Alkaline phosphatase treatment of mitochondrial or bacterial F$_1$ may be an effective way in which to deplete these enzymes of bound nucleotide. If so, this method for gentle removal of bound nucleotide would be preferred over the more laborious chromatographic methods.

In mitochondrial F$_1$, Mg$^{2+}$ forms a complex with bound ADP that inhibits catalytic activity (25). This has been suspected to be the case for CF$_1$-e. Due to the unavailability of nucleotide-depleted CF$_1$-e, it has not been proven conclusively that Mg$^{2+}$ inhibits ATPase activity by forming a complex with bound ADP. Although Mg$^{2+}$ binding to CF$_1$ has been shown to inhibit its catalytic activity (26), the necessity of bound ADP had not previously been demonstrated. Our studies of Mg$^{2+}$ inhibition of ATPase activity (Fig. 3) of CF$_1$ demonstrate that the decrease in the presence of ADP nor of Mg$^{2+}$ alone is sufficient to inhibit. The presence of Mg$^{2+}$ and bound ADP combined is responsible for the observed inhibition.

Earlier studies of the exchange of tightly bound ADP with nucleotide in the medium (27) concluded that CF$_1$ contains one tight binding site for ADP that exchanges and whose exchange is inhibited by Mg$^{2+}$. This site was considered to be the location of the endogenous ADP found in freshly prepared CF$_1$. More recent studies have shown that there are two tight ADP bind-
ing sites (4). It now seems clear that the two tight binding sites for ADP have quite different properties. One will exchange easily for medium nucleotide and the second exchanges very slowly, if at all. This is shown in two ways. CF$_1$-$e$ that contained endogenous tightly bound ADP would not exchange about 1 of the bound ADP for TNP-ADP, and CF$_1$-$e$ that contained 1.44 tightly bound TNP-ADP, but no bound ADP, exchanged only about 1 of the bound TNP-ADP. A similar result has been seen in rat liver mitochondrial F$_{1}$ that has been shown to contain two ADP binding sites, one of which exchanges with medium nucleotide and the second of which does not (28).

It is this nonexchanging, or poorly exchanging, site that appears to enable Mg$^{2+}$ to inhibit the exchange of bound nucleotide from the exchangeable site, as well as being the principle source of Mg$^{2+}$ inhibition of the ATPase activity. It has been suggested before (29) that an ADP binding site separate from the catalytic site is responsible for Mg$^{2+}$-induced inhibition of the ATPase activity, although it has not yet been demonstrated that the poorly exchanging site is not catalytic. In all cases in which Mg$^{2+}$ was present in the exchange medium, the rate of exchange of tightly bound TNP-ADP was inhibited in samples with bound ADP relative to those with little bound ADP. It is even possible that the nonexchanging ADP site is the only site at which a bound MgADP complex inhibits exchange, although this is not yet clear due both to the residual bound ADP in the preparation and the ability of TNP-ADP to bind to the nonexchanging site when it is vacant.

The exchange of TNP-ADP from the tight, easily exchanging ADP site was seen to correlate with an increase in the ATPase activity of the enzyme. The data presented in Fig. 5 and Table V might then suggest that it is the exchange of tightly bound ADP from the easily exchanging ADP tight binding site that activates the enzyme. However, it has also been seen (Fig. 4 and Table III) that nucleotide-depleted CF$_1$-$e$, which has no bound nucleotide to exchange, shows the same increase in activity over time. Thus, it would be one of the remaining sites, either the noncatalytic MgATP binding sites or the loose sites. It was shown by Milgrom et al. (30) that an increase in the ATPase activity of CF$_1$ is correlated with an increase in MgATP bound to the enzyme. It is also possible that the first turnover of the enzyme results in some conformational change that makes the succeeding turnovers faster.

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Differences between Two Tight ADP Binding Sites of the Chloroplast Coupling Factor 1 and Their Effects on ATPase Activity

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