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# Binding and Exchange of Nucleotides on the Chloroplast Coupling Factor $CF_1$

## The Role of Magnesium

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On the soluble part of the coupling factor ($CF_1$), extracted from spinach chloroplasts, three nucleotide-binding sites are identified. Three ADP are bound per $CF_1$ when the enzyme is incubated with ADP either with or without $Mg^{2+}$. Two ADP and one ATP are bound per $CF_1$ when the enzyme is incubated with a limiting concentration of ATP, in the presence of $Mg^{2+}$. At high ATP concentration, in the presence of $Mg^{2+}$, one free ATP exchanges with one bound ADP and two ATP and one ADP remain bound per $CF_1$. When $Mg^{2+}$ is omitted from the incubation medium of ATP and $CF_1$, only two ADP and around 0.5 ATP are bound per $CF_1$.

The three nucleotide binding sites of $CF_1$ fall into two different and independent categories according to the ability of the bound nucleotides to be exchanged with free nucleotides. On one site the bound ADP is difficult to exchange. On the other two sites, the bound nucleotides, ADP or ATP, are readily exchangable.

We propose that the two exchangeable sites form the catalytic part of the enzyme where ATP is hydrolyzed. When ATP concentration is high enough, in the presence of $Mg^{2+}$, one ATP displaces one bound ADP and allows the ATP hydrolysis to proceed. We propose too that the site where ADP is difficult to exchange may represent the 'tight' ADP-binding site, different from the catalytic ones, which becomes exchangeable on the $CF_1$ *in vivo* when the thylakoid membranes are energized by light, as stressed by Bickel-Sandkötter and Strotman [(1976) *FEBS Lett. 65*, 102–106].

Purified $CF_1$ solubilized from chloroplast membranes has no ATPase activity, unless activated by trypsin [1], heat [2] or dithiothreitol [3] but like the membrane-bound enzyme, isolated $CF_1$ can bind nucleotides in a non-covalent and non-energy-requiring process. The maximum number of bound nucleotides varies, depending on the way the complex AdN-$CF_1$ is recovered from the excess of free nucleotides. On the latent ATPase, the number of nucleotide-binding sites has been found to be two [4–6] or three [7–11] after rapid recovery of the AdN-$CF_1$ complex and only one after extensive and slow separation by Sephadex chromatography [7]. A conflicting point is the specificity of these sites for ATP or ADP and the effect of $Mg^{2+}$ on the binding or exchange of nucleotides on $CF_1$. This cation is usually added to the suspending medium of $CF_1$ even if its effect is not explicated. Hochman and Carmeli proposed that the $Mg^{2+}$ salt of the nucleotides is the true substrate for $CF_1$ [12].

The presence of $Mg^{2+}$ increases the affinity of the nucleotides for the sites of the isolated $CF_1$. Higashida and Mukohata [11] found the same number of bound nucleotides with and without $Mg^{2+}$ but, most of the other authors have reported an increase of this number by $Mg^{2+}$ in comparison with the number found in the presence of EDTA [5,7,8]. Hochman and Carmeli [12] studied the binding of $Mn^{2+}$ on $CF_1$ ($Mn^{2+}$ is supposed to be interchangeable with $Mg^{2+}$). They observed that $CF_1$ has one tight site and five loose sites for $Mn^{2+}$ binding and that various diphosphonucleotides or triphosphonucleotides induce the appearance of a second tight site and the disappearance of one of the loose sites. The authors suggested that the two tight sites are at the active site of the enzyme.

The purpose of this paper is to investigate the role of $Mg^{2+}$ on the amount and the nature of the nucleotides bound to the purified latent ATPase (inactive $CF_1$) supplemented with ATP or ADP. We have looked for a correlation between the binding of the nucleotides and of $Mg^{2+}$ to $CF_1$ and between their ability to exchange with free nucleotides and $Mg^{2+}$. ATP, ADP and $Mg^{2+}$ bound to $CF_1$ were directly and simultaneously determined. Exchange and direct binding processes were discriminated by labelling free nucleotides and $Mg^{2+}$ respectively by $^{14}C$ or $^{32}P$ and $^{27}Mg$; the specific radioactivity was measured for AdN as well as $Mg^{2+}$. It was shown that three adenine nucleotides can be bound per $CF_1$: three ADP when $CF_1$ is incubated with ADP in the presence or absence of $Mg^{2+}$ and one ADP plus two ATP when $CF_1$ is incubated with saturating concentrations of ATP, in the presence of $Mg^{2+}$. Some tentative conclusions are drawn from the experimental results concerning the nature and function of the binding and exchange of AdN and $Mg^{2+}$.

## MATERIALS AND METHODS

Coupling factor, $CF_1$ was extracted and purified from spinach leaves following the method of Lien and Racker with 1 mM EDTA [13]. No nucleotide was added at any step of

*Abbreviation.* AdN, adenine nucleotide.

the purification procedure. Only those fractions exhibiting a fluorescence intensity ratio 305 nm/340 nm (excitation at 280 nm) higher than 1.8 were collected and stored at 4 °C in 20 mM Tris/HCl (pH 8), 1 mM EDTA, 2 M $(NH_4)_2SO_4$, no added ATP. The purity of the $CF_1$ was also checked by sodium dodecyl sulfate/polyacrylamide gel electrophoresis [14] and the result gave the normal five-band pattern.

The molar concentration of $CF_1$ in 20 mM Tris/HCl (pH 8), in the absence of nucleotides, was determined spectrophotometrically, in the ultraviolet region, using an absorption coefficient of 0.476 $cm^2$/mg at 280 nm [10] and a molecular weight of 325000 [15]. In the presence of nucleotides, the protein content was estimated by the method of Lowry et al. [16] using purified $CF_1$ as a standard.

Before using the stock suspension of $CF_1$ for incubation with nucleotides, it was freed from ammonium sulfate and dissociable nucleotides, if any, by dialysis without dilution of $CF_1$, in a collodion membrane (Schleicher & Schüll) against 20 mM Tris/HCl (pH 8) and 0.5 mM EDTA.

### *Isolation of the Nucleotide-$CF_1$ Complex*

The AdN-$CF_1$ complex was liberated from the excess of free AdN by the method developed by Penefsky [17] called centrifugation-filtration; the mixture of free AdN and of AdN-$CF_1$ complex was layered on a small syringe tube filled with Sephadex G-50 fine and quickly separated by centrifugation.

### *Titration of Nucleotide*

Radioactive nucleotides were determined with a liquid scintillation system (mark III, Searle Analytice Inc.). Total ATP or ADP concentrations were determined by firefly bioluminescence using a purified luciferin-luciferase mixture (LKB-Wallace ATP monitoring kit 1250-120). AdN bound to $CF_1$ were released by addition of 2 M HCl at 0 °C then the medium was adjusted to pH 8 with 1 M Tris base. ATP and ADP were determined in aliquots of 0.45 ml 0.1 M Tris/$HCH_3COO$ (pH 7.75), 2 mM EDTA, 10 mM $MgSO_4$, 12.5 mM $K_2SO_4$, 80 μM phospho*enol*pyruvate and containing 8–10 μg of $CF_1$. The reaction was initiated by addition of 50 μl of the luciferin-luciferase mixture; then 2 μl of pyruvate kinase (10 mg/ml) was added. Luminescence was measured in a Muka luminomètre. Each determination was repeated at least twice and calibrated by the addition of a known amount of ATP equivalent to the amount titrated.

### *Titration of Magnesium*

Non-radioactive magnesium was determined by atomic absorption with a Perkin-Elmer 290 B atomic spectrophotometer. Aliquots of 0.08 ml, containing 15–20 μM of the AdN-Mg-$CF_1$ complex were routinely used. Calibration was done with a 5 μM $Mg(NO_3)_2$ stock solution.

The isotope $^{27}Mg$ (half-life 9.45 min) was produced by neutronic activation during a $(n, \gamma)$-reaction. The thermal neutrons were provided by the OSIRIS reactor of the Commissariat à l'Energie Atomique. Irradiations lasted 15–30 min. $^{27}Mg$ was titrated in 0.1 ml of AdN-Mg-$CF_1$ complex, freed from the excess of Mg by centrifugation-filtration, after a decay of one or two periods. Gamma peaks at 844 and 1014 keV were sampled with a Plurimat analyzer and calibrated with ultra-pure $Mg(NO_3)_2$ solutions.

### *Chemicals*

The standard solutions of $Mg(NO_3)_2$ were prepared from spectrographic quality MgO (Johnson Matthey Chemicals, Ltd).

5'[$\gamma$-$^{32}$P]ATP, 3 Ci/mmol was obtained from Amersham, [8-$^{14}$C]ADP, 48 Ci/mmol from Schwarz-Mann and [U-$^{14}$C]-ATP, 538 Ci/mol from New England Nuclear.

## RESULTS

### BINDING OF ADP, ATP AND $Mg^{2+}$ ON $CF_1$

#### *Binding of ADP and ATP on $CF_1$. Effect of the $Mg^{2+}$*

When $CF_1$ is incubated with [$^{14}$C]ADP, some radioactive nucleotide is found to bind to $CF_1$. The amount of bound [$^{14}$C]ADP was measured versus the concentration of added [$^{14}$C]ADP after room-temperature incubation for 1 h. In the presence of 1 mM $Mg(NO_3)_2$ (Fig. 1), up to 3 moles of ADP are bound per mole $CF_1$ after isolation of the AdN-$CF_1$ complex by centrifugation-filtration, including the 0.6 mole ADP still present on the $CF_1$ even after extensive dialysis. The half-saturation of the binding sites with ADP is achieved with an initial ADP addition of 20 μM. In the absence of $Mg^{2+}$, essentially the same type of binding curve is observed but the half-saturation is achieved with an addition of 40 μM ADP.

The same type of experiment was performed with ATP added in the incubation medium. After an incubation of [$\gamma$-$^{32}$P, U-$^{14}$C]ATP with $CF_1$, in the absence of $Mg^{2+}$, 2 moles of ADP are found bound per mole $CF_1$ but ATP binding is very weak and reaches 0.5 mole per mole $CF_1$ at the highest external ATP concentration used (Fig. 2). When the same experiment is performed in the presence of 1 mM $Mg^{2+}$, the results are very different (Fig. 3). Between 0 and 50 μM ATP addition, the amount of ADP bound to $CF_1$ increases up to 2 ADP per $CF_1$ and the amount of ATP bound attains 1 ATP per $CF_1$. Above 50 μM ATP addition, the amount of bound ADP decreases progressively to 1 ADP per $CF_1$ and the amount of bound ATP increases up to 2 ATP per $CF_1$. Apparently 1 ATP replaces 1 ADP on the $CF_1$.

The presence of $Mg^{2+}$ seems to enhance the affinity of $CF_1$ for ATP and for ADP. We cannot preclude that $Mg^{2+}$ may stabilize the complex AdN-$CF_1$, during the isolation procedure, without modifying the intrinsic affinity of $CF_1$ for the nucleotides. Nevertheless the nature of the nucleotide bound to $CF_1$ depends on the presence of $Mg^{2+}$ which favors the formation of the complex ATP-$CF_1$.

The plots of nucleotide binding versus concentration of added ATP or ADP show that the number of $^{14}$C-labelled nucleotides bound is always smaller than the total amount of nucleotides bound. This discrepancy indicates that the ADP already bound on $CF_1$ does not exchange extensively with the free nucleotides of the medium. We will discuss this point later.

Contrarily the amount of $^{32}$P fixed is equal to the amount of bound ATP (Fig. 2 and 3). So all of the ATP bound to $CF_1$ comes from the binding of free [$\gamma$-$^{32}$P]ATP from the medium or from the phosphorylation of the bound ADP by free [$\gamma$-$^{32}$P]ATP.

Apart from the effect of $Mg^{2+}$, two series of questions are thus raised by the experiments described: what are the processes which allow ADP to be bound to $CF_1$ when only free ATP is added to the incubation medium, and what mechanism leads to the replacement of bound ADP by ATP on the sites

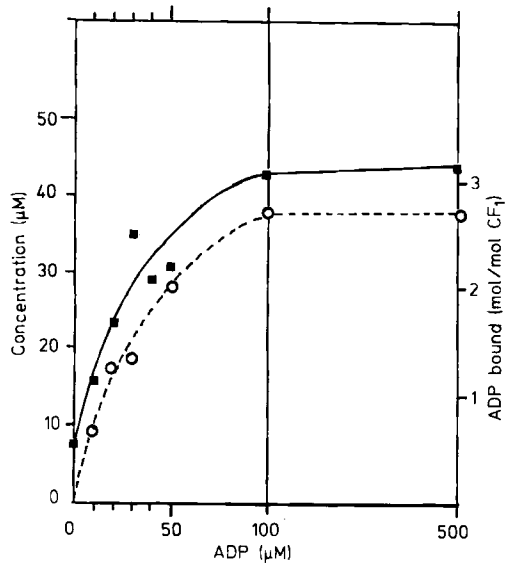


Fig. 1. *Plot of the amount of ADP bound to $CF_1$ versus the initial concentration of added [$^{14}$C]ADP, in the presence of $Mg^{2+}$.* The $CF_1$ (14 μM) was incubated in 20 mM Tris/HCl (pH 8), 0.5 mM EDTA, 1 mM $Mg(NO_3)_2$ and [$^{14}$C]ADP. After 1 h the [$^{14}$C]AdN-$CF_1$ complex was separated from the free [$^{14}$C]ADP by centrifugation-filtration. Bound ADP (■) and bound [$^{14}$C]ADP (○) were expressed by their concentrations (left-hand scale) or the number of molecules per $CF_1$ (right-hand scale)

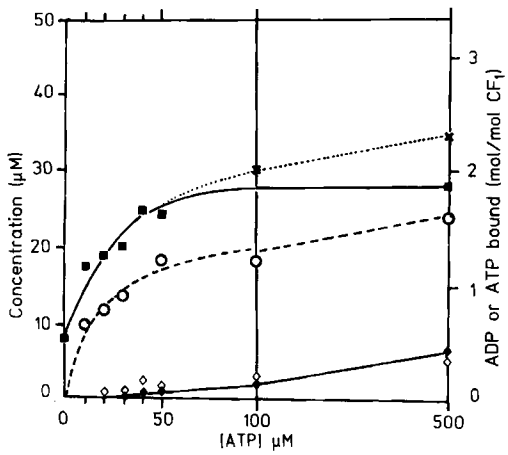


Fig. 2. *Plot of the amount of ADP or ATP bound to $CF_1$ versus the initial concentration of [$\gamma$-$^{32}$P, U-$^{14}$C]ATP, in the absence of $Mg^{2+}$.* The $CF_1$ (14.8 μM) was incubated, during 1 h, in 20 mM Tris/HCl (pH 8), 0.5 mM EDTA and a variable concentration of [$\gamma$-$^{32}$P,$^{14}$C]ATP as indicated on the horizontale scale. Bound nucleotides were expressed by their concentrations (left-hand scale) or the number of moles per $CF_1$ (right-hand scale): ATP (◆); ADP (■); ATP + ADP (×); [$^{14}$C]AdN (○); and [$^{32}$P]AdN (◇)

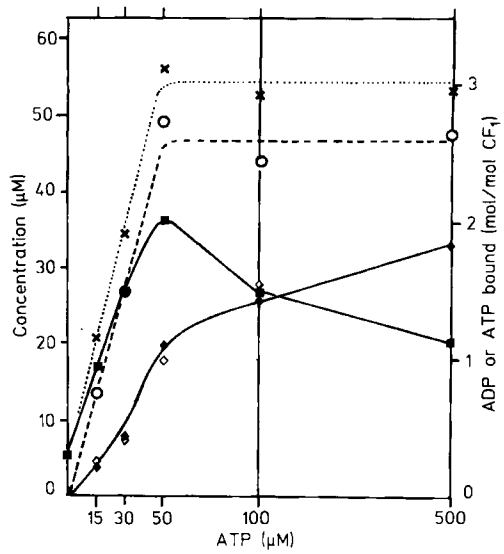


Fig. 3. *Plot of the amount of ADP or ATP bound to $CF_1$ versus the initial concentration of [$\gamma$-$^{32}$P, U-$^{14}$C]ATP, in the presence of $Mg^{2+}$.* The $CF_1$ (18 μM) was incubated in the same medium as in Fig. 2 but in the presence of 1 mM $Mg(NO_3)_2$. The other conditions and symbols on the curves were the same as in Fig. 2

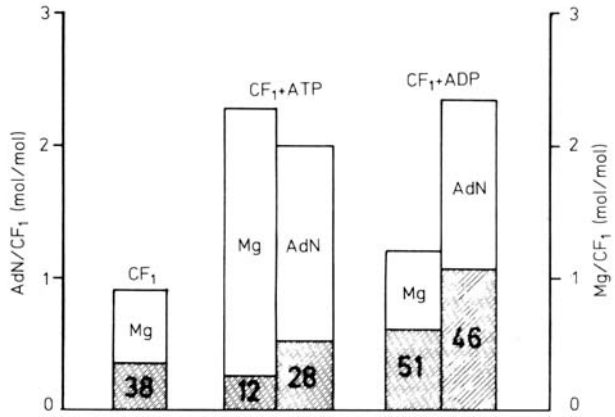


Fig. 4. *Amount of magnesium and AdN bound or exchanged during a short incubation (5 min) with $CF_1$.* Amount of $Mg^{2+}$ and (or not) of AdN bound to $CF_1$ were measured after an incubation during 5 min of 21 μM $CF_1$, extensively dialyzed, with 0.5 mM $^{26-27}Mg(NO_3)_2$ and with or without [$^{12-14}$C]ATP or [$^{12-14}$C]ADP. Exchange of free and bound substrate was measured as following. First 21 μM $CF_1$ was loaded with non-radioactive substrate by a 10-min incubation in the presence of 0.25 mM $^{26}Mg(NO_3)_2$ and (or not) 1 mM [$^{12}$C]ATP or ADP. Then the exchange of bound and free substrate was observed after addition of $^{27}Mg(NO_3)_2$ and with or without [$^{14}$C]ADP or ATP. Total concentration of $Mg^{2+}$ was 0.5 mM and of nucleotides if present, 2 mM during the 5 min of the incubation period. Binding was expressed by the number of moles bound per mole $CF_1$ (left hand scale for AdN, right hand scale for Mg) and exchange by the percentage of the free substrate exchanged with the bound one (numbers in the scored blocks)

of $CF_1$ when the concentration of added ATP is raised over 50 μM in the presence of $Mg^{2+}$?

### *Binding of $Mg^{2+}$ on $CF_1$. Effect of the Nucleotides*

The number of $Mg^{2+}$ ions bound to $CF_1$ has been measured before and after incubation with $Mg(NO_3)_2$ in the presence or absence of ADP or ATP. After extensive dialysis of $CF_1$ and in the absence of added $Mg(NO_3)_2$ or nucleotides, the bound magnesium is sometimes hardly measurable and always less thant 0.6 $Mg^{2+}$ per $CF_1$. After incubation of $CF_1$ with a magnesium salt, the number of cations bound increases to a value close to one $Mg^{2+}$ per $CF_1$ (0.92 $Mg^{2+}$ per $CF_1$ in Fig. 4). If nucleotides are added concomitantly with $Mg^{2+}$, this value increases to 1.25 $Mg^{2+}$ per $CF_1$ in the presence of ADP and 2.3 $Mg^{2+}$ per $CF_1$ in the presence of ATP (Fig. 4). In this last case the amount of bound $Mg^{2+}$ runs parallel to the total amount of nucleotides (ATP + ADP) bound to $CF_1$.

## EXCHANGE OF $Mg^{2+}$, ADP AND ATP BOUND TO $CF_1$

### *Effect of the Nucleotides on the $Mg^{2+}$ Exchange*

The following experiments have been performed to test whether $Mg^{2+}$ and nucleotides bound to $CF_1$ can be exchanged with free $Mg^{2+}$ and nucleotides added in the medium. For this purpose, $CF_1$ is first loaded with non-radioactive substrate $^{26}Mg(NO_3)_2$ in the presence or absence of [$^{12}$C]-ATP or ADP. Then the exchange between bound and free $Mg^{2+}$ or AdN is observed after addition of $^{27}Mg(NO_3)_2$ in the presence or absence of [$^{14}$C]ADP or ATP (Fig.4).

After 5 min the exchange extent for $Mg^{2+}$ and nucleotides respectively is: 12% and 28% in the presence of free $Mg^{2+}$ and ATP; 51% and 46% in the presence of free $Mg^{2+}$ and ADP. In the same time period. 38% of the bound $Mg^{2+}$ is exchanged with the free $Mg^{2+}$ in the absence of added nucleotides.

### *Effect of $Mg^{2+}$ on the Nucleotide Exchange*

The exchange of the $CF_1$-bound nucleotides has been further studied during longer periods from 0.5–22 h. In the experiment reported in Table 1, $CF_1$ saturated with [$^{14}$C]-ADP or [$^{14}$C]ATP, in the presence or absence of $Mg^{2+}$, is freed from the excess of $^{14}$C-labelled nucleotides and transferred to a medium containing free [$\gamma$-$^{32}$P]ATP with or without $Mg^{2+}$. The results of an exchange period of 1 h are summarized below.

$CF_1$ first exposed to [$^{14}$C]ATP exchanges its bound nucleotides with free [$\gamma$-$^{32}$P]ATP more slowly than $CF_1$ exposed first to [$^{14}$C]ADP (compare lines 1–4 with lines 5–8).

The presence of $Mg^{2+}$ during the first exposure of $CF_1$ to the nucleotides stabilizes the complex AdN-$CF_1$ and the exchange rate of the bound nucleotides with the free ATP is slowed down (compare lines 1–2 and 5–6 respectively with lines 3–4 and 7–8).

These results permit us to classify the AdN-$CF_1$ complexes according to their decreasing stability: Mg-ATP-$CF_1$ > ATP-$CF_1$ > Mg-ADP-$CF_1$ > ADP-$CF_1$.

### *Effect of $Mg^{2+}$ on the Free Nucleotides during their Incubation with $CF_1$*

For this purpose free and bound nucleotides were titrated after an incubation of $CF_1$ with ATP. After 1 h, if the concentration of added ATP is less than three times that of $CF_1$, practically all the ATP added to the medium is bound to $CF_1$ as ADP or ATP (lines 3–6 in Table 2). When the concentration of added ATP is over 20 times that of $CF_1$, besides the two ATP and one ADP bound per $CF_1$ in the presence of $Mg^{2+}$, the free ATP is recovered half hydrolyzed into ADP (line 1 in Table 2). If $Mg^{2+}$ is omitted, only 10% of the free ATP is hydrolyzed into ADP (line 2 in Table 2).

### *Origin of the Bound ADP and ATP during Incubation of $CF_1$ with ATP and $Mg^{2+}$*

In addition to ATP, one or two ADP are bound per $CF_1$, depending on the concentration of the added ATP in the presence of $Mg^{2+}$.

Experiments were achieved to test whether ADP bound to $CF_1$ is the result of ATP hydrolysis *in situ* (bound ATP gives bound ADP) or of the fixation of the ADP which has been released in the medium after ATP hydrolysis on the catalytic site(s). The addition in the medium of phospho*enol*-pyruvate plus pyruvate kinase, an ATP-regenerating system which eliminates the free ADP, prevents only 15% of the ADP from being bound to $CF_1$ (Table 2, lines 7–8). The ADP bound to $CF_1$ is thus mostly the result of direct hydrolysis of ATP into ADP and inorganic phosphate ($P_i$) on the site where ATP was bound and where ADP remains afterwards.

We have seen previously (Fig. 3) that when the ATP concentration is increased beyond 50 μM, progressively 2 ATP and 1 ADP are bound per $CF_1$ instead of 2 ADP and 1 ATP at lower ATP concentrations. Apparently 1 ADP is replaced by 1 ATP. Experiments were done to test whether the ATP bound to $CF_1$ is the result of an exchange of bound ADP with free ATP or of the phosphorylation of the bound ADP by free ATP. $CF_1$ was incubated with saturating amounts of [$^{14}$C]ADP and then recovered as a complex [$^{14}$C]-ADP-$CF_1$ by centrifugation filtration in a medium supplemented with [$\gamma$-$^{32}$P]ATP (Table 3). After an exchange period of 0.5–2 h the complex AdN-$CF_1$ was recovered and analyzed for its content in $^{14}$C, $^{32}$P, ATP and ADP bound to the protein. The total amount of bound AdN changes only to a small extent (0.4 AdN/$CF_1$) during the second incubation, i.e. from 2 to 2.4 AdN per $CF_1$ (column 5 in Table 3), but bound ATP increases from 0 to 0.9 ATP per $CF_1$ (column 4 in Table 3) when bound ADP decreases from 2 to 1.4 ADP per $CF_1$ (column 3 in Table 3). In parallel, the amount of $^{14}$C decreases from 1 to 0.4 per $CF_1$ (column 1 in Table 4) and the amount of $^{32}$P increases from 0 to 1 $CF_1$ (column 2 in Table 3). If the [$^{32}$P]ATP bound to $CF_1$ was the result of a direct phosphorylation of the bound [$^{14}$C]ADP by the external [$^{32}$P]ATP, then the increase in the amount of bound $^{32}$P would correlate with no change in the amount of bound $^{14}$C. Since that is not the case, we conclude that bound ATP is the result of an exchange between bound ADP and free ATP. There is no indication of a direct phosphorylation of the bound ADP by the free ATP.

Table 1. *Exchange of the [$^{14}$C]AdN bound to the AdN-$CF_1$ or AdN-Mg-$CF_1$ with free ATP*

$CF_1$ was first allowed to bind [$^{14}$C]AdN in the following medium: 15 μM $CF_1$, 20 mM Tris/HCl (pH 8), when present 1 mM $Mg(NO_3)_2$ and 0.5 mM [$^{14}$C]ATP or [$^{14}$C]ADP. After 2 h the [$^{14}$C]AdN-$CF_1$ or [$^{14}$C]-AdN-Mg-$CF_1$ complex was recovered by centrifugation-filtration directly in the same medium but with 0.5 mM ATP with or without 1 mM $Mg(NO_3)_2$. After 1 h AdN-$CF_1$ complex was recovered again by centrifugation-filtration

| Additions | | [$^{14}$C]AdN bound per $CF_1$ during the first stage | Fraction of the bound [$^{14}$C]AdN exchanged in the second stage |
|---|---|---|---|
| first stage | second stage | | |
| | | | % |
| [$^{14}$C]ATP | ATP + $Mg^{2+}$ | 1.56 | 39 |
| [$^{14}$C]ATP | ATP | 1.56 | 46 |
| [$^{14}$C]ATP + $Mg^{2+}$ | ATP + $Mg^{2+}$ | 2.00 | 20 |
| [$^{14}$C]ATP + $Mg^{2+}$ | ATP | 2.00 | 16 |
| [$^{14}$C]ADP | ATP + $Mg^{2+}$ | 2.04 | 60 |
| [$^{14}$C]ADP | ATP | 2.04 | 83 |
| [$^{14}$C]ADP + $Mg^{2+}$ | ATP + $Mg^{2+}$ | 2.30 | 54 |
| [$^{14}$C]ADP + $Mg^{2+}$ | ATP | 2.30 | 56 |

Table 2. *Effect of the $Mg^{2+}$ and of the addition of an ATP-regenerating system during the incubation of the $CF_1$ with ATP. Nature of the free and bound nucleotides recovered after different times of incubation*
The incubation medium was 20 mM Tris/HCl (pH 8), 17 μM $CF_1$, ATP as indicated, 1 mM $Mg(NO_3)_2$ when present. The regenerating system was 2 mM phospho*enol*pyruvate (*P*Pyr), 20 μg pyruvate kinase in a 0.1 ml volume. Results were given as concentration of free or bound nucleotides and number of molecules per $CF_1$ (numbers between brackets)

| No. | Addition | Time of incubation | AdN bound | | AdN free | |
|---|---|---|---|---|---|---|
| | | | ATP | ADP | ATP | ADP |
| | | h | μM | | | |
| 1 | ATP 400 μM + $Mg^{2+}$ | 1 | 34.5 (2.03) | 19.0 (1.12) | 194 | 147 |
| 2 | ATP 435 μM | 1 | 23.4 (1.38) | 28.7 (1.69) | 351 | 32 |
| 3 | ATP 50 μM + $Mg^{2+}$ | 1 | 19.2 (1.13) | 29.5 (1.74) | 1.05 | 0.75 |
| 4 | ATP 50 μM | 1 | 4.2 (0.25) | 27.8 (1.64) | 2.30 | 16.25 |
| 5 | ATP 30 μM + $Mg^{2+}$ | 1 | 9.1 (0.54) | 17.0 (1.0) | 1.28 | 3.22 |
| 6 | ATP 34 μM | 1 | 1.3 (0.08) | 24.1 (1.42) | 0.90 | 7.7 |
| 7 | ATP 64 μM + $Mg^{2+}$ | 1 | 18.87 (1.11) | 25.53 (1.49) | | |
| 8 | ATP 64 μM + $Mg^{2+}$ + *P*Pyr + pyruvate kinase | 1 | 21.93 (1.29) | 19.38 (1.14) | | |

Table 3. *Exchange and fixation of AdN on a $CF_1$, preloaded with [$^{14}$C]-ADP, during an incubation with [γ-$^{32}$P]ATP*
The medium during the first incubation was 20 mM Tris/HCl (pH 8), 15 μM $CF_1$, 0.5 mM [$^{14}$C]ADP; $Mg^{2+}$ was omitted. After 2 h, the [$^{14}$C]-ADP-$CF_1$ complex was freed from the excess of [$^{14}$C]ADP by centrifugation-filtration and supplemented straight with 0.5 mM [γ-$^{32}$P]ATP 1 mM $Mg(NO_3)_2$. At intervals, as reported, aliquots of 0.1 ml were freed from the excess of AdN by centrifugation-filtration and analyzed for $^{14}$C, $^{32}$P, ADP and ATP

| Time of the exchange | Bound $^{14}$C/bound ADP | Bound $^{32}$P/bound ATP | Bound ADP/$CF_1$ | Bound ATP/$CF_1$ | Total bound AdN/$CF_1$ |
|---|---|---|---|---|---|
| h | | | | | |
| 0 | 1.03 | | 2.04 | 0 | 2.04 |
| 0.5 | 0.35 | 1.10 | 1.44 | 0.79 | 2.23 |
| 1.0 | 0.39 | 0.95 | 1.47 | 0.89 | 2.36 |
| 2.0 | 0.39 | 1.10 | 1.38 | 0.91 | 2.29 |

### *Presence of One Site on $CF_1$ where Bound ADP Is Difficult to Exchange with the Free Nucleotides*

We have already shown that not all the bound nucleotide exchanges with the free nucleotide (Fig. 4 and Table 1).

When $CF_1$ was first loaded with [$^{14}$C]ADP, then freed from the excess of free nucleotides and supplemented with free unlabelled ATP, the bound $^{14}$C-labelled nucleotides are progressively replaced by unlabelled nucleotides. The extent of exchange increases at higher concentrations of added unlabelled nucleotides and for longer times of incubation, with normal ATP. But this exchange is never complete and there remains at least 0.4–0.6 $^{14}$C-labelled nucleotides, most likely [$^{14}$C]ADP, per $CF_1$ (Table 3).

## DISCUSSION

### *Properties of the Different Nucleotide-Binding Sites*

The varibility of the methods used to titrate the nucleotides and the specific properties of $CF_1$ may explain the dispersion of the results published about nucleotide binding. If the determination of the affinity constants of $CF_1$ for the nucleotides appears to be a difficult objective, our data give some information on the nature of the binding sites and on the effect of $Mg^{2+}$.

The first point which seems well established is the presence of at least three nucleotide-binding sites on $CF_1$. In the presence of saturating amounts of ATP and $Mg^{2+}$ we find 2 ATP and 1 ADP bound per $CF_1$, agreeing with the results reported by Harris and Slater [18] for binding of the nucleotides on $CF_1$ *in vivo*.

The second point concerns the exchange of the nucleotides bound on $CF_1$. As already emphasized by Carlier and Hammes [7], the binding sites on isolated $CF_1$, the latent enzyme, fall into two different categories according to the ability of the bound nucleotides to be exchanged with free nucleotides.

### *The Site where Bound ADP is Difficult to Exchange*

One site where the nucleotide is difficult to exchange has been characterized. It is the site where ADP is found bound after extraction and purification of the $CF_1$. This ADP is not desorbed by ammonium sulfate precipitation and Sephadex filtration. Extensive dialysis against 1 mM EDTA containing buffer does not remove all this ADP since 0.4–0.6 ADP remains bound per $CF_1$ after this treatment. Treatment with 50% glycerol, according to Senior [19], does not succeed in removing this ADP. This ADP exchanges very slowly with added free ATP of ADP in the presence of absence of $Mg^{2+}$ but not with the nucleotides bound to the other sites on $CF_1$.

More likely this hardly exchangeable ADP is equivalent to the nucleotide which has been found by Strotmann et al. [20] on isolated $CF_1$ from chloroplasts. This nucleotide becomes exchangeable with free nucleotides only when the chloroplast membranes where the $CF_1$ is buried are energized by the light which induces ATPase activity. We favor a scheme where the 'tight' ADP binding takes place on a site of the active enzyme different from the catalytic ones [21]. This nucleotide binding, as already discussed by Bickel-Sandkötter and Strotmann [22] has no strict requirement for $Mg^{2+}$ since 50% of this nucleotide is exchanged in the light, in the presence of 1 mM EDTA.

### *The Two Sites where the Bound Nucleotides are Readily Exchangeable*

Two other nucleotide binding sites on $CF_1$ are readily exchangeable. In fact, the exchange between bound and free nucleotides observed with $CF_1$ which has been previously

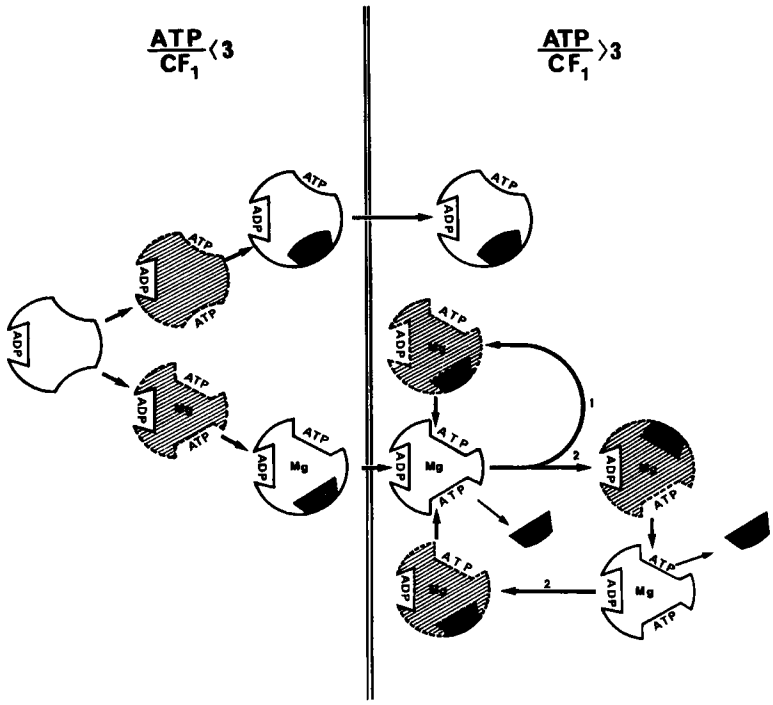


Fig. 5. *Final scheme of the hypothetical role of nucleotide binding sites on $CF_1$.* The contours with interrupted lines represent transitory but short-lived and undetectable states. In the left part of the figure, $CF_1$ is supplemented with ATP at a concentration inferior to three times the $CF_1$ concentration and in the right part at a concentration more than three times the $CF_1$ concentration. $Mg(NO_3)_2$ is absent in the upper part and present in the lower part of the figure. Hydrolysis of ATP proceeds only in the right part of the scheme. In pathway 2, two sites hydrolyze alternatively ATP. In pathway 1, ATP is hydrolyzed always on the same site. The black blocks represent ADP bound to $CF_1$ or free in the medium

loaded with nucleotides concerns mainly these two sites at least for short incubation periods.

This exchange depends on the presence of $Mg^{2+}$ bound on the $CF_1$ or free in the supernatant. $Mg^{2+}$ in all cases stabilizes the complex AdN-$CF_1$ and decreases the exchangeability of the bound nucleotides (Fig. 4 and Table 1). The complex AdN-$CF_1$ is more stable and the bound AdN less exchangeable when $CF_1$ is preloaded with ATP than with ADP (Table 1). That is to be compared with the lower $^1H$-$^2H$ exchange of the peptidic bonds in the Mg-ATP-$CF_1$ than in the Mg-ADP-$CF_1$ complexes [23].

The apparent higher affinity of $CF_1$ for ADP or ATP in the presence of $Mg^{2+}$ than in its absence could be the result of the higher stability of the AdN-$CF_1$ complex when $Mg^{2+}$ is present; the Mg-AdN-$CF_1$ complex is not decomposed during the centrifugation-filtration contrary to the AdN-$CF_1$ complex when $Mg^{2+}$ is absent. This proposal is confirmed by previous analyses of the AdN-$CF_1$ complex with circular dichroism (without separation of the complex) where we did not find any difference between the affinity of $CF_1$ for the nucleotides in the presence and absence of $Mg^{2+}$ (unpublished results).

After addition of ADP, we find at saturation, 2 ADP fixed on these sites in the presence of $Mg^{2+}$ or not. Conversely, in the presence of ATP and $Mg^{2+}$, we find 1 ADP and 1 ATP or 2 ATP bound to these sites, according to the concentration of the nucleotide (Fig. 3). If we omit the $Mg^{2+}$, we find besides 1 ADP around 0.5 ATP bound to these sites (Fig. 2). The simplest hypothesis is that these sites can bind either ATP or ADP. This is supported by the exchange of 1 ADP by 1 ATP when the concentration of added ATP increases from 3–10-times this of the $CF_1$ in the presence of $Mg^{2+}$ (Fig. 3 and Table 3).

### *Final Scheme of the Hypothetical Role of Nucleotide Binding Sites on $CF_1$*

The Fig. 5 shows the hypothetical scheme that we propose to explain the results that we have obtained.

After addition of ATP, even if $Mg^{2+}$ is omitted, we find ADP bound to $CF_1$. This bound ADP comes from the hydrolysis of the bound ATP on the enzyme and not from free ADP which could have been released from the protein after ATP hydrolysis (Table 2). The simplest assumption is that, after ATP addition, ADP is bound on the sites of $CF_1$ where bound ATP has been hydrolyzed.

In the absence of $Mg^{2+}$, this bound ADP coming from hydrolyzed ATP is not replaced by ATP even after addition of a high concentration of ATP (see upper part on Fig. 5). The release and replacement of the ADP on this site needs the presence of $Mg^{2+}$ and the presence of an ATP bound to the other site. (See lower part on Fig. 5.)

We propose that the two exchangeable sites form the catalytic part of the enzyme where ATP is hydrolyzed. We cannot say whether one site of the catalytic part of $CF_1$ hydrolyzes ATP, the second one regulating this process (pathway 1 on Fig. 5) or the two sites hydrolyzes ATP alternately in a flip-flop process (pathway 2 on Fig. 5). In that case the binding of ATP on one site would induce the exchange of the ADP bound on the second by a new molecule of ATP with a concomitant hydrolysis of the ATP bound on the first site. Nevertheless we favor pathway 2 where the two sites play alternately the same role. We have one reason for this choice. $CF_1$ contains two identical $\beta$ subunits. As the two sites involved in the catalytic activities are supposed to be on the $\beta$ subunit, they should be equivalent. In the scheme these sites exercise a cooperative control of the enzyme

activity. Such a cooperativity has been discussed by Grubmeyer and Penefsky for $F_1$ activity [24].

Our results can be compared with those obtained by Grubmeyer and Penefsky [24] on the mitochondrial ATPase, $F_1$. These authors have used an ATP analog, the 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine triphosphate, NTP, which is hydrolyzed 600-fold slower by $F_1$ than ATP. This slow hydrolysis permits one to characterize intermediary steps. With $CF_1$, ATP is only slowly hydrolyzed by the latent enzyme in the presence of $Mg^{2+}$ and practically not in its absence. So we can use ATP with $CF_1$, in the same way as NTP was used with $F_1$, to obtain evidence about AdN-$CF_1$ complexes which are short-lived and not isolatable when the enzyme is fully activated.

In $F_1$ and in $CF_1$, the catalytic part of the enzyme bears two nucleotide-binding sites. In $F_1$, ATP is hydrolyzed on the two sites and in $CF_1$ we cannot exclude that the two sites hydrolyze ATP alternatively. It has been proposed [24] that in $F_1$ the rate-limiting step during steady state hydrolysis is the breakage of the phosphate ester bond or a step subsequent to binding and prior to bond breakage. In $CF_1$ the limiting step is the release of ADP after the bond breakage. The two results can be reconcilied if we remark that the extent of the [$^{32}$P]NTP hydrolysis has been determined by the release of bound $^{32}$P from $F_1$, in a two-step experiment. In the first stage, [$^{32}$P]NTP was bound to $F_1$ in the absence of $Mg^{2+}$; in a second stage, $Mg^{2+}$ and non-radioactive NTP were added and the release of $^{32}$P from $F_1$ was taken as a measure of the induced [$^{32}$P]NTP hydrolysis. It is not excluded that even during the first stage [$^{32}$P]NTP can be hydrolyzed and $^{32}$P-labelled inorganic phosphate can be bound to $F_1$. So the limiting step could be, as in $CF_1$, the release of the products of NTP hydrolysis from $F_1$.

The scheme we propose does not take into account the ATPase activation and the possible role of the ADP bound to $CF_1$ and difficult to exchange (represented on the left part of the enzyme on Fig. 5).

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