Nucleotide Binding and ATPase Activity of Membrane Bound Chloroplast Coupling Factor (CF_1)

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Z. Naturforsch. **43c**, 133–139 (1988); received June 19, 1987

Chloroplasts, CF_0CF_1, Light Triggered ATPase Activity, Regulation, Mechanism of Inactivation

It has been found that the decay of light triggered ATPase activity is paralleled by non-exchangeable type of binding of one ADP per mol of CF_1. Inactivation of light triggered ATPase activity is a continuous process. It can be described mathematically as shown in this paper. Three simplified reaction schemes are described, each of them describing the mode of ATPase inactivation in a mechanistic way. A computer simulation of each model was performed and the results were compared to the observed experimental data.

In the presence of phosphate our results were explained best by a model assuming that there is at first a rapid binding of ADP to "exchangeable" sites. Due to a slowly occurring change in the CF_1 protein with prolonged incubation, an increasing portion of the nucleotides can be found bound in a non-exchangeable way after some seconds of incubation. Our model does not rule out that this reaction sequence might be a part of a catalytic cycle *in vivo*. – Another result was the finding that there is an additional diminution of ATPase activity when phosphate is lacking.

Introduction

In the dark period, following the phosphorylation of a limiting amount of ADP in a thylakoid suspension, an ATPase activity can be observed [1]. This activity can be stabilized by addition of DTE during the illumination step; its decay can be accelerated by addition of ADP in the dark step [2] particularly when added immediately after the light has been switched off.

These results have been explained by Bickel-Sandkötter and Strotmann [3] by assuming two different classes of nucleotide binding sites: tight sites and catalytic sites. This interpretation was confirmed by the finding that activation and inhibition of ATPase activity are paralleled by a release and rebinding of tightly bound ADP [2]. In contrast Boyer and coworkers proposed a model suggesting that alternating states of the nucleotide binding sites are involved in the process of ATP synthesis. They thought that tight binding of nucleotides to the active site is one step within the catalytic process [4, 5].

We measured nucleotide binding and ATPase activity in parallel experiments using DTE/NEM pre-treated thylakoid preparations. We defined three simplified mechanisms, each of them able to explain an inhibitory effect of ADP on ATPase activity. But at first we failed to prove "the correct mechanism" by computer simulation of our data. Therefore we reinvestigated the ADP effect on ATPase activity and showed that high ATPase activity depends on the presence of phosphate in the incubation mixture. In the presence of 5 mM phosphate we found a correlation between tightly binding of ADP and inhibition of ATPase activity. But in terms of our model, ADP binding must be followed by a second (slow) step, a conformational change for instance, to result in an inhibition of ATPase activity.

Abbreviations: DTE, dithioerythritol; PMS, N-methylphenazonium methosulfate; Chl, chlorophyll.

Enzyme: Chloroplast ATPase (EC 3.6.1.34).

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0100–0133 $ 01.30/0

Materials and Methods

Thylakoids from spinach chloroplasts were prepared using the method of Strotmann and Bickel-Sandkötter [6]. Chlorophyll determination was carried out as described by Arnon [7]. Thiol modification of CF_1 was performed as described by Ketcham *et al.* [8]. Using such modified thylakoid preparations ATPase activity was determined from the hydrolysis of [γ-^{32}P]ATP measured in aliquots taken at short intervals. In order to achieve maximal ATPase activity thylakoids were illuminated for 30 sec prior to each ATPase test. In earlier experiments (see Fig. 2 and 3) the reactivation medium did not contain additional phosphate. Organic phosphate was deter-

mined according to the method of Sugino and Miyoshi [9].

Nucleotide binding to CF_1

The total number of bound labelled nucleotides was determined from competition experiments with unlabelled AMP and ADP: The incubation medium contained 30 mM tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, thylakoids corresponding to 60 μg Chl per ml, and varying concentrations of [8-^{14}C]ADP and unlabelled AMP. The specific activity of [8-^{14}C]ADP varied from 1.85 to 2.20 GBq/mmol. Purity of the nucleotides was controlled by thin layer chromatography on PEI-cellulose sheets (layer: 0.1 mm Cel-PEI-MN 300, Macherey-Nagel Cop.) developed with 0.75 M KH_2PO_4, pH 4.1.

The total incubation medium was illuminated in a Beckman centrifuge with top illumination equipment. After a period of illumination, as indicated in the experiment, the sample was centrifuged at $15{,}000 \times g$. The lower part of the pellet not in contact with the aqueous medium was cut and resuspended thylakoids were analyzed for the total number of bound labelled nucleotides. The chlorophyll concentration in the resuspended samples was determined during scintillation counting. The channel ratio of the quench correction program (Tri-Carb 3255, Packard) using an external standard, was gauged to perform not only quench correction but also to calculate the actual chlorophyll concentration of each sample.

The amount of unbound nucleotides dissolved in the aqueous space of the pellet was calculated by measuring the distribution of ^{14}C sorbitol in the samples of a parallel test. As shown by Strotmann and Thiel [10], sorbitol is a marker of the free aqueous space. The nucleotide concentration in the aqueous space was assumed to equal that one of the supernatant. – In order to eliminate unspecific binding, each tested ADP concentration was measured in the presence of 0–10 mM AMP. As there was little affinity of AMP to nucleotide binding sites on CF_1 [11], ADP binding to these sites at sero AMP concentration could be computed.

Non-exchangeably binding of nucleotides to CF_1 was measured by mixing the samples taken from the incubation medium with 2 μM gramicidin and 5 mM unlabelled ADP. Then the thylakoids were washed four times with a medium free from nucleotides in an Eppendorf centrifuge. The resuspended thylakoids were analyzed for non-exchangeable bound labelled nucleotides by liquid scintillation counting.

The following approach aims at elucidating the possible mechanism, leading to inactivation of ATPase activity. There are three simplified mechanisms for the ADP effect on ATPase activity shown below. As it has been shown that an inactivation of ATPase activity is paralleled by binding of one ADP per CF_1 [12] only the binding of the first ADP is mentioned in the models. Even more a branching of the reaction sequence is shown though one should keep in mind that in mathematical terms it would give identical results if the inactive CF_1 = ADP complex occurs as the last step in the hydrolytic reaction sequence. This interpretation implicates that inhibition by ADP means blocking CF_1 because the product remains bound to the enzyme. Those CF_1 conformations inactive in ATPase are designed CF_1^o; CF_1 = ADP means non-exchangeably bound ADP. The paper of Duggleby *et al.* [13] enabled us to perform a computer simulation of our results. We used their models of possible reaction schemes adapted to our problem.

A) In the first model a slow binding of ADP is paralleled by a slow establishment of the inhibitory effect.

$$\mathrm{CF_1} \underset{k_2}{\overset{k_1\cdot[\mathrm{ATP}]}{\rightleftharpoons}} \mathrm{CF_1{-}ATP} \xrightarrow{k^0} \mathrm{CF_1} + \mathrm{ADP} + \mathrm{P_i}$$

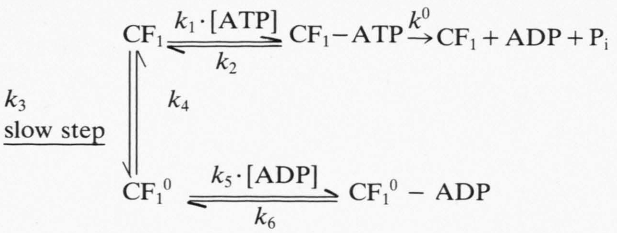
$$\mathrm{CF_1} \underset{k_4}{\overset{k_3\cdot[\mathrm{ADP}]\ \text{(slow step)}}{\rightleftharpoons}} \mathrm{CF_1^0 = ADP}$$

B) In the second one there is a fast binding of ADP to CF_1 followed by a slow conformational change producing an inhibited ATPase.

$$\mathrm{CF_1} \underset{k_2}{\overset{k_1\cdot[\mathrm{ATP}]}{\rightleftharpoons}} \mathrm{CF_1{-}ATP} \xrightarrow{k^0} \mathrm{CF_1} + \mathrm{ADP} + \mathrm{P_i}$$

$$\mathrm{CF_1} \underset{k_4}{\overset{k_3\cdot[\mathrm{ADP}]}{\rightleftharpoons}} \mathrm{CF_1{-}ADP} \underset{k_6}{\overset{\text{slow step}\ k_5}{\rightleftharpoons}} \mathrm{CF_1^0 = ADP}$$

C) In the third model a slow conformational change in the CF_1 protein produces a state able to bind ADP at an inhibitory site.



Any of these models would result in the observation that the actual rate of ATPase activity changes during the inactivation period. This can be described mathematically as follows:

$$v = v_{end} + (v_{start} - v_{end}) \exp. (-k'_{app}\, t)$$

where v_{end} is the final rate of ATPase activity

v_{start} is the ATPase activity in the absence of ADP

k'_{app} is the apparent first order rate constant.

Due to our experimental technique we had to determine the substrate concentrations in the samples stopped after distinct periods of incubation. We were unable to determine the actual reaction rate, we rather measured average rates during short incubation times. Therefore we had to cope with the following experimental errors: i) The initial substrate concentration was difficult to be estimated. ii) Hydrolysis of ATP at a very slow rate took place without any activation of ATPase. Therefore we had to expect inaccuracies in determining v_{start} and v_{end}. These considerations indicated that k'_{app} was the only parameter we could estimate without significant experimental error [14]. According to usual models of thermodynamics of enzyme catalyzed reactions [15] the initial rate of light triggered ATPase mathematically may be described in terms of a first-order reaction. This was proved to hold true for our experiments by linear plots of ln(ATP-concentration) *versus* the incubation time (see Fig. 2). The progress curves of ATPase activity were integrated over different amounts of added ADP in experiments with DTE treated CF_1 preparations. The slopes of these plots indicate the apparent first order rate constant of ATP hydrolysis.

From the above models the calculation of the apparent first order rate constants can be derived as shown in the legend of Fig. 1. From our experiments we knew that the rates of slow and fast steps differ at least in one order of magnitude if we used as low nucleotide concentrations as we did in our ATPase tests. We adjusted the initial ATP concentration to two times the K_m value [16, 17]. So we were able to compute the shape of k'_{app} *versus* the ADP concentration as shown in Fig. 1.

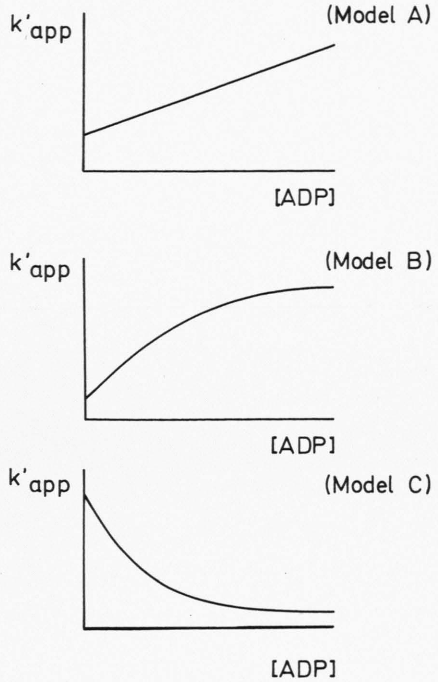


Fig. 1: Computed plots of k'_{app} *versus* the ADP concentration. The following apparent first order rate constants have been used when computing the shapes of plots of k'_{app} *versus* the ADP-concentration corresponding to the three models:

Model A

$$k'_{app} = \frac{k_4 \cdot \left(1 + \frac{[ATP]}{K_m} + \frac{[ADP] \cdot k_3}{k_4}\right)}{1 + \frac{[ATP]}{K_m}}$$

Model B

$$k'_{app} = \frac{k_6 \cdot \left(1 + \frac{[ATP]}{K_m} + \frac{[ADP] \cdot k_3 \cdot (k_5 + k_6)}{k_4 \cdot k_6}\right)}{1 + \frac{[ATP]}{K_m} + \frac{k_3 \cdot [ADP]}{k_4}}$$

Model C

$$k'_{app} = \frac{k_3}{1 + \frac{[ATP]}{K_m}} + \frac{k_4}{1 + \frac{[ADP] \cdot k_5}{k_6}}$$

Results

Using the method of light to dark activation of ATPase activity [1], introduced by Strotmann in our laboratory, we measured the decay of ATPase activity and binding of ^{14}C-labelled ADP in parallel experi-

ments. As shown in Table I, we found that there is a very fast binding of ADP. The nucleotides are bound in an exchangeably manner to one site of the enzyme showing a high affinity to ADP. It appears to us that with prolonged incubation time bound ADP becomes non-exchangeably bound and that this process is paralleled by a decay of ATPase activity.

From sets of 15 to 18 ATPase experiments performed with one thylakoid preparation we calculated the slopes of the linear plots of ln(ATP-concentration) *versus* the incubation time. As an example primary experimental data of three such traces of ATP concentrations are given in Fig. 2. In Fig. 3 we plotted k'_{app} *versus* the ADP concentration and found the shape of this plot corresponding to the one of model C. This result indicates a slow transition in the activated ATPase. This results in a state which binds ADP with high affinity at a site which adopts its non-exchangeable state after the nucleotide has been bound. The result is an enzyme which is inactive in the tested ATPase reaction.

It is reported that the rate of decay of the light-triggered state of ATPase in chloroplasts is decreased in the presence of phosphate [18]. Moreover we observed that the extent of ADP inhibition depends whether ADP is added prior to ATP, together with ATP or after the addition of ATP. For instance: If ADP was added prior to 100 μM ATP we determined a K_i value of 2.9 μM. In contrast to this low

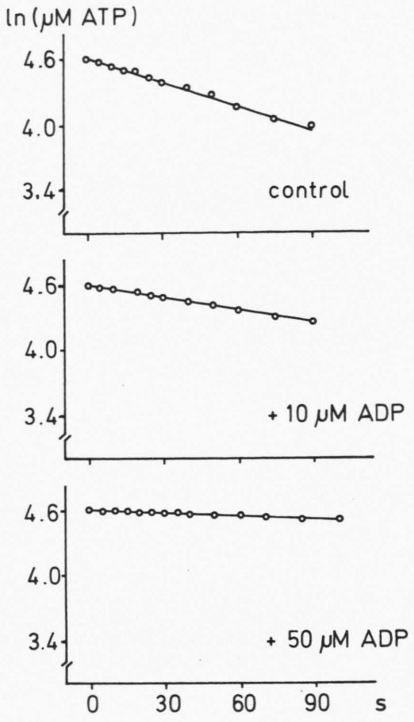


Fig. 2. Effect of added ADP on the rate of ATP hydrolysis. – No added phosphate. – The ATP concentration was monitored during an ATPase experiment by taking aliquots at indicated intervals. After reactivation of ATPase activity the thylakoid preparation was injected into a medium containing 100 μM ATP and additional 0.1 to 50 μM ADP. Three such traces are shown as an example.

Table I. Kinetics of ADP binding to CF_1 during an ATPase test. In the ATPase experiment shown in this figure the binding of 5 μM [8-^{14}C]ADP to high affinity sites in the dark step has been analyzed. Two different binding types: exchangeable and non-exchangeable can be distinguished. ATPase activity was activated by illumination (white light, 800 W/m^2) for 5 min in a syringe. Light to dark transition was performed by injecting it into a darkened reaction chamber containing ADP at a final concentration of 5 μM. At indicated incubation times in the dark [γ-^{32}P]ATP (final concentration = 30 μM) was injected using a second syringe. The initial rate of ATPase activity was determined by taking aliquots from the chamber at intervals of 5 sec. These aliquots have been analyzed for free labelled phosphate using the following method: 0.2 ml of the sample, containing 0.3 M $HClO_4$, were mixed for 15 sec with 1 ml molybdate medium (10 mg/ml ammoniumheptamolybdate and 1 mmol/ml $HClO_4$ saturated with a mixture of 2-methylpropan-1-ol : toluene (1:1, v:v)) and another 1 ml of the above mixture of the organic solvents. A sample of the supernatant was added to 4.5 ml of liquid szintillator (Ready Solve EP, Beckman) and was analyzed for $^{32}P_i$. 100% ATPase activity in this test was 29.3 μmol of ATP hydrolyzed per mg chlorophyll and per hour.

S	nmol ADP bound per mg Chl		non-exchangeable / total	μmol ATP hydrol. / mg Chl · hour	Inhibition [%]
	non-exchangeable	exchangeable			
0				29.3	
1	0.022	0.358	0.058	27.4	6.6
2	0.058	0.351	0.142	24.7	15.7
3	0.077	0.365	0.174	23.3	20.5
4	0.099	0.366	0.213	21.4	26.8
5	0.174	0.362	0.325	21.0	28.3

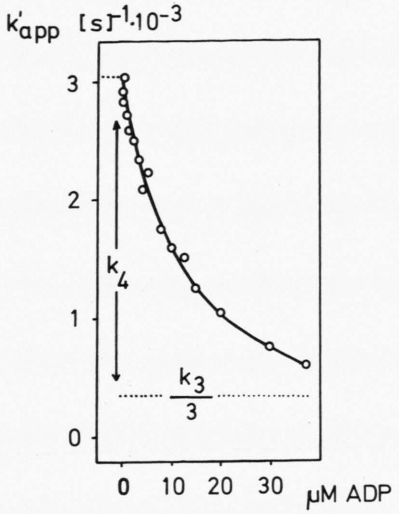


Fig. 3. ADP effect on the apparent first order rate constant of ATPase reaction. – No added phosphate. – A series of ATPase experiments has been performed using 16 different "inhibitory" ADP concentrations in the dark step. The chlorophyll content in this medium was adjusted to 25 μg/ml. When the light was switched off 0.2 μM gramicidin and 150 μM labelled ATP mixed with 0.1 to 50 μM ADP were added. The values of k_4 and k_3 can be estimated from this plot as indicated. The lower intersection with the y-achsis normally gives $k_3/1 + \text{ATP} \cdot K_m^{-1}$; but in our experiments ATP was twice the K_m value. From the data shown in this figure the following values were calculated: $k_3 = 10^{-1}$ s^{-1} and $k_4 = 2.5 \times 10^{-3}$ s^{-1}.

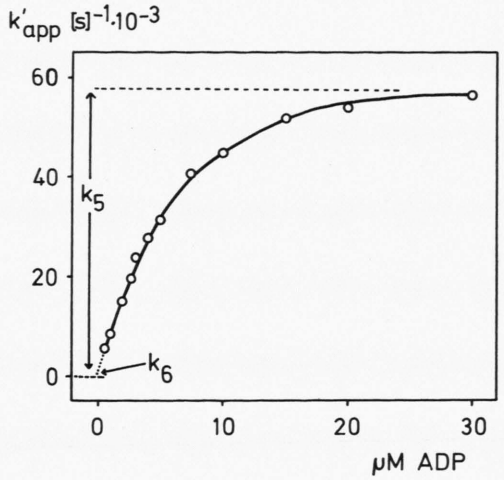


Fig. 4. ADP effect on the apparent first order rate constant of ATPase reaction. – Addition of 5 mM P_i. – Experimental conditions were the same as described under Fig. 2, except that the 30 sec of re-activation were performed in a medium containing additional 5 mM P_i. The values of k_5 and k_6 can be estimated from this plot as indicated; the value of k_6 was found to be sero within the experimental error. k_5 was calculated to be 58×10^{-3} s^{-1}.

value we found a K_i of 57 μM if ADP was added together with 50 μM ^{32}P-labelled ATP after the light-triggered ATPase had been incubated for 10 sec with 50 μM unlabelled ATP.

In discussions with Boyer's group they suggested us that these differences were due to denaturation of the coupling factor. Therefore we reinvestigated our results using an incubation medium containing additional 5 mM P_i. As shown in Fig. 4 the plot of k'_{app} now was shaped like predicted for model B. This means that in the presence of P_i there is a fast binding of ADP to CF_1. This CF_1–ADP complex can undergo a conformational change resulting in an inactive CF_1^o = ATP complex.

In Fig. 5 the effect of phosphate on ATPase activity is shown. Though P_i is a product of the ATPase reaction, an addition of phosphate resulted in a stimulation of the initial rate of ATP hydrolysis (Fig. 5a). The apparent K_m value for ATP (75 μM) was not affected by addition of free phosphate

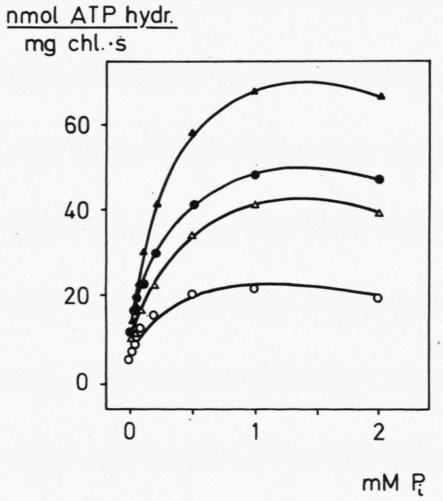
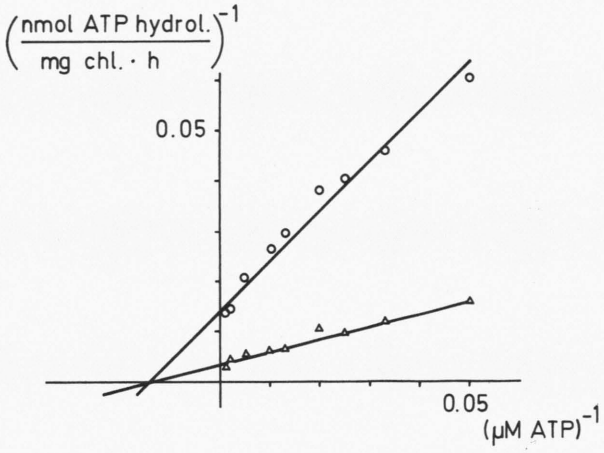


Fig. 5. (a) Phosphate effect on the initial rate of light triggered ATPase activity. ATPase activity of the coupling factor has been activated and tested as described above. Chlorophyll concentration was adjusted to 40 μg/ml. 0.2 μM gramicidin was added when the light was switched off. A mixture of phosphate and ATP was added 5 sec later. The initial hydrolysis activity was monitored for another 15 sec. – In four independent experiments 50 μM (○), 100 μM (△), 200 μM (●), and 1 mM (▲) ATP have been added.

(Fig. 5b). Half of the maximal effect was reached by addition of 170 μM P_i.



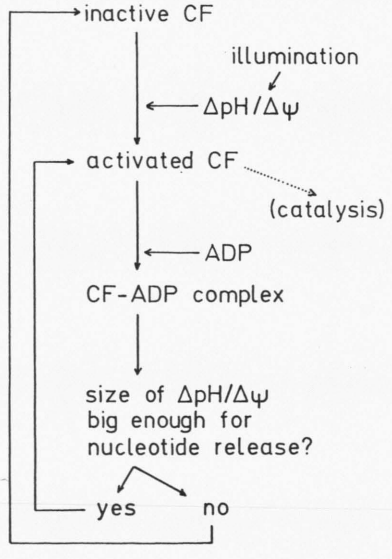
(b) Double reciprocal plot of 1/hydrolysis-rate *versus* 1/ATP-concentration. ATPase activity with (△) and without (○) addition of 2 mM phosphate has been monitored. Both graphs indicate an apparent K_m value of 75 μM ATP.

Discussion

Schumann and Strotmann [12] showed in their paper an experiment in which the rate of light triggered ATPase activity was measured as a function of pre-illumination time. In a parallel experiment the release of membrane bound labelled ADP during the light activation step was measured. They found the time courses of these two processes to be identical. Based on this observation it was suggested that binding and release at one non-exchangeable binding site per mol of CF_1 controls the catalytic activity of chloroplast ATPase [1, 2, 12]. It was shown that illuminated CF_1 contains a total of nearly one mol of nucleotides bound to high affinity sites and that the portion, bound in a non-exchangeable way, increases with increasing concentration of added uncouplers [19]. These two sets of information suggested us to measure the kinetics of those two types of nucleotide binding and the deactivation of ATPase activity in parallel tests. In our experiments we used 5 μM ADP, thus making sure that binding sites in their high affinity state predominantly are involved in the observed reaction. As shown in Tab. I, our results indicate that the decrease of ATPase activity is paralleled by the occurrence of an increasing number of non-exchangeably bound nucleotides. From our data we could not judge whether there were two types of binding sites involved in this reaction [20] or a transformation from exchangeable to non-exchangeable type of binding took place at the same site.

In further studies we wanted to find out by which sequence of reactions light triggered ATPase activity is inhibited. As nucleotide binding to high affinity sites took place very rapidly, we thought that the inactivation of the ATPase might be due to a slowly occurring process, a conformational change in the protein for instance. If there is a regulatory site on CF_1 "inhibitory" nucleotide binding and slow inactivation could proceed simultaneously. If "inhibitory" nucleotide binding takes place at a catalytical site, binding and deactivation must proceed successively anyway. The question was what to come first, binding of ADP or the deactivation step. We tried to find this out by using a mathematical model. The final result was that the slowly occurring inactivation step was preceded by high affinity nucleotide (ADP) binding. In good agreement with results from Strotmann's lab Fig. 4 shows that release of high affinity bound ADP from the deactivated CF_1 does not take place ($k_6 = 0$).

With respect to regulation of the ATPase activity our experimental approach is focussed on understanding the mode of nucleotide binding exclusively. Our results can be illustrated by the following scheme:



We found that there is a transformation of the CF_1–ADP complex to an inactive state of CF_1 caused by a slow process. Such kind of mechanism

regulating the enzymic activity is essential for plants because it prevents hydrolysis of ATP during the night [21]. Nevertheless it is questionable whether the mechanisms of ATP synthesis and hydrolysis on CF_1 completely differ from those ones on the mitochondrial enzyme (F_1) which is quite similar with regard to subunit composition. Therefore we wondered whether our results would fit a "Boyer-type" [5] catalytic mechanism. From the mathematical view we found an agreement indeed. We had to make the following assumptions: (i) ADP is bound to a catalytical active site and only can be released from this site if a sufficient ΔpH or $\Delta\psi$ is present. This would agree with earlier results from this lab [11]. (ii) If ADP can be released, CF_1 remains active and can undergo another catalytic cycle. If the nucleotide remains bound to its site, the catalytic activity of the enzyme drops below a value, detectable by our test. – Our experimental approach is incompetent to decide which catalytical mechanism takes place. Moreover from computer calculations we had to learn another point: Our computer approach is unable to distinguish between two mechanisms, both resulting in a decreasing ATPase activity in our test. The first mechanism is a slow transition in each enzyme as described above; the second one is a successive inactivation of the enzymes in our test. This means that a slowly decreasing number of active enzymes would result in a plot like that one shown for model B in Fig. 1.

In our first experiments dealing with this topic, the ATPase re-activation medium did not contain additional phosphate. The data from these experiments indicated a completely different reaction mechanism (Fig. 3). In agreement with Feldman and Boyer [22] we think that this result is due to a de-activation of those enzymes lacking phosphate. This interpretation is supported by the finding that addition of phosphate, though a product of the ATPase reaction, stimulates the initial rate of ATP hydrolysis (Fig. 5). The affinity of the ATPase to ATP was not affected by the addition of phosphate. One half of the maximal activation of ATPase activity was achieved with 170 μM phosphate. This phosphate concentration resembles the value of the apparent K_m value for phosphate in ATP synthesis. These findings suggest that phosphate binding to its catalytical site activates ATPase activity. Our data confirm those of Feldman and Boyer [22] who stated that "the activity of CF_1 is inhibited when the enzyme with tightly bound ADP but without P_i at a catalytical site is exposed to Ca^{2+} or Mg^{2+}".

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