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ENERGY-DEPENDENT EXCHANGE OF ADENINE NUCLEOTIDES ON CHLOROPLAST COUPLING FACTOR (CF_1)

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SUMMARY

1. [^{14}C]ADP is incorporated into washed broken chloroplasts in the light. The bound labelled nucleotides which cannot be removed by washing are almost exclusively related to coupling factor CF_1. [^{14}C]ADP binding exhibits a monophasic concentration curve with a K_m of 2 μM.

2. By illumination of the chloroplasts, previously incorporated labelled nucleotides are released. A fast release is obtained in the presence of unlabelled ADP and ATP, indicating an energy-dependent exchange. A slow and incomplete release is induced by light in the absence of unlabelled adenine nucleotides. Obviously, under those conditions, an adenine nucleotide depleted CF_1 conformation is established.

3. Re-binding of [^{14}C]ADP by depleted membranes is an energy-independent process. Even after solubilization of adenylate-depleted CF_1, [^{14}C]ADP is incorporated into the protein. By re-binding of ADP in the dark, CF_1 is converted to a non-exchangeable form.

4. Energy-dependent adenine nucleotide exchange on CF_1 is suggested to include three different conformational states of the enzyme: (1) a stable, non-exchangeable form which contains firmly bound nucleotides, is converted to (2), an unstable form containing loosely bound adenine nucleotides. This conformation allows adenylate exchange; it is in equilibrium with (3) a metastable, adenylate-depleted form. The transition from state (1) to state (2) is the energy-requiring step.

INTRODUCTION

Chloroplast-coupling ATPase (CF_1) has been shown to contain bound adenine nucleotides [1–10]. In de-energized chloroplasts and in the isolated protein, the nucleotide-CF_1 complex is rather stable [1–3, 9]. A rapid exchange of the bound nucleotides with free adenylates occurs upon illumination of the thylakoid membranes [3, 9, 10]. Exchange can also be induced by an acid-base transition, suggesting that a

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

ΔpH is the driving force for this process [9]. Most probably, the exchange is caused by an energy-dependent conformational change of coupling factor. Conformational changes involving CF_1 have been suggested based on different experimental approaches [11–14].

As the result of adenine nucleotide exchange in chloroplasts, free ATP is released into the medium [9]. In a recent paper [10], we demonstrated that adenine nucleotide exchange and photophosphorylation depend in exactly the same manner on external factors, including light intensity, pH, uncoupler and electron transport inhibitor concentrations. Therefore, we might conclude that the exchange reaction is a process closely related to the mechanism of photophosphorylation. This has previously been claimed by the conformational hypothesis as developed by Boyer [15, 16] and Slater [3, 17].

In this paper, a more detailed investigation of the adenine nucleotide exchange is presented. The results show that exchange can be divided into two partial processes, release of the bound nucleotides and re-binding of free adenine nucleotides. The first reaction requires energy whereas the latter does not. By pre-illumination of chloroplasts in the absence of free adenine nucleotides a metastable adenylate-depleted CF_1 conformation is formed. This form retains its ability of re-binding of adenine nucleotides for several minutes, even after solubilization of the membrane-bound coupling factor. By re-binding of free adenine nucleotides, the stable non-exchangeable complex is re-established. The results can be fully explained by a sequence of equilibrium reactions.

EXPERIMENTAL

Chloroplast isolation from spinach leaves was carried out as described earlier [9]. The isolation medium contained 0.3 M sucrose, 50 mM NaCl, 1 mM $MgCl_2$ and 10 mM Tricine buffer, pH 7.8. The chloroplasts were washed once with isolation medium and further three times with a medium which contained 50 mM NaCl and 2 mM Tricine buffer, pH 7.8. Finally the chloroplasts were resuspended in the same medium and a chlorophyll content of 2 mg/ml was adjusted. Photophosphorylation of those chloroplasts exhibited rates between 80 and 120 μmol/mg chlorophyll per h under optimum conditions, i.e. 60–80 % of once washed broken chloroplasts.

Incorporation of [^{14}C]adenine nucleotides into chloroplasts was performed in a medium consisting of 25 mM Tricine buffer, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM methylviologen, and [8-^{14}C]ADP (Amersham-Buchler, Braunschweig) at the indicated concentrations. The medium did not contain inorganic phosphate. Post-illumination binding experiments were carried out in the same medium; however [^{14}C]ADP was added after pre-illumination of the samples. Light intensity was $1.8 \cdot 10^5$ ergs/cm^2 per s. The temperature was 20 °C.

After incubation, the samples were immediately centrifuged for 2 min at 15 000 $\times g$ and washed three to four times with a medium containing 50 mM NaCl and 25 mM Tricine buffer, pH 7.8. The [^{14}C]adenine nucleotide contents of the chloroplasts were determined either by direct measurements of the chloroplast suspensions or after solubilization by 10 M urea. Measurements were performed in Unisolve 1 scintillator (Koch-Light Laboratories Ltd.) using the liquid scintillation spectrometer Tricarb model 3320 (Packard).

Light-induced release of membrane-bound [^{14}C]adenine nucleotides was carried out as described in a previous paper [10]. For chlorophyll determination the method of Arnon [18] and for protein measurement the method of Lowry et al. [19] were applied.

RESULTS

In Table I, the incorporation of [^{14}C]ADP into washed broken chloroplasts in the dark and light is shown. After incubation, the excess free [^{14}C]ADP was removed by washing. From an aliquot of the pre-labelled membranes, CF_1 was recovered and the [^{14}C]adenine nucleotide content of the isolated protein was measured. The results show that [^{14}C]ADP was bound by the membranes in the light and that the bound label is related to CF_1. Since the [^{14}C]/CF_1 ratio is comparable in the whole membranes and the enzyme isolated from the same membranes, we may conclude that (i) the CF_1-adenylate complex is stable even after solubilization of the membrane protein, and (ii) virtually no unspecific binding of [^{14}C]ADP by the thylakoid membranes occurs. This result confirms earlier data reported by us [9].

In Fig. 1, incorporation of [^{14}C]ADP into thylakoid membranes as a function of [^{14}C]ADP concentration is shown. In the light, the binding curve reveals a monophasic process with a K_m of 2 μM. Maximum binding in this experiment is 1 mol ADP per mol CF_1. Similar results were obtained with isolated CF_1 by Roy and Moudrianakis [1] in long time incubation experiments. However, in this case, in

TABLE I

INCORPORATION OF [^{14}C]ADP INTO BROKEN CHLOROPLASTS IN THE DARK AND LIGHT AND [^{14}C]ADENINE NUCLEOTIDE CONTENTS OF CF_1 ISOLATED FROM THE SAME CHLOROPLASTS

Isolated washed broken chloroplasts were incubated for 1 min in the presence of 22.9 μM [8-^{14}C]-ADP as described under Experimental. The chlorophyll content during incubation was 1 mg/ml. After 3 washes from an aliquot of the chloroplast suspensions, CF_1 was isolated as described in a previous paper [9]. Purity was controlled by disc-electrophoresis [9]. The protein contents of the preparations were 0.213 mg/ml (from dark-treated membranes) and 0.194 mg/ml (from illuminated membranes), respectively.

[^{14}C]Adenine nucleotide content:	Chloroplasts	
	nmol/mg chlorophyll	[^{14}C]AdN/CF_1*
Dark treated	0.028	0.02
Light treated	0.660	0.51
	Isolated CF_1	
	nmol/mg protein	[^{14}C]AdN/CF_1**
From dark treated membranes	0.071	0.02
From light treated membranes	1.617	0.52

* CF_1/chlorophyll = 1/860 [20].

** Molecular weight of CF_1 : 326 000 [23].

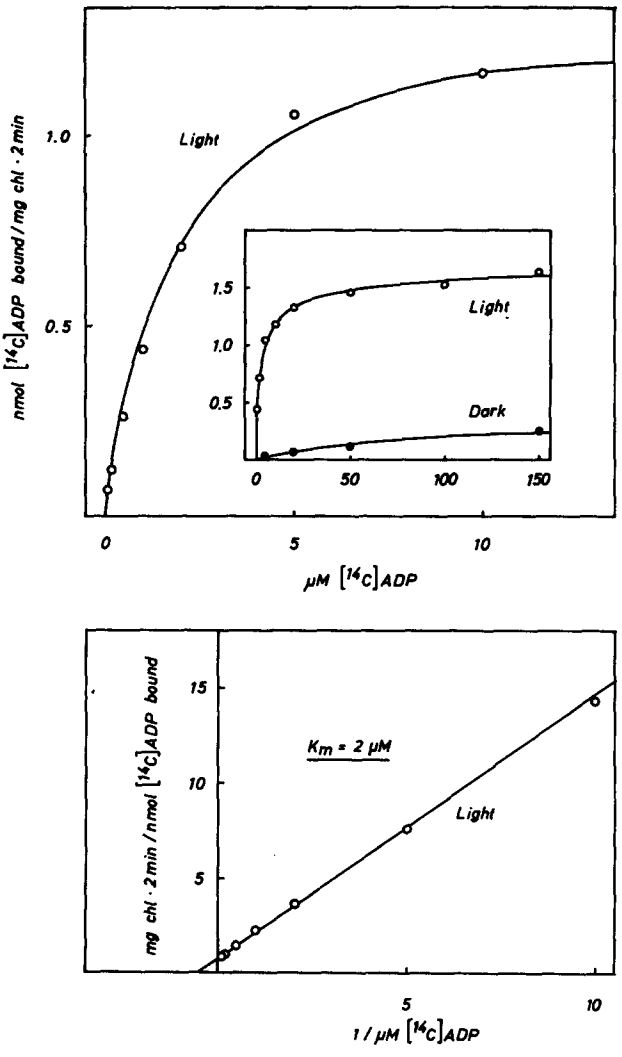


Fig. 1. Incorporation of [^{14}C]ADP into broken chloroplasts in the light and dark as a function of [^{14}C]ADP concentration. The chlorophyll content during incubation was 0.793 mg/ml. Incubation time was 2 min.

addition to the high affinity site, a second ADP binding site with a K_m of 35 μM was observed.

In an experiment shown in Fig. 2, [^{14}C]ADP pre-labelled membranes were incubated in the light in the absence and presence of unlabelled ATP, ADP and AMP. A rapid light-induced release of bound label was found in the presence of ATP and ADP, indicating an exchange reaction. However, even in the complete absence of free adenine nucleotides a slower and incomplete release of bound labelled nucleotides took place. AMP did not increase the rate and extent of the background release. In the absence of ATP or ADP, light obviously induced a CF_1 form which is partially depleted from the bound adenine nucleotides.

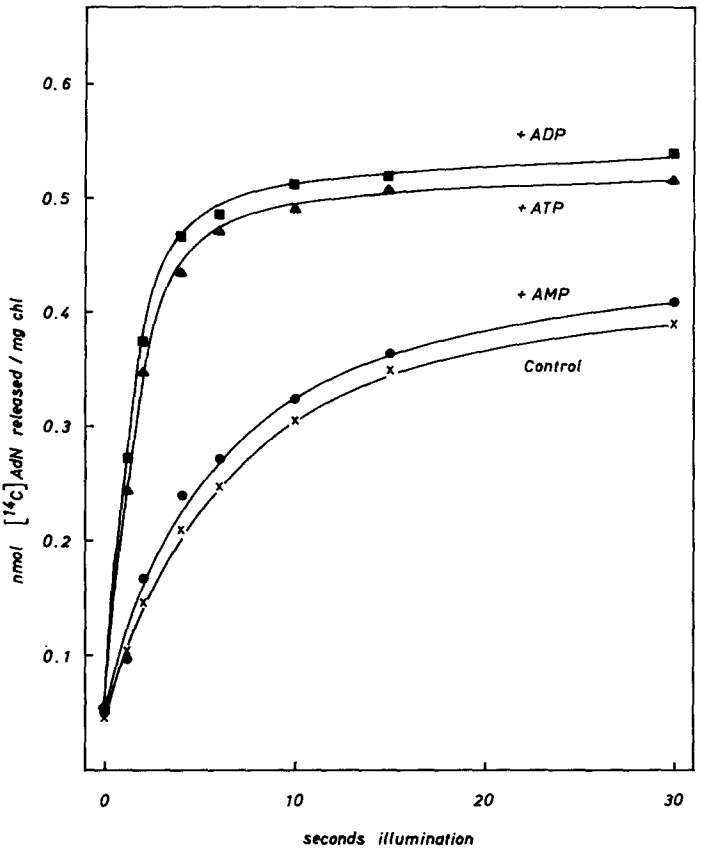


Fig. 2. Time course of light-induced release of bound ^{14}C-labelled adenine nucleotides from chloroplasts in the absence and presence of AMP, ADP and ATP. Pre-labeling of the chloroplasts was performed in the light with 22.9 μM [8-^{14}C]ADP. Release was carried out in a medium containing 25 mM Tricine buffer, pH 8.0, 50 mM NaCl, 1 mM $MgCl_2$ and 0.5 mM methylviologen. AMP, ADP and ATP were added at a concentration of 0.1 mM. The chlorophyll content was 0.308 mg/ml. The experimental details of release incubation are described in ref. 10.

In the following experiments re-binding of [^{14}C]ADP by adenylate-depleted membranes was studied. In Fig. 3, chloroplasts were pre-illuminated in an adenine nucleotide free medium and [^{14}C]ADP was added immediately after pre-illumination. Post-illumination binding of [^{14}C]ADP is a function of illumination time. The kinetic is comparable to the creation of light-dependent ΔpH rather than the electrical field which is established much faster [24]. In a parallel experiment, pre-illumination was carried out in the presence of the uncoupler FCCP. Under those conditions, virtually no [^{14}C]ADP was bound.

In Fig. 4, post-illumination binding of [^{14}C]ADP after varying dark intervals is presented. In a parallel series FCCP was added after the light was turned off. The results demonstrate that the ability of pre-illuminated chloroplasts to bind [^{14}C]ADP persists for rather a long time in the dark. Half times between 1 and 6 min were obtained. Since the energized state of the thylakoids decays in a few seconds after the

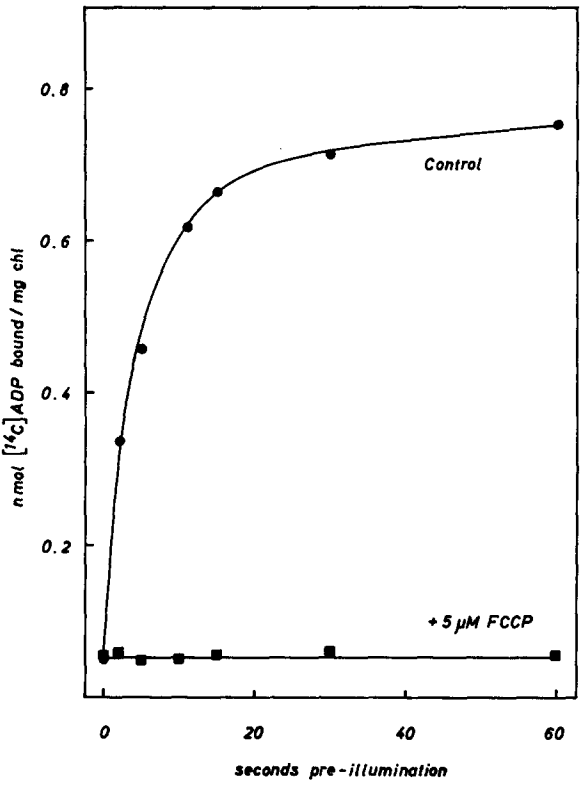


Fig. 3. [^{14}C]ADP binding by chloroplasts as a function of pre-illumination time. Pre-illumination was carried out in an adenine nucleotide-free medium in the absence and presence of 5 μM FCCP. FCCP was added in ethanol solution (final concentration 10 %). The controls contained the same amount of ethanol, which did not affect photophosphorylation. Immediately after turning off the light, [^{14}C] ADP was added at a concentration of 15.7 μM. The chlorophyll content was 0.750 mg/ml.

light is turned off, re-binding of [^{14}C]ADP by depleted membranes obviously does not need energy. This is definitely confirmed by the fact that uncoupler addition, at a point where depletion was finished, had no effect on the kinetics of subsequent ADP binding.

The depleted form of CF_1 retains its ability to re-bind [^{14}C]ADP even after solubilization of the coupling factor. After light or dark pre-treatment of the chloroplast in nucleotide-free medium, CF_1 was rapidly removed from the membranes. The CF_1-containing supernatant was then supplied with [^{14}C]ADP. The time between the end of pre-treatment and the addition of [^{14}C]ADP to the solubilized coupling factor was 7 min. CF_1 was subsequently isolated from the supernatants and excess free [^{14}C]ADP was separated from the protein by Sephadex G-25 chromatography. In the protein fractions of the pre-illuminated samples, a significantly higher ^{14}C activity was detected than in CF_1 from membranes which were kept in the dark (Table II).

In Fig. 5, unlabelled ADP was added to a chloroplast suspension immediately after pre-illumination in a nucleotide-free medium. At different times in the dark,

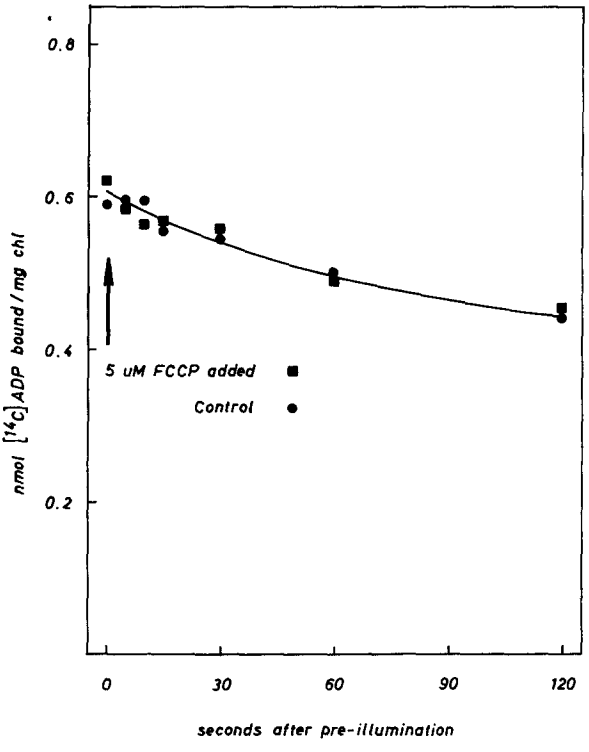


Fig. 4. Time course of post-illumination binding of [^{14}C]ADP by chloroplasts. Immediately after pre-illumination (1 min), either FCCP in ethanol solution (final concentration 5 μM) or the same amount of ethanol (final concentration 10 %) was added. At the indicated times in the dark, [^{14}C]-ADP was added at a concentration of 16.1 μM. The chlorophyll content was 0.842 mg/ml.

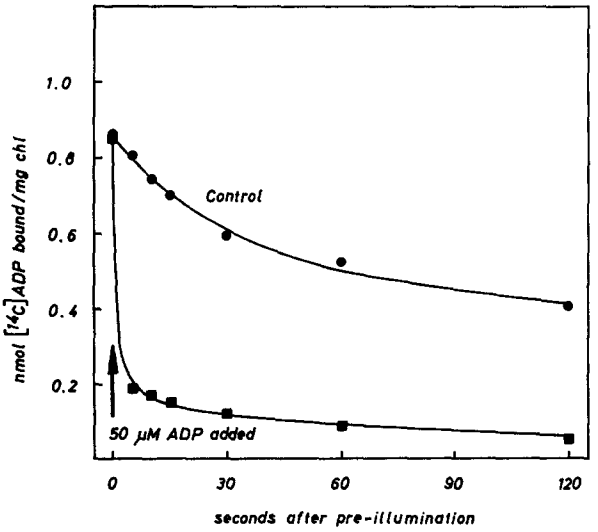


Fig. 5. Time course of post-illumination binding of [^{14}C]ADP as affected by the addition of unlabelled ADP at light-to-dark transition. Pre-illumination: 1 min. At the indicated times in the dark, [^{14}C]ADP was added at a concentration of 22.9 μM. In the control labelled and unlabelled ADP of the same concentrations were added simultaneously at the indicated times. The chlorophyll content

TABLE II

INCORPORATION OF [^{14}C]ADP INTO CF_1 SOLUBILIZED FROM PREILLUMINATED AND DARK KEPT MEMBRANES

Chloroplasts were incubated in a medium containing 25 mM Tricine buffer, pH 8.0, 50 mM NaCl, 5 mM EDTA and 0.5 mM methylviologen, either in the light or in the dark for 1 min. Because of the high Na^+ concentration, CF_1 remains attached to the membranes although EDTA is present [20]. Immediately after incubation, the chloroplasts were collected by centrifugation (1 min at 15 000 × *g*) and resuspended in 0.3 M sucrose+2 mM Tricine buffer, pH 7.8. By this treatment, CF_1 is detached from the membranes [20]. After centrifugation (see above) the light-green supernatants were supplied with [^{14}C]ADP (final concentration 15.7 μM). From the supernatants CF_1 was isolated [9]; in order to remove free [^{14}C]ADP, the protein solutions were passed through a Sephadex G-25 column [9]. The protein peak was well separated from the free nucleotides. The ^{14}C contents of the fractions were measured as described under Experimental.

CF_1 from	Protein content (mg/ml)	[^{14}C]AdN content (nmol/mg protein)
Illuminated membranes	0.0701	2.11
Dark kept membranes	0.1087	0.87

[^{14}C]ADP was added and the incorporation of labelled ADP was followed. In a control experiment, ADP and [^{14}C]ADP were simultaneously added. In both series, the final concentrations and the specific activities of ADP were the same.

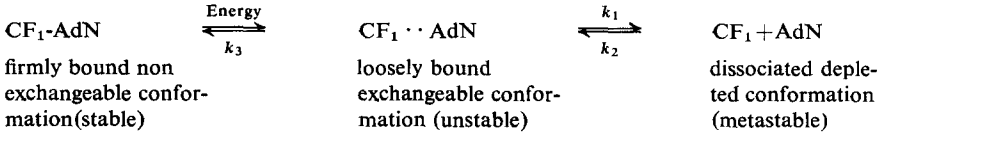
In the case when unlabelled ADP was previously added, the subsequent incorporation of [^{14}C]ADP was rapidly decreased, indicating that the free binding sites were irreversibly occupied by the unlabelled substrate molecules. Obviously, by this reaction, the depleted CF_1 form is instantaneously re-converted to the non-exchangeable conformation.

DISCUSSION

Adenine nucleotide interaction with CF_1 in intact thylakoids can be studied either by forward or by back-exchange experiments, using [^{14}C]adenine nucleotides. In both systems an appreciable exchange is obtained only in energized chloroplasts. This result confirms earlier observations [3, 9, 10]. ADP and ATP, not AMP, are exchangeable substrates.

Adenine nucleotide exchange can be divided into two partial reactions, the energy-dependent release of bound nucleotides and the energy-independent re-binding of free adenylates. The two reactions can be separated temporally. In the absence of exchangeable free nucleotides, illumination of the chloroplasts induces a partially adenylate-depleted metastable CF_1 form which is able to bind ADP in the absence of an energized state of the membrane. Even after solubilization, the depleted CF_1 conformation retains this ability. On the other hand, after re-binding of free nucleotides in the dark, CF_1 is converted to the non-exchangeable ground conformation. The results presented in this paper can be explained by assuming three different conformational states of CF_1, which are characterized by their adenylate binding properties and their stability. The conformations are transferred into each other by

equilibrium reactions; one reaction requires energy from photosynthetic electron transport or a ΔpH, respectively:



If $k_3 > k_1 \simeq k_2$, we may expect an equilibrium situation far on the side of firmly bound nucleotides in the deenergized state of the membrane. By energization, the equilibrium would be shifted to the right side. In the presence of external adenine nucleotides the unstable conformation would appear predominantly and allow an effective exchange. When the energized state decays, CF_1 would return to the stable non-exchangeable form. In the absence of free nucleotides, an equilibrium between the unstable and metastable depleted CF_1 form would be adjusted in energized chloroplasts. When energy supply is interrupted, the adenylate-depleted conformation would be slowly converted to the stable non-exchangeable form due to the extreme dilution of the released adenine nucleotides in the medium.

The actual meaning of energy-dependent exchange of tightly bound adenine nucleotides in the mechanism of photophosphorylation cannot yet be definitely estimated. On one hand, bound ADP is phosphorylated to ATP by added inorganic phosphate in the light [9] and adenylate exchange exhibits the same dependence on pH, light intensity, uncoupler and DCMU concentration as steady state phosphorylation [10]. These results suggest a close relationship between the two processes. On the other hand, the K_m for tight binding of ADP is lower than the apparent K_m for ADP in photophosphorylation by a factor of at least 10 [21, 22]. Moreover the rate of adenine nucleotide exchange is about 50 to 100 times lower than the steady state rate of phosphorylation.

These data seem to indicate that adenine nucleotide exchange as reported in this paper and by other authors does not reflect the actual turnover of substrate nucleotides at the catalytic site of CF_1. Probably the exchange of firmly bound adenine nucleotides monitors an energy-dependent conformational change of the protein which is not directly involved in the energetics of ATP synthesis. This conformational transition might be an initial step in the process by which the ATP synthetase is transformed from the inactive to the active phosphorylating condition.

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