ENERGY-DEPENDENT RELEASE OF ADENINE NUCLEOTIDES TIGHTLY BOUND TO CHLOROPLAST COUPLING FACTOR CF_1

H. STROTMANN, Susanne BICKEL and B. HUCHZERMEYER

Botanisches Institut der Tierärztlichen Hochschule Abteilung für Biochemie der Pflanzen
3 Hannover, Bünteweg 17, West Germany

1. Introduction

Chemiosmotic coupling has been widely accepted as the basis of understanding of the mechanism of photophosphorylation. One of the most significant evidence was contributed by Jagendorf and Uribe [1], who demonstrated acid–base induced synthesis of ATP from added ADP and P_i in the absence of light-dependent electron transport. However, the mechanism of ATPase reaction involved in the final step of energy transformation, seems to be unsatisfactorily solved by the chemiosmotic hypothesis.

Chloroplast coupling ATPase (CF_1) was shown to contain tightly bound adenine nucleotides which participate in photophosphorylation [2–4]. Recently in analogy to oxidative phosphorylation, a conformational hypothesis was put forward by Harris and Slater [4]. In their model the energy requiring process of the phosphorylation cycle is believed to be an electron transport-induced conformational change of CF_1 which leads to release of tightly bound ATP. By assuming certain equilibria between free and CF_1-bound adenine nucleotides and P_1, the authors predicted that tightly bound ATP may be formed from ADP and P_i without further energy requirement.

In the present paper energy-dependent release of adenylates tightly bound to CF_1 is demonstrated. Energy can either be supplied by light-dependent photosynthetic electron transport or by an acid–base transition in the dark. The results indicate that chemiosmotic and conformational hypotheses may not be regarded as alternative [5] but additive concepts for the comprehension of the mechanism of photophosphorylation.

2. Materials and methods

Chloroplasts were prepared from freshly harvested spinach leaves in a medium consisting of 300 mM sucrose, 50 mM NaCl, 1 mM $MgCl_2$, and 10 mM tricine buffer, pH 7.8. The isolated chloroplast were washed once with isolation medium and further three times with a medium which contained 50 mM NaCl, 1 mM $MgCl_2$, and 2 mM tricine buffer, pH 7.8. Finally they were resuspended in the latter medium and a chlorophyll concentration of 2 mg/ml was adjusted.

$[^{14}C]$ ADP pre-loading of the washed isolated chloroplasts was performed in a medium which contained 25 mM tricine buffer, pH 7.8, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM methylviologen, and 22.5 μM carrier-free $[8-^{14}C]$ ADP (Amersham-Buchler, Braunschweig). The chloroplast concentration was equivalent to 1 mg chlorophyll/ml. The sample was illuminated for 1 min with white light $(1.8 \times 10^5$ ergs/cm^2 sec). After turning off the light, the chloroplasts were collected by centrifugation and usually washed three times with a medium consisting of 25 mM tricine buffer, pH 7.8, and 50 mM NaCl. During washing a chlorophyll concentration of 0.5 to 1 mg/ml was maintained. The washed $[^{14}C]$ ADP pre-loaded chloroplasts were resuspended in the latter medium before use for further experiments.

CF_1 was isolated from pre-loaded chloroplasts by a method which essentially corresponded to the procedure described in a previous paper [6]. From salt-washed membranes almost pure CF_1 can be recovered by treatment with a chelating agent in the presence of excess Na^+ and subsequent sucrose washing [6]. In the experiments described here, the pre-loaded and

washed chloroplasts were taken up in a medium consisting of 25 mM tricine buffer, pH 7.8, 50 mM NaCl, and 5 mM EDTA. After centrifugation, extraction of CF_1 was achieved by resuspension of the chloroplast pellet in 300 mM sucrose, buffered by 2 mM tricine, pH 7.8. The CF_1 containing supernatant was obtained by centrifugation at 150 000 g for 1 h. Purity of the preparation was controlled by disc-electrophoresis [7].

[^{14}C] Adenine nucleotide content of washed preloaded membranes was determined by measurement of radioactivity of 50 μl chloroplast suspension in 'Unisolve 1' scintillator (Koch-Light Laboratories Ltd.) using the liquid scintillation spectrometer 'Tri-carb, model 3320' (Packard). The chlorophyll quench was determined by an internal standard. Extraction of tightly bound adenine nucleotides was performed with 10 M urea [2]. By this method usually 90 to 95% of the bound nucleotides were solubilized.

Separation of adenine nucleotides was either performed by ion exchange column chromatography on Dowex 1 × 8 [8], or by thin-layer chromatography on cellulose plates with n-butanol/acetone/acetic acid/5% ammonia (45:15:10:20, v/v) as the eluant [9].

For chlorophyll determination the method of Arnon [10], and for protein measurement the method of Lowry et al. [11] were applied.

3. Results and discussion

Before using for experiments, the isolated chloroplasts were washed three times with a hypotonic salt solution (see Materials and methods). This treatment was applied in order to remove membrane associated adenylate kinase. The washed chloroplasts were found to be completely free from adenylate kinase activity. On the other hand, the coupling factor CF_1 remained attached to the thylakoid membrane (cf. [6]) and the chloroplasts retained at least 50% of their phosphorylation activity in a non-cyclic system.

When washed chloroplasts were incubated in the light in the presence of [^{14}C] ADP at micromolar concentrations, they were found to incorporate ^{14}C-label. A considerable portion of the label could not be removed by subsequent washes (fig.1), indicating a tight interaction between adenylates and thylakoid membranes. The amounts of tightly bound
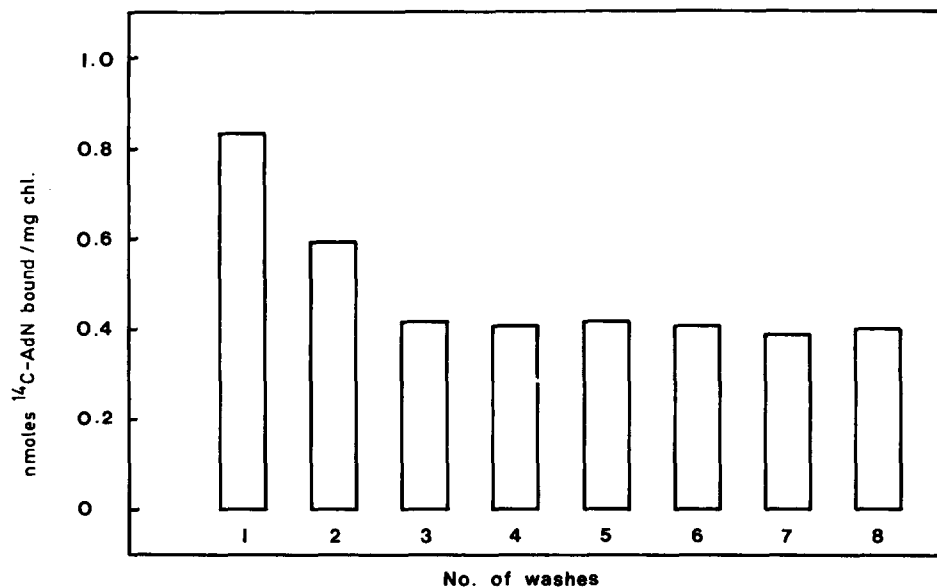

Fig.1. Contents of [^{14}C] adenine nucleotides bound to thylakoid membranes as affected by washing. [^{14}C] ADP pre-loading was performed as described in Materials and methods. After each washing step aliquots of the membrane pellets were analyzed for total ^{14}C content.

Table 1

Contents of tightly bound [^{14}C]adenine nucleotides in thylakoid membranes
and CF$_1$ isolated from the same membranes

	[^{14}C]Adenine nucleotide content
Thylakoid membranes	0.507 nmol/mg chlorophyll
Molar ratio [^{14}C]AdN/CF$_1$ [a]	0.39
Isolated CF$_1$	1.136 nmol/mg protein
Molar ratio [^{14}C]-AnD/CF$_1$ [b]	0.37

[^{14}C]ADP pre-loaded membranes were washed three times and directly anlyzed for ^{14}C content. From an aliquot CF$_1$ was isolated as described under Materials and methods. Purity of the CF$_1$ preparation as judged from disc-electrophoresis, was about 90%. In order to remove possible free adenine nucleotides, the protein solution was passed through a Sephadex G-25 column (2.5 cm diameter, 12 cm length) with 10 mM tricine buffer, pH 7.8 as eluent. CF$_1$—adenylate complex appeared in fractions 12 to 14 (each 1.5 ml). Negligible amounts of free labelled nucleotides were found in fractions 25 to 35. The protein fractions were combined and subjected to freeze drying. The dry sample was re-dissolved in 0.5 ml distilled water. Aliquots were used for protein determination and ^{14}C measurement.

[a]CF$_1$/chlorophyll ratio: 1/860 [6].

[b]Mol. wt. of CF$_1$: 326 000 [12].

[^{14}C]adenine nucleotides obtained in such experiments, varied between 0.4 and 1.2 nmol/mg chlorophyll.

In an experiment shown in table 1, CF$_1$ was isolated from [^{14}C]ADP pre-labelled membranes and the contents of ^{14}C-bound to the membranes and to CF$_1$, respectively, were compared. For both cases almost the same molar ratios ^{14}C/CF$_1$ were computed. This result shows that tightly bound ^{14}C of [^{14}C]ADP pre-loaded membranes is nearly exclusively related to CF$_1$. Obviously there are no tight binding sites except for CF$_1$, and the inner space of the thylakoid system does not retain free labelled nucleotides.

Table 2

Distribution of tightly bound [^{14}C]adenine nucleotides in [^{14}C]ADP pre-loaded thylakoid membranes.

	Tightly bound [^{14}C]adenine nucleotides (nmoles/mg chlorophyll)	(%)
Total	0.441	100
AMP	0.121	27.4
ADP	0.202	45.8
ATP	0.118	26.8

[^{14}C]ADP pre-loaded and thrice washed membranes were extracted by 10 M urea and centrifuged at 150 000 g for 1 h. The supernatant was analyzed by thin-layer chromatography.

Therefore [^{14}C]ADP pre-loaded membranes seem to be a useful experimental material for the study of CF$_1$-mediated reactions related to photophosphorylation.

In table 2 the adenine nucleotide distribution of pre-labelled membranes is shown. ADP was found to be the main tightly bound nucleotide species, yielding about 50% of the total amount. The rest consisted of AMP and ATP, respectively. The distribution was similar in CF$_1$ recovered from pre-loaded membranes.

If [^{14}C]ADP pre-loaded thylakoids were again incubated in the light, part of the tightly bound label appeared in the medium. In an experiment shown in fig.2, incubation was performed in the light and in the dark with four different substrate additions to the incubation medium. A 30 sec illumination induced almost comparable release of tightly bound labelled adenine nucleotides in the absence of any phosphorylation substrate and in the presence of P$_i$. Addition of unlabelled ADP or ADP plus phosphate increased light-dependent release of ^{14}C. In the substrate-free control preferently ADP was released, in the presence of P$_i$ a large amount of ATP was formed. With ADP alone about equal ratios of ATP, ADP, and AMP were liberated. The percentage of ATP was increased by additional supply of P$_i$.
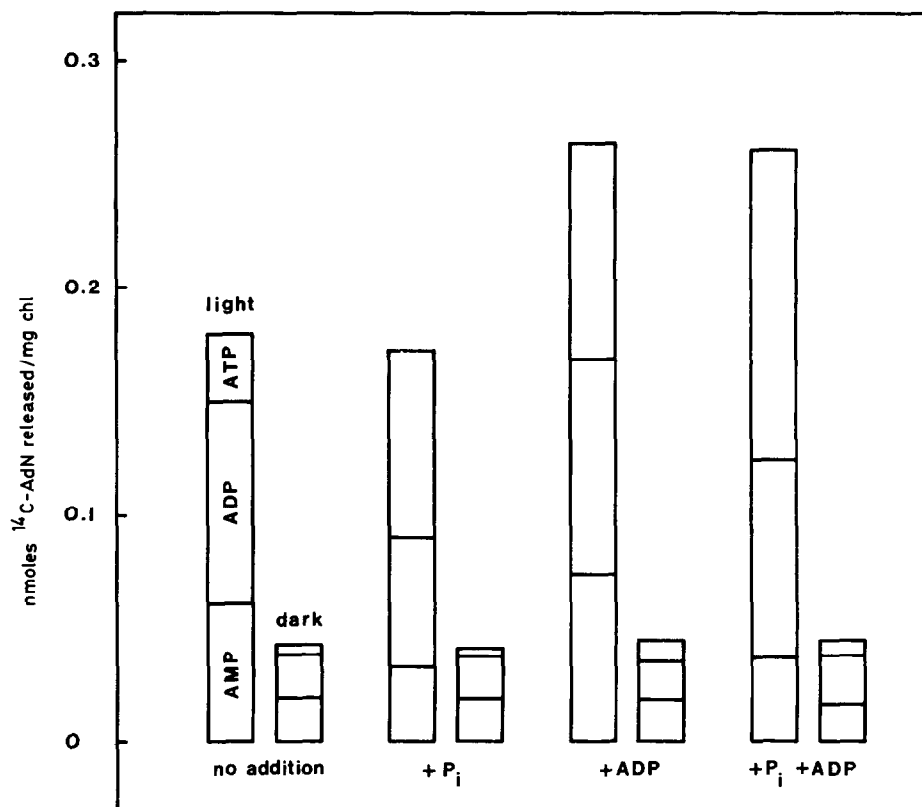
Fig.2. Release of tightly bound ^{14}C-adenine nucleotides in the light and dark in the presence of unlabelled substrates. Release experiments with [^{14}C]ADP pre-labelled thylakoids were performed in small semi-transparent centrifugation tubes, which contained a final vol of 0.3 ml. The incubation medium contained 25 mM tricine buffer, pH 8.0, 50 mM NaCl, 1 mM MgCl$_2$, 0.5 mM methylviologen and where indicated, 1 mM P$_i$ and 0.1 mM ADP. The reaction was started by the addition of chloroplasts (final chlorophyll concentration 0.238 mg/ml). The tubes were either kept in the dark for 60 sec or after 30 sec in the dark, illuminated for 30 sec with white light (1.8 × 10^5 ergs/cm^2 sec). Illumination was performed in a Beckman 'Microfuge 152' centrifuge with special equipment. After the indicated dark and light periods, respectively, the centrifuge was immediately switched on for 1 min. The supernatants were analyzed by ion exchange chromatography.

The results clearly show that tight CF$_1$ —adenylate complexes either dissociate or undergo nucleotide exchange when the membranes are energized by light-induced electron transport. Depending on the addition of phosphorylation substrates, different patterns of released adenine nucleotides are obtained. A detailed presentation on this subject will be given in another paper. Energy dependent release of tightly bound adenine nucleotides is inhibited by DCMU and by the uncouplers methylamine and FCCP in exactly the same concentration dependency as photophosphory-lation.

Following the experimental conditions reported by Jagendorf and Uribe [1], the effect of acid—base transition on the release of tightly bound adenine nucleotides was investigated. In table 3, light-induced and acid—base induced release in the presence of ADP plus P$_i$ are compared. The result demonstrates that light-dependent electron transport can be fully replaced in this respect by a pH jump. The control experiments show that an actual pH change is required rather than a low pH (during the acid stage) alone. The percentage of ATP formed by acid—base transition is low as compared to light-induced release. For the present this is referred to our unsufficient experimental conditions. Irrespective of the ATP yield,

Table 3

Light-induced and acid–base-induced release of tightly bound
[^{14}C]adenine nucleotides in the presence of ADP and P$_i$

Treatment	Total	(nmoles [^{14}C]AdN released/mg chl.)		
		AMP	ADP	ATP
Light	0.651	0.058	0.182	0.411
Dark	0.085			
Acid–base	0.615	0.137	0.374	0.104
Acid	0.179			
Base	0.088			

Light-induced release was measured as described in the legend to fig.2. Acid–base
transition experiments were performed essentially as described by [1]. The pre-
loaded chloroplasts were collected by centrifugation and resuspended in 'acid
medium' which contained 10 mM malein–glycine buffer, pH 4.0, and 30 μM
DCMU. The chlorophyll content was 0.486 mg/ml. 0.2 ml of the suspension was
poured into 0.4 ml 'basic medium' consisting of 110 mM tricine buffer, pH 8.3,
5.5 mM MgCl$_2$, 2.2 mM P$_i$ and 0.22 mM ADP. After 2 min the chloroplasts were
rapidly spun down by centrifugation and the supernatants were analyzed for [^{14}C]
adenine nucleotides by thin-layer chromatography. For 'acid' control tricine buffer
was omitted from 'basic medium'. For 'base' control the pre-loaded chloroplasts
instead of acid treatment were directly taken up in a medium which contained all
constituents present in the final stage.

a pH jump is able to alter the binding properties for
adenylates of CF$_1$ in a manner comparable to light-
induced electron transport.

Energy-dependent change of adenine nucleotide
binding is one of the essential events in the concept
developed by Harris and Slater [4]. It is suggested
to be brought about by a change in CF$_1$ protein con-
formation. Energy dependent conformational changes
of CF$_1$ have been concluded from ^{3}H-incorporation
experiments [13] and studies with covalently-bound
fluorescent dyes [14].

With a view to both chemiosmotic and conforma-
tional hypotheses, the intrinsic sequence of reaction
steps involved in coupling between electron transport
and photophosphorylation may be described as
follows:

Electron transport → creation of a ΔpH across the
thylakoid membrane → conformational change of
CF$_1$ → alteration of adenine nucleotide binding sites
→ release of ATP.

Acknowledgement

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