Biochimica et Biophysica Acta, 766 (1984) 334–342
Elsevier

BBA 41564

A STUDY ON THE EXCHANGE OF TIGHTLY BOUND NUCLEOTIDES ON THE MEMBRANE-ASSOCIATED CHLOROPLAST ATP SYNTHASE COMPLEX

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(Received February 29th, 1984)

Key words: Photophosphorylation; ATPase; Nucleotide binding; Photosynthesis

Light-induced exchange of tightly bound ADP on the membrane-associated chloroplast coupling factor 1 (CF_1) was concluded to be a two-step mechanism involving a loose enzyme-ADP complex (Strotmann, H., Bickel-Sandkötter, S. and Shoshan, V. (1979) FEBS Lett. 101, 316–320). Rapid binding of [^{14}C]ADP to the coupling factor after deenergization of thylakoids which were illuminated in the presence of [^{14}C]ADP was suggested to reflect the conversion of loosely bound to tightly bound ADP. Experimental data of the present paper support the assumption of an intermediate enzyme form with loosely bound ADP: (a) the amplitude of the rapid binding phase is independent on the concentration of uncoupler added in the light; (b) the amplitude is virtually unaffected by dilution of the medium [^{14}C]ADP concentration; (c) high concentrations of unlabeled ADP are required to reduce the rapid binding phase while binding of medium [^{14}C]ADP is inhibited by unlabeled ADP in the micromolar range. These results exclude the possibility that the rapid initial formation of tightly bound [^{14}C]ADP on deenergization might be caused by an energized nucleotide-free enzyme form which is able to pick up [^{14}C]ADP from the medium at a higher rate than the deenergized nucleotide-free form. At saturating [^{14}C]ADP concentrations in the light, the amount of the loose enzyme-ADP complex is about 35%, while 65% of the coupling factors contain a tightly bound ADP. Dissociation of the loose complex is slow in the absence of medium nucleotides but accelerated if ADP is present, suggesting that ADP binding to another site of the enzyme promotes release of the former ADP molecule. The significance of the loosely bound nucleotide in the catalytic mechanism is discussed.

Introduction

Membrane-associated chloroplast coupling factor (CF_1) contains at least two different classes of nucleotide binding sites: the so-called tight site binds one molecule of ADP which can be released or exchanged with medium ADP only on energization of the thylakoid membranes [1–4]. Since the exchange is slow compared to photophosphorylation [4,5], and medium ADP rather than tightly bound ADP is phosphorylated at the onset of illumination [6], this type of binding site is probably a noncatalytic site. It was suggested that binding and release of nucleotides at this particular site may control the catalytic activity of chloroplast ATPase [7–12].

Furthermore, one or more catalytic sites exist where ADP is phosphorylated and ATP is hydrolyzed, respectively. Boyer and coworkers proposed that tight binding of nucleotides on the active site is an intermediate step in the catalytic process [13,14]. However, Aflalo and Shavit [15] found that phosphorylation is possible without the par-

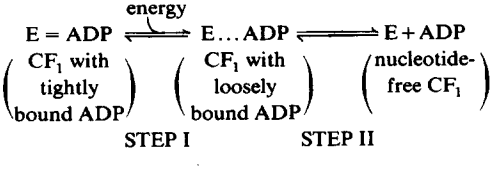
Abbreviations: CF_1, chloroplast coupling factor 1; Chl, chlorophyll; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

ticipation of tightly bound nucleotides as intermediates.

On the basis of equilibrium-binding experiments, Bickel-Sandkötter and Strotmann [12,16] concluded that at the same time either a tight site or a catalytic site is occupied by a nucleotide molecule. Their model also includes the possibility that the tight binding site may not be physically separate but may be a different form of the active site. Regulation of enzyme activity by tight ADP binding was directly demonstrated in the case of light-triggered ATP hydrolysis: light-induced release of tightly bound nucleotides is kinetically related to the evolution of ATPase activity [7]. On the other hand, ADP binding to the nucleotide-free, activated enzyme leads to inactivation of the enzyme [7–9]. However, ATP or some ADP analogues [11,17] which also bind at the tight site do not deactivate the ATPase.

Binding of radioactively labeled nucleotides to the empty 'tight' site by chloroplasts kept in the dark is an irreversible process, and the rate of binding is slow. In the light, a rapid equilibrium between tight binding and release of bound nucleotides exist. Strotmann and coworkers [5] introduced a quenching technique to follow the levels of tightly bound labeled ADP in the light: by the addition of an uncoupler together with excess unlabeled ADP, energy-dependent release of bound ADP is interrupted and further binding of labeled medium ADP is prevented (isotope dilution). Using this technique, levels of tightly bound nucleotides at light-to-dark transitions were studied. The increase of ADP binding upon turning off the light was found to proceed in a biphasic manner. From these results a sequence of two reversible reactions, a fast (step I) and a slow one (step II) was concluded (Scheme I). In step I the forward reaction is driven by energy from the transmembrane protonmotive force. Probably the nucleotide-free form E is the enzyme species, which is active in light-triggered ATP hydrolysis [5,7]:

Scheme I



Additional experimental data suggest that the nucleotide-free form may exist in two different conformations, an energized form (E^+) and a deenergized form (E^-).

(1) Tight nucleotide binding by the nucleotide-depleted enzyme is greatly enhanced by illumination of the chloroplasts (Ref. 7 and Strotmann, H., personal communication).

(2) Trypsin is less active in the proteolysis of membrane-associated ATPase in the dark after illumination than in the light [18].

(3) The active site of ATPase in energized chloroplasts exhibits a much higher affinity for ADP than for ATP [20], while in deenergized chloroplasts a higher affinity for ATP is observed [20,21]. From these results the enzyme conformation involved in ATP synthesis was concluded to be different from that which catalyzes ATP hydrolysis [7].

Based on intermediate $P_i : H_2O$ oxygen exchange studies, Sherman and Wimmer [19] arrived at the same conclusion.

If the assumption of two different forms is correct, the above-cited model (Scheme I) is insufficient to explain the whole interplay between enzyme activation, ATP synthesis and ATP hydrolysis. Therefore, in the present study the problem was reinvestigated. Particular attention was directed to the existence and functional meaning of the proposed intermediate form carrying a loosely bound nucleotide.

Materials and Methods

Broken chloroplasts were isolated from spinach leaves as described [22]. Chlorophyll concentrations were determined according to Arnon [23]. Incubation of the chloroplasts was carried out in small glass vessels under stirring with a magnetic stirrer. The assay medium contained Tricine buffer (25 mmol/l, pH 8), NaCl (50 mmol/l), $MgCl_2$ (1 mmol/l), phenazine methosulfate (50 μmol/l) and thylakoids (chlorophyll concentration, 0.15–0.17 mg/ml). The samples were illuminated from the top (200 W/m^2, white light). For measurements of [^{14}C]ADP binding to the nucleotide-depleted membranes, samples were preilluminated in the absence of nucleotides for 30 s and [8-^{14}C]ADP (2.1 GBq/mmol; Amersham-Buchler) was added

together with an FCCP solution in the light. FCCP was added as an ethanolic solution; the final concentration of ethanol was 5%. Binding of labeled ADP was terminated by the addition of excess unlabeled ADP (isotope dilution); final concentration of ADP was 5 mmol/l.

Steady-state levels of bound labeled ADP in the light were measured by illumination of thylakoids in the presence of [^{14}C]ADP. After 30 s, the FCCP solution was added without or together with unlabeled ADP. The amount of tightly bound [^{14}C]ADP was determined after washing of the chloroplasts as described in Ref. 22; radioactivity was measured in liquid scintillation cocktail (Unisolve I, Koch-Light Lab.). The chlorophyll concentration of each sample was measured according to Ref. 23.

Results

When thylakoids are illuminated in a nucleotide-free medium, tightly bound nucleotides are released from the coupling factor [1–4]. For rebinding of ADP, membrane energization is not a necessary prerequisite, but it was shown that the rate of rebinding is accelerated by energization [7]: if [^{14}C]ADP is added after the proton gradient has decayed, rebinding is slow and monophasic (k_{bind} = 0.01–0.02 $s \cdot l \cdot \mu mol^{-1}$) [7]. However, if [^{14}C]ADP is added immediately after preillumination, the binding kinetics are biphasic. The amplitude of the initial rapid phase is increased by an internal buffer and decreased by an uncoupler (FCCP) [7]. Since FCCP was unable to abolish the rapid phase completely, it was suggested that a high-energy conformation of the enzyme (E^+) is responsible for this fast phase of binding. The E^+ form may be transferred to a low-energy conformation E^- within a few seconds after deenergization of the membranes. E^- might then be responsible for the slow binding of ADP. However, the concentration of FCCP employed in this experiment (5 μmol/l) was presumably not sufficient for immediate uncoupling.

In the following, the effect of FCCP concentration was therefore studied more in detail in a slightly modified type of experiments. Chloroplasts were preilluminated in the absence of medium nucleotides and after 30s [^{14}C]ADP together with FCCP was added while illumination was continued. [^{14}C]ADP binding was terminated by the addition of excess unlabeled ADP. Fig. 1 shows that the binding kinetics are biphasic when 5 μmol [^{14}C]ADP/l and 5 μmol FCCP/l were added. After a rapid incorporation of labeled ADP during the first second, an exponential binding curve is obtained.

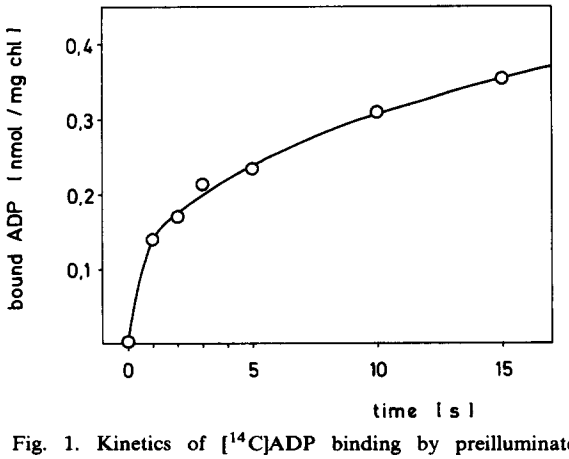


Fig. 1. Kinetics of [^{14}C]ADP binding by preilluminated thylakoids upon deenergization with FCCP. Thylakoids (0.155 mg Chl/ml) were illuminated for 30 s in the absence of [^{14}C]ADP. FCCP (5 μmol/l) and [^{14}C]ADP (5 μmol/l) were added together in the light. Incorporation of labeled [^{14}C]ADP was terminated at the indicated times by the addition of unlabeled ADP (5 mmol/l).

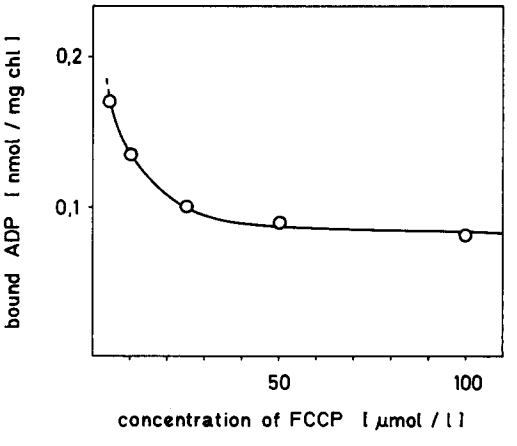


Fig. 2. Amplitude of the FCCP-induced initial rapid phase of [^{14}C]ADP binding in the light as a function of FCCP concentration. Thylakoids (0.15 mg/ml) were illuminated for 30 s in the absence of [^{14}C]ADP. [^{14}C]ADP (5 μmol/l) was added together with varying concentrations of FCCP. Unlabeled ADP was added 1 s later.

The amount of [^{14}C]ADP bound in the initial rapid phase is decreased by increasing the FCCP concentration (Fig. 2). A constant value is attained at about 30 μmol FCCP/l. Under those conditions the binding curve is still biphasic (not shown); the amount of ADP bound in the rapid phase (extrapolated to infinite FCCP concentration) is about 0.05–0.07 nmol per mg Chl. Since high concentrations of FCCP may have some side effects, the experiments were repeated with other uncouplers (ammonium chloride and methylamine) with similar results (data not shown).

In Fig. 3 the amount of [^{14}C]ADP after 1 s is plotted as a function of [^{14}C]ADP concentration. In this experiment two concentrations of FCCP (5 and 25 μmol/l, respectively) were employed. Even at higher [^{14}C]ADP concentrations, the binding kinetics are similar to the curve shown in Fig. 1; the initial rapid phase is completed within 1 s, while the slow phase is clearly exponential (not shown). At higher concentrations of FCCP, the amount of rapidly bound [^{14}C]ADP is reduced. The reduction can be equalized by increasing the concentration of [^{14}C]ADP.

The results clearly show that in the energized state of the membrane a form of the enzyme (E^+) exists which is able to bind and to release ADP rapidly. Upon deenergization, the nucleotide-free form E^+ may either rapidly incorporate ADP (at high ADP concentration) or convert to a slowly binding form E^-.

If [^{14}C]ADP is already present in the medium during illumination, a biphasic increase of bound labeled ADP is observed on light-to-dark transitions [5]. A similar biphasic increase in binding is obtained when an uncoupler is added in the light instead of turning off the light (Fig. 4). In variance to the originally given interpretation [5], this effect might also be explained by assuming two forms of the nucleotide-free ATPase which bind ADP at different rates. If this explanation were correct, increasing amounts of uncoupler added in the light should accelerate the decay of E^+ to E^- and thus reduce the amplitude of the rapid phase. In the experiment shown in Fig. 5, varying concentrations of FCCP (5–100 μmol/l) were added to chloroplasts illuminated in the presence of [^{14}C]ADP. Excess unlabeled ADP was added together with the FCCP in order to obtain the steady-state level of bound [^{14}C]ADP in the light. In a parallel series the unlabeled ADP was added 2 s after the addition of FCCP. The difference between these two levels yields the amplitude of the rapid phase.

The data show that the rapid binding phase is not affected by increasing the concentration of the uncoupler. Its amplitude is, therefore, independent of the velocity of deenergization of the membrane. Both the amount of tightly bound ADP (E = ADP)

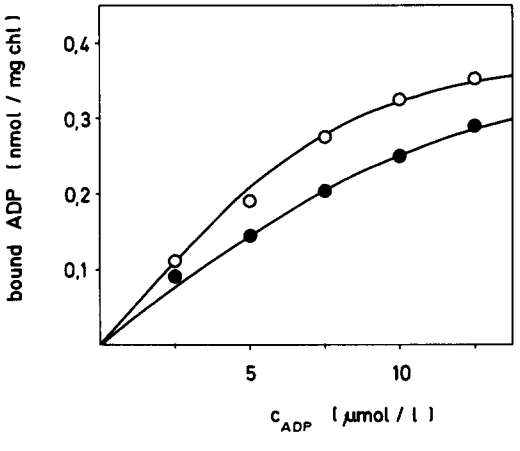


Fig. 3. Amplitude of the initial rapid phase of [^{14}C]ADP binding in the light as a function of [^{14}C]ADP concentration. Conditions as in Fig. 2, except that [^{14}C]ADP concentration was varied and FCCP concentration was kept at 5 μmol/l (○) or 25 μmol/l (●).

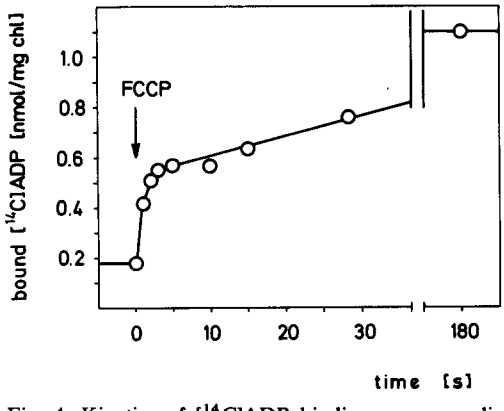


Fig. 4. Kinetics of [^{14}C]ADP binding on uncoupling in the light. Thylakoids (0.17 mg/ml) were illuminated in the presence of [^{14}C]ADP (2.5 μmol/l). After 30 s, FCCP (10 μmol/l) was added in the light. Unlabeled ADP (5 mmol/l) was added at the indicated times.

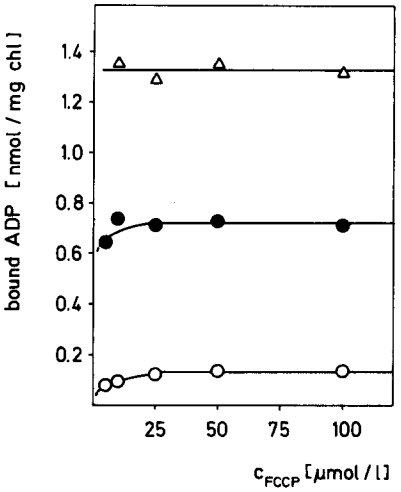


Fig. 5. Effect of FCCP concentration on the rapid and slow phases of [^{14}C]ADP binding. Conditions as in Fig. 4; ○, addition of FCCP together with ADP (light level); ●, addition of ADP 2 s after FCCP (light level + rapid phase); △, addition of ADP 30 min after FCCP (light level + rapid phase + slow phase).

in the light and the amplitude of the fast phase of ADP binding after turning off the light depend on the concentration of [^{14}C]ADP in the medium [5]. In an experiment using FCCP for deenergization (Fig. 6) light levels of tightly bound [^{14}C]ADP and the amplitudes of the fast phase as functions of medium [^{14}C]ADP concentrations were measured.

The data can be treated as normal adsorption isotherms. The calculated K_d value for ADP in both reactions is about 3 µmol/l (Fig. 6B). That means the ratio between them does not depend on the concentration of medium ADP. At infinite ADP concentration, the sum of both is 1.1 nmol per mg Chl; the same value was found for the entirety of tight binding sites (determined by incubation with [^{14}C]ADP of preilluminated chloroplasts for 15 min in the dark without quenching). The light level of tightly bound ADP at ADP saturation is about 0.71 nmol/mg Chl.

If rapid binding of [^{14}C]ADP after deenergization were due to uptake of medium [^{14}C]ADP by a rapidly binding energized form E^+, a sudden dilution of the [^{14}C]ADP of the medium, e.g., from 5 to 1.25 µmol/l together with deenergization of the membranes should yield a lower amplitude of the rapid phase (see Fig. 6A). Such an experiment is shown in Table I. Dilution was attained by addi-

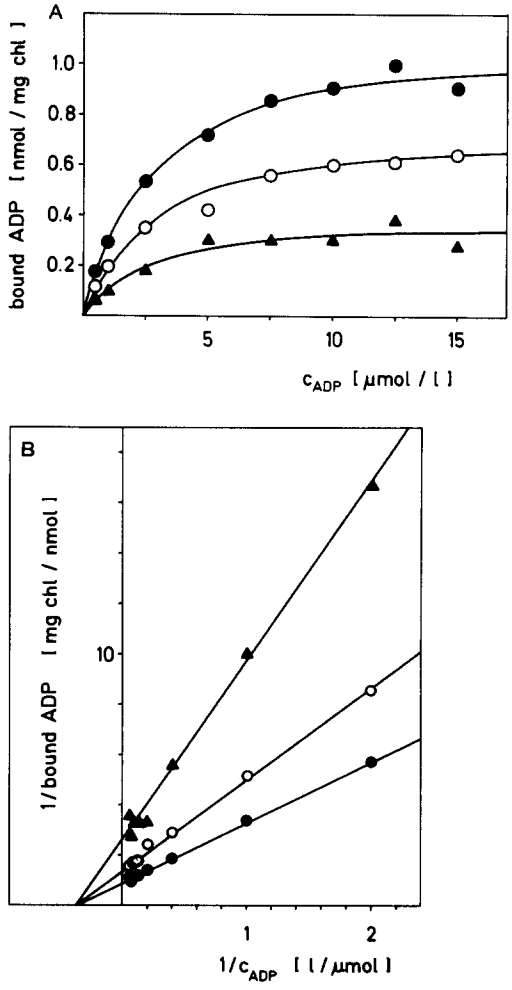


Fig. 6. (A) Level of bound [^{14}C]ADP in the light and 2 s after uncoupling as a function of [^{14}C]ADP concentration. Chloroplasts (0.12 mg/ml) were illuminated in the presence of varying concentrations of [^{14}C]ADP. Either FCCP + unlabeled ADP (○) (light level) or FCCP alone (●) was added in the light. In the latter case, unlabeled ADP was added 2 s later (light level + rapid phase). FCCP concentration, 50 µmol/l; ▲, difference between (○) and (●) (= rapid phase). (B) Double-reciprocal plot of A.

tion of different volumes of FCCP and the amplitude of the rapid phase was determined as in Fig. 6. The results clearly show that the effect of dilution is marginal.

In the next experiment, different amounts of unlabeled ADP were added together with FCCP in order to dilute the specific radioactivity of [^{14}C]ADP in the medium. If labeled ADP molecules were taken up from the medium after the addition of the uncoupler, increasing amounts of

TABLE I

EFFECT OF DILUTION ON THE AMPLITUDE OF FCCP-INDUCED RAPID BINDING OF [^{14}C]ADP

In a volume of 200 μl, thylakoids equivalent to 25 μg Chl were illuminated in the presence of 5 μmol [^{14}C]ADP/l. FCCP solutions (50–600 μl) were added after 30 s in the light; final FCCP concentration was 50 μmol/l. Unlabeled ADP was added 2 s later in order to prevent further [^{14}C]ADP binding. Total binding of [^{14}C]ADP was determined in the same way but without addition of unlabeled ADP after a reaction time of 15 min; the value was 1.1 nmol/mg Chl. The amount of tightly bound [^{14}C]ADP in the light was 0.15 nmol/mg Chl as determined by simultaneous addition of FCCP and unlabeled ADP in the light. Theoretical values assuming free equilibrium were calculated as described in the Discussion section.

Concn. of medium [^{14}C]ADP after dilution (μmol/l)	Dilution factor	[^{14}C]ADP bound (nmol/mg Chl)	Theoretical value (nmol/mg Chl)
5	1	(0.68) [a]	0.68
4	1.25	0.68	0.63
3.3	1.5	0.66	0.58
2.5	2	0.66	0.52
1.67	3	0.65	0.43
1.25	4	0.63	0.38

[a] Extrapolated value.

unlabeled ADP should lead to a progressive apparent inhibition of binding of [^{14}C]ADP. For example, a 1 : 1 dilution of the specific activity of medium [^{14}C]ADP (by the addition of the same concentration of unlabeled ADP) should reduce the uptake of ^{14}C-labeled ADP molecules from the medium by 50%.

With low concentrations of unlabeled ADP (up to 5 μmol/l vs. 2.5 μmol [^{14}C]ADP/l), a decrease of bound [^{14}C]ADP by about 2/3 can be detected (Fig. 7B) the half maximum concentration being about 2 μmol ADP/l. However, high concentrations of unlabeled ADP were required to decrease [^{14}C]ADP binding further (half saturation at about 1 mmol/l). Above 4 mmol ADP/l, no further reduction of the level of bound [^{14}C]ADP is obtained. This amount indicates the steady-state level of tightly bound [^{14}C]ADP in the light [5].

Hence, the different binding phases (Fig. 4) exhibit different sensitivities against isotope dilution. The phase of ADP binding which is highly sensitive to isotope dilution (half maximal effect at 2 μmol/l) corresponds to the slow phase of Fig. 4;

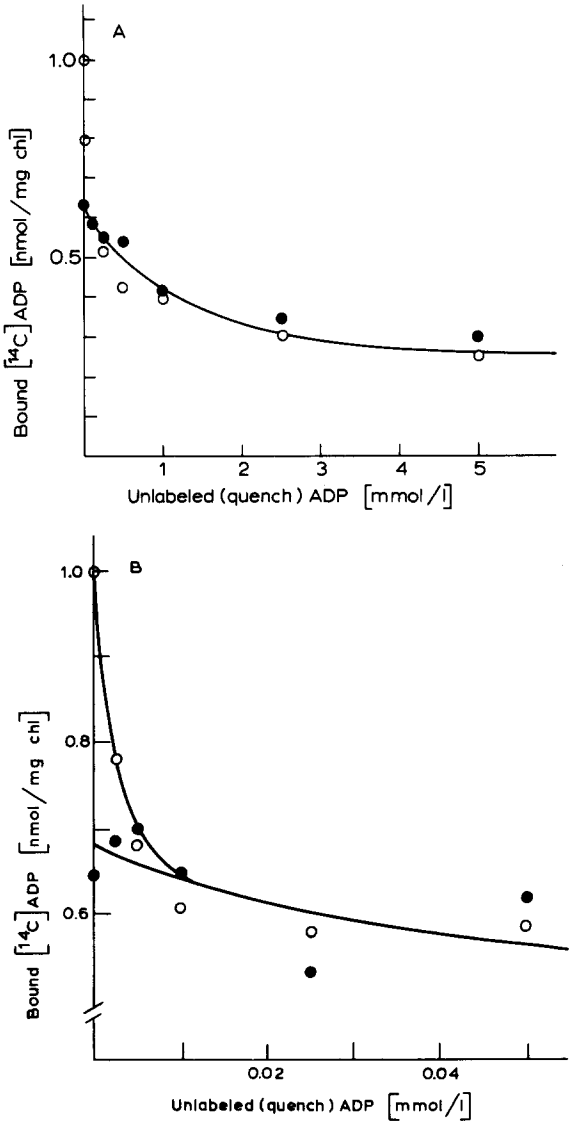


Fig. 7. Levels of tightly bound [^{14}C]ADP after quenching with FCCP and different concentrations (A,B) of unlabeled ADP. Thylakoids (0.16 mg/ml) were illuminated in the presence of 2.5 μmol [^{14}C]ADP/l. ○, FCCP (50 μmol/l) was added after 30 s together with varying concentrations of unlabeled ADP; ●, addition of unlabeled ADP (5 mmol/l) 2 s after the addition of FCCP.

during this phase ADP was clearly taken up from the medium.

The phase which is less sensitive to isotope dilution of medium ADP corresponds to the rapid binding phase of Fig. 4. Therefore, the source of ADP bound during this phase is not medium

ADP. In order to measure the effect of unlabeled ADP of the rapid phase, slow binding of [^{14}C]ADP was prevented by a second addition of excess unlabeled ADP 2 s after the addition of FCCP/ADP (Fig. 7). Thus the amount of tightly bound [^{14}C]ADP in these samples represents the amplitude of the initial rapid binding phase (cf. Fig. 4). Small concentrations of unlabeled ADP in the FCCP quench have no effect on the rapid binding phase, while at higher concentrations of ADP the same curve is obtained as for total binding of [^{14}C]ADP. This result indicates that the slow binding phase rather than the rapid one is inhibited by low concentrations of unlabeled ADP.

Discussion

The experimental data show that illumination of thylakoids in the presence of [^{14}C]ADP creates a CF_1-ADP intermediate which after deenergization converts rapidly to a non-dissociable complex. In this case labeled ADP from the medium cannot be the source for the rapid increase of bound labeled ADP because this reaction is not affected by reducing the effective medium [^{14}C]ADP concentration either by dilution of the total concentration or by isotope dilution. The difference in the levels of tightly bound ADP in the light and one second after deenergization, therefore, represents the amount of an enzyme form with loosely bound ADP (E $\cdots$ ADP), as suggested previously [5].

From the amplitudes it may be estimated that at high medium ADP concentrations in the light, about 35% of the total enzyme molecules contain a loosely bound ADP, and 65% a tightly bound ADP while almost none of the enzyme molecules are free from ADP (Fig. 6). Since tight ADP binding was found to inactivate the membrane-bound ATPase [7,10] the enzymes containing loosely bound ADP are probably catalytically active in ATP formation [12,16]. This is in agreement with the interpretation of Gräber et al. [4] that only a certain percentage (10–20%) of the ATPase molecules are active at the same time.

Bickel-Sandkötter and Strotmann [12] found that at the same time, only one ADP is either tightly bound (representing an inactive enzyme molecule) or loosely bound (representing a catalytic intermediate in ATP formation). The different modes of binding do not necessarily imply that the sites have to be physically different [12,16]. The experiments in this paper (Fig. 7 and Table I) suggest that the site occupied with loosely bound ADP is converted to the site containing a tightly bound ADP.

At low ADP concentration an increasing percentage of nucleotide-free enzyme molecules may exist in illuminated chloroplasts. In an ADP-free medium probably all of the ATPases are present in this form (E^+) which is ready to bind ADP at a high rate, but is converted to a slowly binding conformation (E^-) when the chloroplasts are deenergized. The ratio of rapid-to-slow binding at the transition from energized to deenergized conditions depends on the velocity of the transition (caused by different concentrations of uncoupler) on one hand and the concentration of ADP on the other hand. Thus at high ADP and low FCCP concentration, tight binding of ADP precedes the formation of the deenergized enzyme form (E^-), while during the addition of a low concentration of ADP together with a high concentration of FCCP, the formation of E^- is faster than the rapid formation of E = ADP (Fig. 6). During the quenching in the light with FCCP and excess unlabeled ADP, all of the nucleotide-free sites are rapidly occupied by ADP. Furthermore, the loosely bound labeled ADP is chased by unlabeled quench ADP (Fig. 7). At the first sight exchange of loosely bound [^{14}C]ADP for medium ADP could be a simple replacement reaction, which includes a dissociation and a reassociation reaction at the same site. Provided that dissociation of [^{14}C]ADP from the loose binding site were a prerequisite for the binding of unlabeled ADP to the same site, decrease of the medium [^{14}C]ADP concentration by dilution should decrease the amount of the loose enzyme-ADP complex, while the amount of tightly bound [^{14}C]ADP remains unaffected (the light level of tightly bound nucleotides is 0.15 nmol/mg Chl; see Table I).

Based on these assumptions, the amount of loose enzyme-ADP complex ($c_{E\cdots ADP}$) can be calculated by

$$c_{E\cdots ADP} = \frac{c_{ADP}}{c_{ADP} + K_d} c_E^{tot}$$

where c_{ADP} is the total concentration of labeled ADP after dilution (neglecting the small decrease by loose and tight binding) and c_E^{tot} is the sum of the concentrations of free enzyme and loose complex. The dissociation constant K_d can be computed from the data shown in Table I:

$$K_d = c_{ADP}\frac{c_{E^+}}{c_{E\cdots ADP}} = 4\ \mu mol/l$$

The amount of tightly bound [^{14}C]ADP after dilution and quenching after 2 s is therefore:

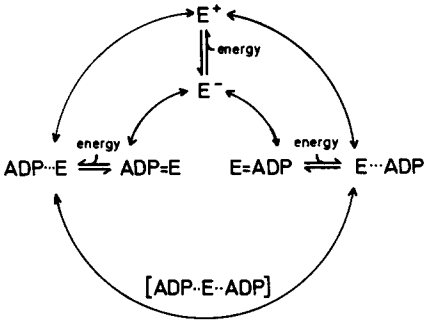
$$c_{E-ADP} = c_{E\cdots ADP} + 0.15\ nmol/mg\ Chl$$

The theoretical values calculated by this equation are included in Table I. They clearly predict a decrease of the concentration of bound ADP by dilution. The experimental data do not confirm this expectation. Therefore, it is assumed that the loose complex dissociates slowly in the absence of unlabeled ADP.

Since unlabeled ADP is able to abolish the rapid phase of binding if added together with uncoupler (Fig. 7), it is assumed that dissociation of the loose complex is accelerated if unlabeled ADP interacts with the coupling factor containing a loosely bound [^{14}C]ADP. ADP binding by a second site seems to promote the release of loosely bound ADP from the former site. Therefore, the model of nucleotide-CF_1 interactions (see Scheme I) was extended as shown in Scheme II.

The nucleotide-free enzyme can exist in two different conformations (E^+ and E^-; upper part of the scheme). Tight ADP binding by E^+ is faster than by E^-. The conversion of E^- to E^+ [7] as well as the conversion of E = ADP (containing tightly bound ADP) to E ··· ADP (containing loosely bound ADP) [5] are energy-dependent steps.

The form E^+ has two identical binding sites for ADP but only one of them is converted to a tight binding site. E = ADP and ADP = E (as well as E ··· ADP and ADP ··· E) shall design the two equivalent enzyme forms resulting from ADP binding by E^+. Starting from E = ADP (the predominant form of ATPase existing under deenergized conditions) energization would lead to equilibration between all of the indicated enzyme forms. The equilibrium concentration of the single



Scheme II

forms would depend on the protonmotive force and the concentration of ADP in the medium [10,12]. At high protonmotive force (the conditions employed in the here reported experiments) in the absence of medium ADP, the equilibrium would be shifted towards E^+. Upon deenergization, this form is converted to E^- which binds ADP in a relatively slow process. If ADP is supplied in the light together with deenergization, part of E^+ can rapidly react with the nucleotide to form tightly bound ADP (E = ADP), while another portion of E^+ is converted to E^- which slowly binds ADP. If ADP is present in the light, the equilibrium is shifted to E ··· ADP. At high ADP concentrations, the ratio between these forms depends on the light intensity [12]. E ··· ADP forms E = ADP as soon as energization is interrupted. E ··· ADP may be regarded as a highly reactive enzyme form on which the nucleotide undergoes rapid exchange with medium ADP. Several experimental results suggest that this exchange is related to the catalytic turnover of substrates in photophosphorylation [16].

The results reported here indicate that the exchange involves a flip-flop cooperation of two identical sites. Release of the loosely bound nucleotide is promoted by binding of a nucleotide at the second site.

The same conclusion was drawn from results of kinetic analysis of the release of tightly bound adenine nucleotides. The dissociation of bound nucleotides was found to be accelerated by medium ADP at the onset of illumination, and this effect is enhanced by phosphate [24]. The interactions of ADP with the coupling factor described in this

paper are influenced by phosphate, too. For example, a larger amount of loosely bound [^{14}C]ADP is released during the quench if phosphate (together with low concentrations of unlabeled ADP) is added to the quench solution (unpublished data). On the other hand, the amount of [^{14}C]ADP which is rapidly bound after deenergization in the presence of [^{14}C]ADP is increased by phosphate [25,26]. These facts indicate that the affinity of the coupling factor for ADP is higher if phosphate and magnesium ions are present in the medium. During the addition of ADP and phosphate together with FCCP, ATP is formed on the enzyme (Ref. 25 and unpublished data).

In some respect, the flip-flop cooperativity reported here shows similarities to a proposed mechanism for photophosphorylation, the so-called 'energy-linked binding change mechanism' [13,14]. According to this hypothesis, newly formed tightly bound ATP is present on one site of the enzyme, while ADP and phosphate from the medium is bound to another site. In an energy-dependent step, the conformation of the enzyme is changed so that ADP and phosphate become tightly bound while ATP can dissociate from the former site. It was found that the retention of ATP on its site depends on the concentration of ADP and phosphate in the medium: by decreasing the ADP or P_i concentration, release of ATP was retarded as was demonstrated by the increase of ^{18}O incorporation from [^{18}O]H_2O into ATP [27]. Accordingly, the release of ATP is accelerated by high medium concentrations of ADP and phosphate.

Acknowledgements

I wish to thank Professor H. Strotmann for his continuous interest and support during the preparation of this paper. I acknowledge helpful discussions with Professor N. Shavit and Dr. C. Giersch and excellent technical help by Karin Brendel and Martina Sanders.

References

1 Roy, H. and Moudrianakis, E.N. (1971) Proc. Natl. Acad. Sci. USA 68, 464–468
2 Magnusson, R.P. and McCarty, R.E. (1976) J. Biol. Chem. 251, 7417–7422
3 Strotmann, H., Bickel, S. and Huchzermeyer, B. (1976) FEBS Lett. 61, 194–198
4 Gräber, P., Schlodder, E. and Witt, H.T. (1977) Biochim. Biophys. Acta 461, 426–440
5 Strotmann, H., Bickel-Sandkötter, S. and Shoshan, V. (1979) FEBS Lett. 101, 316–320
6 Rosing, J., Smith, D.J., Kayalar, C. and Boyer, P.D. (1976) Biochem. Biophys. Res. Commun. 72, 1–8
7 Schumann, J. and Strotmann, H. (1981) in Photosynthesis II. Electron Transport and Photophosphorylation (Akoyunoglou, G., ed.), pp. 881–892, Balaban International Science Services, Philadelphia, PA
8 Bar-Zvi, D. and Shavit, N. (1980) FEBBS Lett. 119, 68–72
9 Dunham, K.R. and Selman, B.R. (1981) J. Biol. Chem. 256, 212–218
10 Strotmann, H., Bickel-Sandkötter, S., Franek, U. and Gerke, V. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 187–196, Elsevier/North-Holland
11 Schumann, J. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 223–230, Elsevier/North-Holland
12 Bickel-Sandkötter, S. and Strotmann, H. (1981) FEBS Lett. 125, 188–192
13 Rosen, G., Gresser, M., Vinkler, C. and Boyer, P.D. (1979) J. Biol. Chem. 254, 10654–10661
14 Boyer, P.D. and Kohlbrenner, W.E. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 231–240, Elsevier/North-Holland
15 Aflalo, C. and Shavit, N. (1982) Eur. J. Biochem. 126, 61–68
16 Bickel-Sandkötter, S. (1983) Biochim. Biophys. Acta 723, 71–77
17 Bar-Zvi, D. and Shavit, N. (1982) J. Bioenerg. Biomembr. 14, 467–478
18 Bakker-Grunwald, T. and Van Dam, K. (1974) Biochim. Biophys. Acta 347, 290–298
19 Sherman, P.A. and Wimmer, M.J. (1982) J. Biol. Chem. 257, 7012–7017
20 Franek, U. and Strotmann, H. (1981) FEBS Lett. 126, 5–8
21 Bennun, A. and Avron, M. (1965) Biochim. Biophys. Acta 109, 117–127
22 Strotmann, H. and Bickel-Sandkötter, S. (1977) Biochim. Biophys. Acta 460, 126–135
23 Arnon, D.I. (1949) Plant Physiol. 24, 1–15
24 Strotmann, H. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. II, pp. 477–484, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague
25 Schumann, J. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. II, pp. 543–546, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague
26 Shoshan, V. and Strotmann, H. (1980) J. Biol. Chem. 255, 996–999
27 Hackney, D.D., Rosen, G. and Boyer, P.D. (1979) Proc. Natl. Acad. Sci. USA 76, 3646–3650