Biochimica et Biophysica Acta, 723 (1983) 71–77
Elsevier Biomedical Press

BBA 41282

LOOSE AND TIGHT BINDING OF ADENINE NUCLEOTIDES BY MEMBRANE-ASSOCIATED CHLOROPLAST ATPase

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(Received September 27th, 1982)

Key words *Photophosphorylation, ATPase, Adenine nucleotide binding, Thylakoid membrane, Proton-motive force; (Spinach chloroplast)*

Steady-state binding of adenine nucleotides by thylakoid membranes is measured by employing a centrifugation technique. By this method tightly bound nonexchangeable nucleotides can be discriminated from loosely bound, exchangeable nucleotides. Nucleotide binding requires membrane energization and is highly specific for medium ADP. In illuminated chloroplasts almost no exogenous AMP and only some ATP are incorporated, most being recovered as tightly bound nucleotides. In light-triggered chloroplasts, however, which are capable of hydrolyzing ATP, a high level of exchangeable nucleotides is found on the membranes. The sum of tightly bound and loosely bound nucleotides originating from medium ADP is about one per CF_1. The ratio between them decreases with increasing proton-motive force. Exchangeable nucleotides most probably represent the ligands involved in the catalytic process, as suggested from substrate specificity and the effect of a competitive inhibitor of photophosphorylation, naphthoyl ADP. This compound in a low concentration range supresses loose binding but not tight binding of medium ADP. Under phosphorylating conditions (presence of ADP, P_i and light), some of the tightly bound nucleotides exist as ATP even in the presence of a hexokinase system. The results are discussed in the context of the regulation of chloroplast ATPase by tight nucleotide binding.

Introduction

Chloroplast ATPase (i.e, the CF_0-CF_1 complex) catalyzes the formation of ATP driven by a transmembrane proton potential. The catalytic site(s) as well as the so-called 'tight' nucleotide-binding site(s) are located on the CF_1 part [1–4]. Release or exchange of tightly bound nucleotides takes place in membrane-associated CF_1 only when the thylakoids are energized by illumination [3–5], acid-base transition [6], or an externally applied electrical field [7].

ATP-hydrolyzing activity of ATPase in situ is latent and requires preillumination of the membranes (light-triggered ATPase) [8,9]. Light activation is related to release of tightly bound ADP from CF_1 [10], suggesting that the nucleotide-depleted ATPase is the active form of the enzyme. On the other hand, deactivation of ATPase [11] is attained by spontaneous rebinding of ADP [10,12,13]. During illumination in the presence of ADP, simultaneous release and binding take place and a dynamic equilibrium between the two forms of the enzyme is established [14]. Gräber et al. [7] proposed release of tightly bound nucleotides to be a prerequisite for activation also in the process of photophosphorylation. This could be supported

Abbreviations CF_1, chloroplast coupling factor 1, Chl, chlorophyll, FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, naphthoyl ADP, 3′(2′)-*O*-(1-naphthoyl)adenosine 5′-diphosphate, naphthoyl ATP, 3′(2′)-*O*-(1-naphthoyl)adenosine 5′-triphosphate, Tricine, *N*-tris(hydroxymethyl)methylglycine

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by the finding that the steady-state level of tightly bound nucleotides is inversely related to the rate of phosphorylation when external factors such as light intensity and uncoupler concentration are varied [15].

In illuminated chloroplasts a species of exchangeable bound nucleotides can be discriminated from the tightly bound ones. The former were assumed to represent interactions at the catalytic site [16]. In this paper further experimental evidence for this notion is presented. While the steady-state level of tightly bound nucleotides decreases, that of the exchangeable nucleotides increases with increasing light intensity, so that the sum of both remains constant and amounts to about one molecule per molecule of CF_1 [16]. Two possible interpretations have been proposed [16]: (1) If two separate but interdependent catalytic and regulatory sites are involved, one has to assume that nucleotide binding at the regulatory (= tight) site excludes binding at the catalytic site of the same enzyme molecule and vice versa; or (2) the same site might exist in two different states as a tight or a catalytic site, depending on the conditions. This problem is also tackled experimentally in the present communication.

Experimental Procedure

Chloroplasts were prepared from freshly harvested spinach leaves as described in Ref. 5. Steady-state binding of ADP was accomplished by difference measurement in the supernatants in an ADP-regenerating (hexokinase) medium as described in Ref. 16, binding of ATP in an ATP-regenerating (pyruvate kinase) medium, containing 25 mM Tricine buffer (pH 8), 50 mM KCl, 5 mM $MgCl_2$, 5 mM P_i, 50 μM *N*-methylphenazonium methosulfate, 5 mM phospho*enol*pyruvate, 50 U/ml pyruvate kinase (salt-free) and 0.1–10 μM [8-^{14}C]ATP (Amersham Buchler, spec. act. 50 μCi/μmol). The final chlorophyll concentration was about 0.4 mg/ml and the total volume of the reaction mixture 1 ml. The experiments were performed at room temperature.

The employed quenching-centrifugation technique has been described and its accuracy in a concentration range of medium nucleotides up to about 20 μM has been demonstrated in Ref. 16. In order to determine also the patterns of bound nucleotides, this method was modified in the following way: The centrifugation tubes contained from bottom to top 50 μl of 40% perchloric acid, a layer of silicone oil (AR 20/AR 200 = 9 : 3, Wacker Chemie, München, 50 μl) and 250 μl of the chloroplast-containing incubation mixture. After 30 s illumination the chloroplasts were centrifuged through the silicone oil layer into the perchloric acid phase while illumination was continued. Separation of extracted nucleotides was performed by ion-exchange column chromatography on Dowex 1x8, 200–400 mesh [17]. Elution was achieved by a discontinuous HCl gradient with 8 ml each of 10 mM (AMP), 65 mM (ADP) and 200 mM HCl (ATP). The values were corrected for the medium attached to the chloroplasts during centrifugation.

Binding of [^{14}C]ATP by light-triggered chloroplasts was carried out in the dark after 2 min preillumination followed by the addition of [^{14}C]ATP in a medium as described above which additionally contained 10 mM dithioerythritol and only 1 mM instead of 5 mM $MgCl_2$. In order to interrupt ATPase activity, the quenching solution contained in addition to ADP/FCCP [14] 5 mM EDTA [18] The experiments were performed in the presence of 50 U/ml pyruvate kinase (salt-free) and 5 mM phospho*enol*pyruvate

Results

Fig. 1 shows the results of the steady-state binding of ^{14}C-labeled adenine nucleotides by illuminated chloroplasts as determined by the described difference measurement technique [16]. The curves show the sums of bound nucleotides, which include tight as well as loose interactions. As demonstrated by this experiment, binding of AMP is of the order of maximally 0.1 nmol/mg Chl. AMP binding in the light does not exceed that in the dark (0.08 nmol/mg Chl, not shown), and is of the same order of magnitude as dark binding of ADP (0.2 nmol/mg Chl.) [16]. Since the chloroplast preparations used here were extensively depleted of adenylate kinase by repeated washes [6], formation of ADP from AMP by the activity of this enzyme can be ruled out. For this reason binding of AMP as well as dark binding of ADP

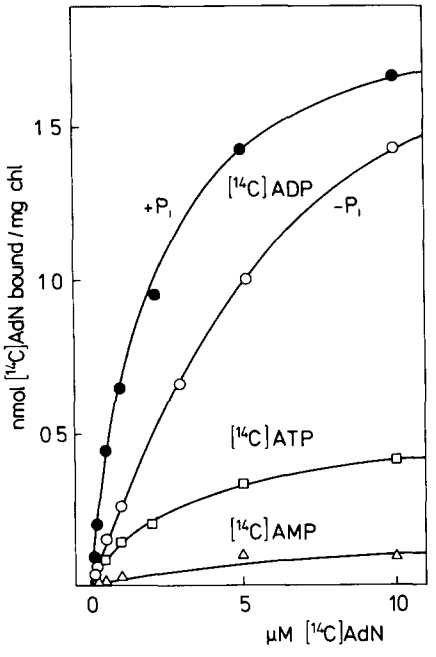

Fig 1 Steady-state binding of ^{14}C-labeled adenine nucleotides ([^{14}C]AdN) by broken chloroplasts Experimental conditions as in Table I P_i concentration was 5 mM, where added Binding of [^{14}C]ADP and [^{14}C]ATP was performed in the corresponding regenerating system

might represent unspecific interactions with the thylakoid membranes. In contrast, binding of ADP in the light is several-fold higher than in the dark [16] and approaches 1.7 nmol/mg Chl at ADP saturation in this experiment. As an average CF_1 concentration in chloroplasts 1.3 nmol/mg Chl has been determined [19]. However, this value is not constant and depends on the growth conditions for the plants [20] In winter spinach (probably due to a higher relative chlorophyll content) a lower and in summer spinach a higher CF_1/chlorophyll ratio are found. Similar changes are observed in maximum binding of ADP related to chlorophyll. Fig. 1 shows the results of an experiment performed with chloroplasts from summer spinach, whereas in Table I chloroplasts from winter plants were used (maximum ADP binding 1 nmol/mg Chl) Thus, specific light-dependent steady-state binding may be of the order of one molecule per molecule of CF_1 (see also Ref. 16) The presence of P_i increases the apparent affinity for ADP but not maximum binding (Fig 1) Binding of ATP by illuminated membranes is much lower (0.4 nmol/mg Chl at ATP saturation) than ADP binding.

TABLE I

CHROMATOGRAPHIC ANALYSIS OF ^{14}C-LABELED ADENINE NUCLEOTIDES BOUND TO ILLUMINATED THYLAKOID MEMBRANES

The medium contained 25 mM Tricine buffer (pH 8 0), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM P_i, 50 μM *N*-methylphenazonium methosulfate, 30 U/ml salt-free hexokinase and 10 mM glucose as well as the indicated concentrations of [^{14}C]ADP The chlorophyll concentration was 0 4 mg/ml, illumination time was 30 s Values are expressed as nmol ^{14}C-labeled adenine nucleotide bound/mg Chl

	[^{14}C]ADP concentration		
	5 μM	10 μM	20 μM
Total	0 434	0 713	0 810
Tight sum	0 084	0 134	0 238
AMP	0 018	0 030	0 070
ADP	0 023	0 054	0 091
ATP	0 044	0 051	0 077
Loose sum	0 350	0 579	0 572

By employing the FCCP/ADP quench [14], only tightly bound nucleotides are measured. By substraction from the total amount those nucleotides are obtained which can be replaced by the excess unlabeled ADP from the quenching solution ('loosely bound'). Under phosphorylation conditions most of the membrane-associated nucleotides are exchangeable (under the employed experimental conditions about 70%, Table I). However, the ratio between loosely and tightly bound nucleotides is variable between the experiments even under the same experimental conditions. Since the medium contains hexokinase and glucose and the loosely bound nucleotides may exchange with the medium rapidly (even during centrifugation through the silicone oil layer), the actual composition of the pattern of loosely bound nucleotides cannot be determined On the other hand, tightly bound adenine nucleotides are inaccessible to added hexokinase or pyruvate kinase, respectively [21], and thus their pattern can be reliably determined by chromatographic separation. Most of them consist of ADP as well as ATP (Table I).

TABLE II

EFFECT OF P_i ON STEADY-STATE BINDING OF [^{14}C]ADP AND [^{14}C]ATP

Assay medium as described in Table I and in Experimental Procedure The ^{14}C-labeled nucleotides were present at a concentration of 10 μM Values are expressed as nmol ^{14}C-labeled adenine nucleotide bound/mg Chl

Conditions		Total	Loose	Tight			
				Sum	AMP	ADP	ATP
[^{14}C]ADP	$-P_i$	0 565	0 252	0 313	0 065	0 217	0 031
	$+P_i$	0 696	0 434	0 262	0 000	0 152	0 110
[^{14}C]ATP	$-P_i$	0 338	0 127	0 211	0 021	0 104	0 086
	$+P_i$	0 262	0 052	0 210	0 021	0 174	0 015

In Table II the effect of 5 mM P_i on steady-state binding of ADP and ATP (both 10 μM) is compared. In the case of ADP total binding is higher if P_i is present due to the increased apparent affinity as shown in Fig. 1 The ratio of exchangeable to nonexchangeable nucleotides is much higher in the presence (1.7) than in the absence of P_i (0.8). In the latter case most of the tightly bound nucleotides consist of ADP Added ATP is bound in much lower amounts (cf. Fig. 1) and most of the nucleotides are tightly bound. In the presence of P_i, the ratio of loose to tight nucleotides is only 0.25 while in the absence of P_i it is 0.6 (Table I). P_i does not affect the amount of tightly bound nucleotides but the pattern again is different In the presence of P_i tightly bound nucleotides consist of more than 80% ADP, while in the absence of P_i about 40% are found to be ATP

In Fig 2, tight and loose ADP binding as a function of the concentration of the naphthoyl esters of ADP and ATP is shown. Naphthoyl ADP as well as naphthoyl ATP are not phosphorylated or hydrolyzed, but act as competitive inhibitors of ATP synthesis [22]. As demonstrated in Fig 2 (upper part), naphthoyl ADP strongly affects loose binding of ADP while naphthoyl ATP is a poorer inhibitor. Both analogs do not change the level of tightly bound nucleotides significantly in the employed concentration range. The $c_{I_{50}}$ values (Fig 2) are comparable to the corresponding values determined in photophosphorylation [22,28]. ATP-hydrolyzing activity of ATPase can be induced by preillumination in the presence of a thiol compound (light-triggered ATPase) A high rate of ATP hydrolysis is obtained in the dark period following energization of the membranes [23]. In Table III dark binding of added [^{14}C]ATP by

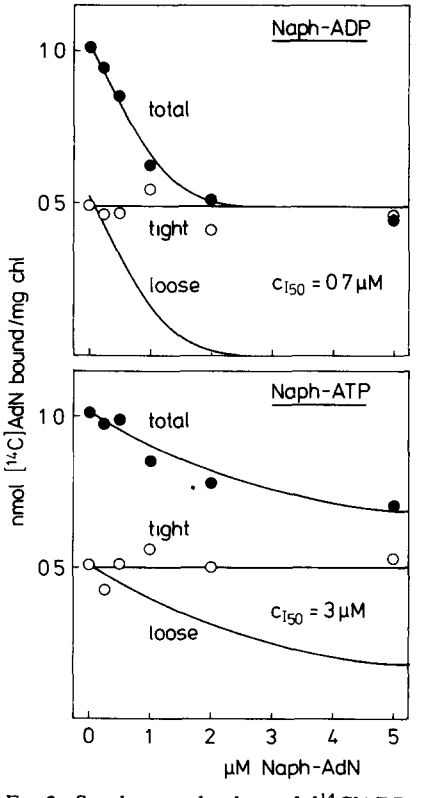

Fig 2 Steady-state binding of [^{14}C]ADP as a function of naphthoyl ADP (upper part) and naphthoyl ATP (lower part) concentrations, respectively Experimental conditions as in Table I, the concentration of [^{14}C]ADP was 5 μM AdN, adenine nucleotide, Naph, naphthoyl

TABLE III

DARK BINDING OF ATP BY LIGHT-TRIGGERED CHLOROPLASTS IN COMPARISON WITH ATP BINDING IN THE LIGHT

[^{14}C]ATP was added immediately after preillumination, separation of the chloroplasts from the medium was achieved after 30 s in the dark The control chloroplasts were illuminated for 30 s and centrifuged in the light Other conditions as in Table I Values are expressed as nmol ^{14}C-labeled adenine nucleotide bound/mg Chl

Conditions	[^{14}C]-ATP (μM)	Total	Tight	Loose
[^{14}C]ATP + 5 mM P_i + 10 mM dithioerythritol, 2 min preillumination, binding in the dark	10	0 501	0 038	0 463
	20	0 708	0 064	0 644
[^{14}C]ATP + 5 mM P_i binding in the light	10	0 260	0 220	0 040
	20	0 372	0 296	0 076

light-triggered membranes is compared with binding during illumination. The table shows that in the former case the total amount of bound nucleotides is 2-times higher than in the light. Moreover, in contrast to the situation in illuminated chloroplast (see also Table II), light-triggered membranes contain more than 90% of the nucleotides in a loosely bound form.

In Fig. 3 (upper part) steady-state levels of bound labeled adenine nucleotides as a function of FCCP concentration are shown when [^{14}C]ADP is present in the medium. In order to attain quantitative exchange of the endogenous bound nucleotides, chloroplasts were illumiated for 60 s before adding FCCP at the indicated concentrations. The levels of bound nucleotides were determined after a further 30 s of illumination. The total amount of bound nucleotides is obviously not affected by the uncoupler, whereas the fraction of tightly bound nucleotides increases and that of exchangeable nucleotides decreases with increasing FCCP concentration Uncoupling leads to the same result as reduction of light intensity [16]. In contrast, the

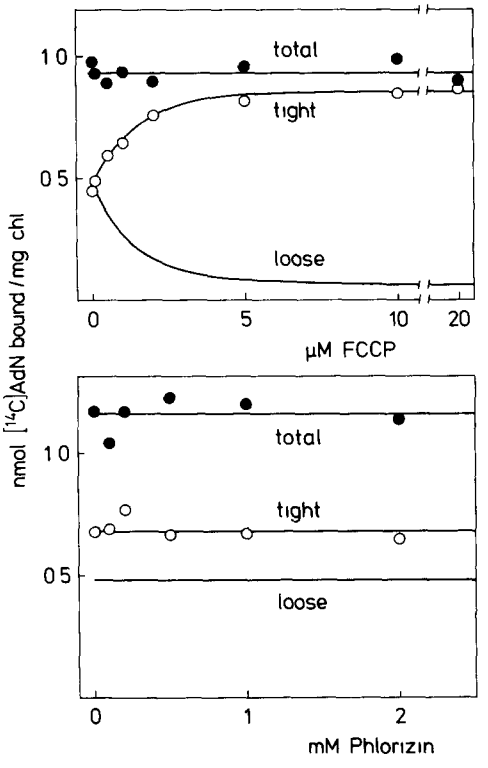

Fig 3 Steady-state levels of bound labeled adenine nucleotides as a function of FCCP (upper part) and phlorizin concentrations (lower part) [^{14}C]ADP was present at 5 μM The samples were illuminated for 60 s, prior to the addition of FCCP or phlorizin at the indicated concentrations After a further 30 s illumination, the chloroplasts were separated from the medium by centrifugation in the light AdN, adenine nucleotide

energy-transfer inhibitor phlorizin affects neither tight nor loose binding of nucleotides (Fig. 3, lower part), thus confirming the previous notion that phlorizin acts in the catalytic step, rather than in binding of the ligands [4].

Discussion

The designed technique for detection of tightly bound and exchangeable adenine nucleotides, respectively, by thylakoid membranes may provide some insight into the properties of nucleotide-binding sites of the membrane-associated ATPase, although it is limited in several respects.

(1) One limitation concerns its sensitivity. Since binding is calculated from difference measurements in the supernatants, accurate data are ob-

tained only in a certain low concentration range (up to 20 μM). However, K_m values for ADP in phosphorylation and for ATP in ATP hydrolysis are normally between 10 and 100 μM [16,22] and the K_d for catalytic nucleotide binding is supposed to be considerably lower [16]. Moreover, the apparent K_d for tight nucleotide binding is of the order of 2–5 μM [5,24]. Thus, the measured concentration range is relevant with regard to catalytic as well as tight nucleotide binding.

(2) The method allows measurement of the composition of tightly bound nucleotides. For the reason given in Results, determination of the actual pattern of loosely bound nucleotides is, however, not possible

(3) Labeled adenine nucleotides tightly bound to thylakoid membranes have been shown to be exclusively present on CF_1 [5]. It is impossible to demonstrate this for the exchangeable nucleotides by direct experimental proof All the results presented here and elsewhere [16] suggest, however, that the measured exchangeable nuceotides are related to the catalytic process at the chloroplast ATPase:

(a) Virtually no binding takes place in deenergized membranes, indicating that the catalytic site is inaccessible to medium nucleotides under those conditions [16]. This has also been concluded from steady-state kinetics of photophosphorylation at varying light intensities [16]

(b) In energized membranes loose adenine nucleotide binding is highly specific for ADP. The results shown in Fig. 1 coincide with the known facts that AMP is neither a substrate nor an inhibitor of phosphorylation and ATP is only a poor competitive inhibitor of ATP formation with a K_i of about 4 mM [22]. However, in light-triggered deenergized chloroplasts the affinity for ATP is greatly increased [22]. The activated ATPase is supposed to undergo a conformational change on deenergization of the chloroplasts, which affects the active site, so that ATP becomes a suitable ligand. This can be directly confirmed by an increase in the amount of bound nucleotides from medium ATP in light-triggered chloroplasts (Table III). Unlike in the light, under those conditions exchangeable nucleotides can be detected on the membranes (Table III).

(c) Furthermore, the apparent K_d for 'loose' ADP binding is decreased by P_i. This result could indicate an interrelation between nucleotide and phosphate binding at the active site. An increase in apparent affinity for ADP at the tight site by P_i has been reported in [25] However, this effect is obvious only at low light intensity. At high light intensity – as used in the experiment described here – no effect or even a decrease in the level of bound ADP by P_i is observed, which is in accordance with the result shown in Table II.

(d) Naphthoyl ADP, a competitive inhibitor of ADP phosphorylation in chloroplasts [22] and mitochondria [26], specifically reduces loose binding of ADP in energized chloroplasts. As in steady-state phosphorylation, the ATP analog is less inhibitory.

The functional role of tightly bound nucleotides has recently been investigated by several groups It has been established that release of those nucleotides needs membrane energization, but rebinding to the 'empty' sites is a spontaneous, energy-independent reaction [5] Release of nucleotides is related to activation of ATPase [10] and rebinding of ADP in the dark leads to deactivation of the enzyme [10,12,13]. In the light, an equilibrium between nucleotide-containing and nucleotide-free ATPase molecules is adjusted which depends on the magnitude of the proton-motive force [14]. It has been demonstrated that the steady-state level of tightly bound nucleotides is inversely related to phosphorylation activity, suggesting that in phosphorylation as in ATPase reaction the active enzyme molecules are essentially free from tightly bound nucleotides while the inactive ones contain them [15].

In view of this interpretation the interplay between tightly bound and catalytically bound exchangeable nucleotides may be reasonably understood. At a given ADP concentration, the steady-state level of catalytic nucleotides is the inverse of that of tightly bound nucleotides when light intensity [16] and uncoupler concentration (Fig. 3) are varied Accordingly, the catalytic site of nonactive ATPase molecules appears to be inaccessible for medium nucleotides. Since the sum of both species is about one per CF_1 at ADP saturation, the conclusion may be drawn that at a given time only one site is occupied by a nucleotide molecule although more sites may be present

on CF_1 [27] In the hypothesis of Boyer and coworkers [21], a certain species of tightly bound ATP is involved in the catalytic cycle. This type of nucleotide, which is supposed to undergo a high turnover rate, is probably not included in the tightly bound nucleotides as measured by the technique described here, otherwise it should have been affected by naphthoyl ADP which competitively inhibits the catalytic reaction. Thus, the majority of tightly bound nucleotides may be actually considered to be present on a regulatory site and tight binding can be taken as a measure of the activation state of ATPase.

It is still an open question as to whether the regulatory site is physically separate from the catalytic site or only a different conformation of the same site. The latter notion gains some probability from the fact that tightly bound ATP is found in the light when ADP and P_i as well as a hexokinase system are present in the medium Moreover, ADP is the main species of tightly bound nucleotides in the light when the medium contains ATP and an ATP-regenerating pyruvate kinase system Accordingly, the tightly bound nucleotides cannot originate from the bulk medium directly. The tight site may either possess some cryptic catalytic activity or is formed from a catalytic site by a conformational change. The nucleotide molecule which is present at the moment of the change could be enclosed and prevented from further reaction

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft. The author gratefully acknowledges helpful discussions with Professor Strotmann.

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