Biochimica et Biophysica Acta, 681 (1982) 451–458
Elsevier Biomedical Press

BBA 41147

MODULATION OF THE CHLOROPLAST ATPase BY TIGHT BINDING OF NUCLEOTIDES

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(Received February 15th, 1982)

Key words: Nucleotide binding; ATPase; Enzyme regulation; Nucleotide analog; (Chloroplast)

Inactivation of the chloroplast ATPase upon tight nucleotide binding was studied with several adenine nucleotide analogs. Compared with ADP, the other nucleoside diphosphates were less effective in the following order: IDP > ϵ-ADP > 1-oxido-ADP > GDP. The nucleotide analogs compete with ADP for binding to the tight nucleotide-binding site(s) on the ATPase and also prevent further inactivation by ADP. Ado*PP*[NH]*P* also causes inactivation but has a lower affinity than ADP. [^{3}H]GDP binds tightly to the ATPase, but the resulting enzyme-GDP complex is more readily dissociable than the enzyme-ADP complex. Although both nucleotides appear to bind to the same site, the catalytic and binding properties of the corresponding nucleotide-enzyme complexes differ. Binding of GDP also decreases the rate and extent of the spontaneous decay of the activated enzyme. PP_i decreases the rate of inactivation caused by ADP and also the level of tightly bound ADP. Based on these results, we suggest that two different conformations of the ATPase exist which contain tightly bound ADP. The active conformation is converted to the inactive conformation in the absence of a continued supply of energy by illumination or ATP hydrolysis.

Introduction

The chloroplast ATP synthetase as well as other energy-transducing ATPases contains high-affinity binding sites for adenine nucleotides ($K_d \approx 10^{-6}$ M) (for a review, see Ref. 1). These nucleotide-binding sites, located on the α- and β-subunits [2], exchange the bound nucleotides with nucleotides in the medium, upon energization of thylakoid membranes by light [3,4], an artificial H^+ gradient [5], or an externally imposed electrical gradient [6]. Although the tight nucleotide-binding sites were intensively examined and characterized both with the soluble and membrane-bound CF_1, very little is known about their role in the reactions catalyzed by this enzyme complex [1]. The rates of association and dissociation of the tightly bound nucleotides seem too slow to be part of the catalytic process [1]. Other workers have suggested that more than one class of bound nucleotides may exist. The kinetic behavior of one class of rapidly labeled tightly bound [^{32}P]ATP arising from $^{32}P_i$ was consistent with its participation as a catalytic intermediate in photophosphorylation [7]. However, recent results [8–14] seem to indicate that the major role of tightly bound ATP and ADP is the modulation of the ATP synthetase activities.

Energized thylakoid membranes catalyze ATP hydrolysis and a P_i-ATP exchange reaction in the dark. Much faster rates of hydrolysis and exchange are obtained if thiol reagents are present during the preillumination (activation) step [15–17]. The active conformation of the ATPase decays slowly in the dark in the absence of the substrate

Abbreviations: CF_1, chloroplast coupling factor 1; ϵ-ADP, 1-N^6-ethenoadenosine diphosphate; ado*PP*[NH]*P*, 5′-adenylyl imidodiphosphate; Tricine, *N*-tris(hydroxymethyl)methylglycine; Chl, chlorophyll.

ATP [16,17]. Addition of low concentrations of ADP, after the activation step but before the addition of ATP, results in the inactivation of the ATPase and P_i-ATP exchange activities [18]. Recently, we have reported that tight binding of ADP results in the inactivation of the ATPase and P_i-ATP exchange activities in isolated thylakoid membranes [8,9]. Similar results were also reported by Schumann and Strotmann [12] and Dunham and Selman [13]. We have also shown that inactivation occurs only after complete hydrolysis of the added ATP [8] and that the nucleotide remaining bound to the ATPase is mainly ADP [9]. The rate of decay of the active ATPase conformation, with and without added ADP, was decreased in the presence of P_i [16,18]. P_i also decreases the rate and extent of ADP binding [12,14].

We describe here the effects of the nucleoside triphosphate analog Ado*PP*[NH]*P*, of nucleoside diphosphates other than ADP, and of PP_i on the inactivation of the ATPase and on nucleotide binding to the tight binding site(s). The results indicate that the tight nucleotide-binding site(s) are probably involved in the inactivation of the ATPase and are distinct from the catalytic site(s).

Materials and Methods

Chloroplasts were isolated from fresh market lettuce leaves by standard procedures [19]. Once or thrice washed chloroplasts were used in binding and activity assays. [2-^{3}H]ADP and [8-^{3}H]GDP were purchased from Amersham, and [γ-^{32}P]ATP was obtained as described previously [8].

1-Oxido-ADP was prepared and purified according to the method of Mantsch et al. [20] and ϵ-ADP according to that of Secrist et al. [21]. Tripolyphosphate and all other unlabeled nucleotides were purchased from Sigma Co. GDP was also purchased from P-L Chemicals and Calbiochem-Behring Corp. The IDP, 1-oxido-ADP and Ado*PP*[NH]*P* samples contained less than 0.2% ADP as contaminant. GDP was further purified on Dowex 1×8, eluted with a linear gradient of NaCl (0–0.3 M) in 10 mM HCl, and desalted by passage through Sephadex G-10.

P_i released from PP_i was determinated colorimetrically [22] and chlorophyll concentration as described earlier [23].

P_i-ATP exchange and ATP hydrolysis

Chloroplast thylakoid membranes were activated in small test tubes with constant stirring, by illumination for 3 min with a beam of a strong white light (80 000 lx) filtered through a water layer. The activation mixture contained, in 0.85 ml, the following components (in μmol): Tricine (pH 8.0), 20; NaCl, 20; $MgCl_2$, 10; dithiothreitol, 10; phenazine methosulfate, 0.03; and chloroplasts containing 50–70 μg chlorophyll. Other compounds were added in 50 μl immediately after activation or as otherwise indicated. P_i-ATP exchange assays were initiated by the addition of 5 μmol ATP and 5 μmol $^{32}P_i$ (containing about $2 \cdot 10^6$ cpm) in a volume of 100 μl, and were terminated after 5 min by addition of trichloroacetic acid to a final concentration of 3%. The rate of exchange, with and without added nucleoside diphosphates, was constant for at least 10 min. ATP hydrolysis assays were initiated by the addition of 5 μmol [γ-^{32}P]ATP (containing $0.5–1.0 \cdot 10^6$ cpm) and terminated after 1 min by the addition of trichloroacetic acid. $^{32}P_i$ esterified or released was determined by the isobutanol/xylene extraction method [24].

Nucleotide binding

Thrice washed chloroplasts were activated as described above. [2-^{3}H]ADP or [8-^{3}H]GDP ($2.5 \cdot 10^5$ cpm/nmol) at the indicated concentrations was added after the activation step. Binding was quenched after incubation in the dark by addition of 5 μmol of unlabeled nucleotide as indicated. Bound nucleotides were determinated as described in Ref. 25.

Results

Effect of nucleotide binding

Tight binding of ADP was shown to inactivate P_i-ATP exchange and ATP hydrolysis by the membrane-bound ATPase in activated thylakoid membranes [8–14]. The specificity of the tight nucleotide-binding sites on membrane-bound CF_1 and the level of nucleotide binding were correlated with their effect on the inactivation of the enzyme. Ado*PP*[NH]*P*, a nonhydrolyzable analog of ATP, inactivates the P_i-ATP exchange activity with a $K_{0.5}$ of about 70 μM (Fig. 1). For inactivation,

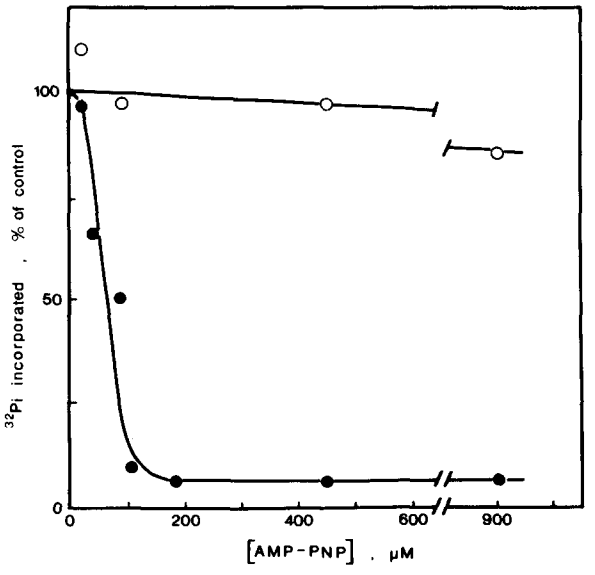

Fig. 1 Inhibition of P_i-ATP exchange. Ado*PP*[NH]*P* (AMP-PNP) at the indicated concentrations was added in the dark to activated chloroplasts (56 μg Chl). $^{32}P_i$ + ATP (5 mM each) were added immediately (○) or after 1 min in the dark (●). Control activity was 28.6 μmol $^{32}P_i$ incorporated/mg Chl per h.

thylakoids were preincubated with Ado*PP*[NH]*P* prior to the addition of ATP and P_i, the substrates for the P_i-ATP reaction. Addition of Ado*PP*[NH]*P* together with ATP had no effect. The level of contamination of this lot of Ado*PP*[NH]*P* with ADP was determined to be not more than 0.2%, and therefore the contaminant could not be responsible for the large degree of inactivation observed. AMP, up to 2.5 mM, does not inactivate the membrane-bound ATPase. AMP also had no effect on the inactivation of the enzyme by ADP, suggesting that it does not bind to the tight binding site as does ADP.

Several other nucleoside diphosphates were tested as shown in Fig. 2. Only ADP causes full inactivation of the P_i-ATP exchange and ATPase activities. The effectiveness of the nucleotides tested was in the following order: ADP > IDP > ε-ADP > 1-oxido-ADP > GDP. GDP causes partial inactivation (5–35%) which may be due to ADP contamination. The ADP content (0.6–1.5%) of GDP samples obtained from several commercial sources was reduced to less than 0.2% by ion-exchange chromatography. Analysis of the effect of different GDP samples showed that the degree of inhibition was correlated with their ADP content.

When the analogs were incubated with the activated enzyme in the presence of ADP before ATP and P_i were added, they effectively protected the enzyme against the ADP-induced inactivation (Fig. 3). Since the nucleotides (e.g., ε-ADP) partially inactivated the enzyme, full protection by each nucleotides was obtained against the effect of ADP but only to the level of inactivation by the analog itself. None of the analogs was inhibitory if added directly to the assay reaction mixture without prior incubation with the membranes.

Since GDP is the least inhibitory and also the

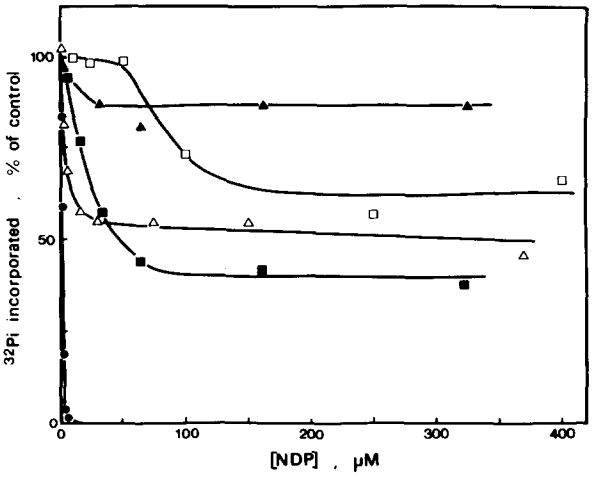

Fig. 2 Inhibition of P_i-ATP exchange by various nucleoside diphosphates (NDP). Nucleotides were added immediately after activation. $^{32}P_i$ + ATP was added after 1 min in the dark. ●, ADP; ▲, GDP; □, 1-oxido-ADP; △, ε-ADP; ■, IDP.

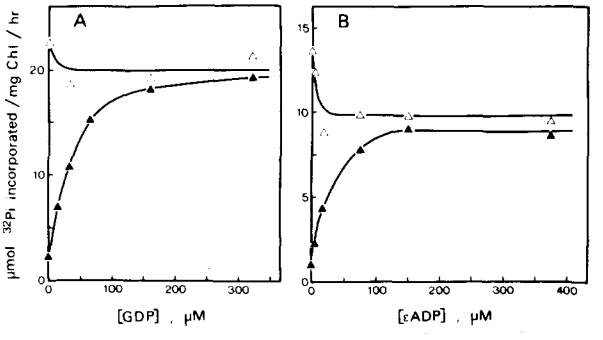

Fig. 3 Effect of GDP and ε-ADP on the inhibition of P_i-ATP exchange by ADP. GDP or ε-ADP alone (△) or together with 5.6 μM ADP (▲) were added immediately after activation. $^{32}P_i$ + ATP was added after 1 min. (A). GDP, (B) ε-ADP.

best protective nucleotide against inactivation of the enzyme by ADP, we chose this pair of nucleotides to study the reversibility of their interaction with the nucleotide-binding site(s) (Table I). The inactivation of the membrane-bound ATPase by ADP can be fully reversed by reenergization followed by the immediate addition of ATP (and P_i) (sample 1,B). Delaying the addition of ATP results in reinhibition of the ATPase. As shown, incubation of the enzyme with GDP and ADP together results in partial inactivation (by ADP) according to the relative concentrations of the two nucleotides (sample 2,A; and Fig. 3A). Addition of GDP after the interaction with ADP has already occurred (sample 3,A) does not affect the degree of inactivation caused by ADP. Similarly, ADP does not inactivate the enzyme when added after the interaction with GDP has occurred (sample 4,A). Full reactivation is achieved by reenergization followed by the immediate addition of ATP and P_i. Reactivation was independent of the previous order of exposure of activated enzyme to nucleotides. However, delaying the addition of ATP and P_i after reenergization results in the inactivation of the enzyme in a manner which depends on the concentrations of both ADP and GDP. Thus, the interaction between the tight nucleotide-binding site(s) and nucleoside diphosphates under nonenergized conditions is practically irreversible.

In Fig. 4 we show that ADP and GDP appear to compete for binding to the tight binding site on the activated enzyme, with calculated K_d values of 1.3 and 25 μM, respectively. The levels of ADP and GDP remaining bound to activated and nonactivated membranes, upon quenching of their binding with unlabeled ATP or ADP, are given in Table II. After quenching with ATP or ADP, nonactivated membranes retain similar levels of bound nucleotide diphosphates. In general, these levels are higher than those retained on activated membranes. However, with activated membranes,

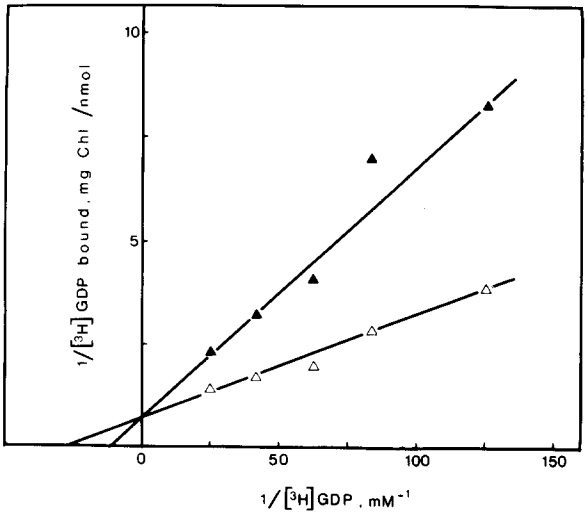

Fig. 4 Inhibition of [^{3}H]GDP binding by ADP. [^{3}H]GDP was added alone (△), or together with 3.3 μM ADP (▲), immediately after energization. Binding was quenched by the addition of 5 mM unlabeled GDP 1 min later, and assayed as described in Materials and Methods.

TABLE I

IRREVERSIBLE INTERACTION OF GDP AND ADP WITH THE CHLOROPLAST ATPase.

Nucleotides at the indicated concentrations were added to the activated thylakoids at the beginning of stage A (samples 1 and 2) or as indicated (samples 3 and 4). $^{32}P_i$ + ATP was added at the end of each of the consecutive stages (A–C) and activity was assayed. (A) Dark, 120 s; (B) light, 10 s; (C) dark, 60 s. Control activities without added nucleotide diphosphates: (A) 23.3, (B) 31.2, (C) 31.6 μmol $^{32}P_i$ incorporated/mg Chl per h.

Sample	Compound added	P_i-ATP exchange assayed after stage (% of control)		
		A	B	C
1	ADP (5.6 μM)	4	110	6
2	ADP+GDP (27.7 μM)	38	93	41
3	ADP immediately, GDP after 60 s	10	98	43
4	GDP immediately, ADP after 60 s	83	95	46

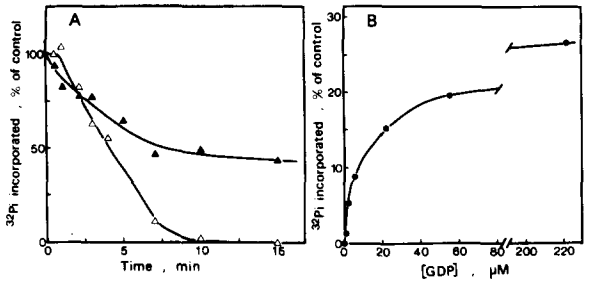

Fig. 5 Effect of GDP on the decay of the activated ATPase. Chloroplasts were activated as described in Materials and Methods. (A) △, no addition; ▲, 30 μM GDP was added immediately after activation. $^{34}P_i$ + ATP was added after the time intervals indicated. Control activity was 20 μmol P_i incorporated/mg Chl per h. (B) GDP at the indicated concentrations was added immediately after activation. $^{32}P_i$ + ATP was added after 15 min in the dark. Control activities (without GDP added) were 13 and 0 μmol P_i incorporated /mg Chl per h, immediately after activation and after 15 min decay, respectively.

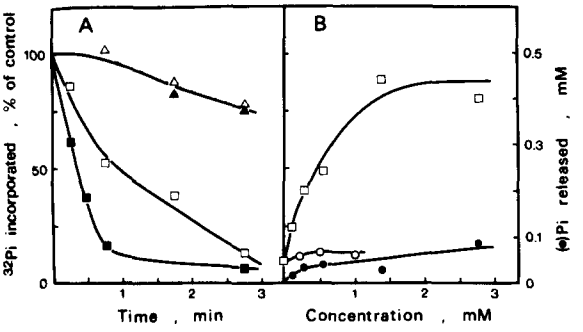

Fig. 6 Effect of PP_i on the inactivation of the ATPase by ADP. (A) The following compounds were added 10 s after activation: ▲, none; ■, 5.6 μM ADP; □, 5.6 μM ADP + 278 μM PP_i; △, 278 μM PP_i. $^{32}P_i$ + ATP was added after the indicated time in the dark, and activity assayed as described in Materials and Methods. (B) 5.6 μM ADP was added together with PP_i (□) or P_i (○), and incubated with activated chloroplasts for 1 min in the dark before the addition of $^{32}P_i$ + ATP. ●, PP_i hydrolysis was determined at the indicated concentrations after 1 min incubation in the dark. Control activities were (A) 19 and (B) 33 μmol P_i incorporated/mg Chl per h.

quenching with ATP was more effective than with ADP. Moreover, after quenching with ATP, the level of bound [^{3}H]GDP was significantly lower than that of [^{3}H]ADP. Quenching with unlabeled GDP was as effective as with ADP (not shown). These results indicate that although both nucleotide diphosphates appear to bind to the same nucleotide-binding site(s), the CF_1-GDP complex is more readily dissociable (with ATP) than the inactive CF_1-ADP complex.

Since GDP protects against inactivation by ADP, we also examined whether GDP affects the rate of decay of the ATPase activated state. As shown in Fig. 5A, GDP decreases the rate of decay. In the absence of GDP, complete decay of the activated state occurs after about 10 min, while in the presence of 30 μM GDP, the decay after this interval is only about 50%. As shown in Fig.

TABLE II

LEVELS OF TIGHTLY BOUND [^{3}H]ADP AND [^{3}H]GDP UPON QUENCHING WITH ATP AND ADP.

Thylakoids were preilluminated for 2 min with 10 mM dithiothreitol (activated thylakoids) or without dithiothreitol (nonactivated). The labeled nucleotide was added immediately after illumination. After 1 min in the dark, the reaction was quenched by addition of 5 mM of the unlabeled nucleotide as indicated. Bound nucleotides were determined as described in Materials and Methods. Each value shown is the mean of three different experiments done in duplicate. ATPase activity was 29.2 and 3.1 μmol ATP hydrolyzed/mg Chl per h for the activated and nonactivated chloroplasts, respectively. ATP/ADP, ratio of bound nucleotides after quenching with ATP or ADP.

Nucleotide added, (μM)	Amount of bound nucleotide (nmol/mg Chl)					
	Activated thylakoids			Nonactivated thylakoids		
Quenched with:	ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP
[^{3}H]ADP, (5)	0.10	0.16	0.6	0.25	0.22	1.1
[^{3}H]GDP, (40)	0.05	0.18	0.3	0.24	0.24	1.0

5B, the activity remaining after a relatively long period (15 min) of incubation with GDP depends on the nucleotide concentration. The K_s for GDP in preventing the ATPase decay (15 μM) is comparable to the K_d for GDP binding (25 μM). The apparent difference between these two constants may be due to the different experimental conditions in the two assays (incubation time).

Effect of PP_i

P_i decreases the rates of decay of the activated ATPase, the inactivation by added ADP, and the binding of ADP to the enzyme. As shown in Fig. 6A, PP_i also decreases the rate of inactivation of the enzyme by ADP, but does not affect the maximal degree of inactivation. Fig. 6B shows the dependence of this effect on PP_i concentration, and also that PP_i is much more effective than P_i. Since our chloroplast thylakoid preparations have pyrophosphatase activity, we determined the P_i released during the incubation period. Fig. 6 also shows that the amounts of P_i released by PP_i hydrolysis (after a 1 min incubation) cannot be responsible for the decrease in the rate of inactivation.

Table III shows that in the presence of PP_i, less [^{3}H]ADP is bound to the activated enzyme. Like P_i, PP_i decreases the initial rate of ADP binding and also reduces the total amount of bound ADP. Tripolyphosphate, up to 5 mM, has no effect on the rate and extent of inactivation by ADP. Tripolyphosphate by itself does not inhibit the ATPase or the P_i-ATP exchange reaction and is not hydrolyzed by the activated thylakoid membranes (not shown).

TABLE III

EFFECT OF PYROPHOSPHATE ON [^{3}H]ADP BINDING TO ACTIVATED THYLAKOIDS

5.6 μM [^{3}H]ADP was added alone or with 450 μM PP_i to activated thylakoids 10 s after activation. Reactions were terminated by the addition of 5 mM ATP at the indicated time in the dark, followed by 5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) 30 s later, bound nucleotides were assayed as described in Materials and Methods.

Time (s)	[^{3}H]ADP bound, (nmol/mg Chl)	
	$-PP_i$	$+PP_i$
5	0.05	0.03
10	0.10	0.07
60	0.22	0.15
120	0.29	0.17

Discussion

Modulation of the membrane-bound ATPase appears to be controlled by the binding of nucleoside diphosphate to the tight nucleotide-binding site(s) on CF_1. On the basis of the evidence presented above and elsewhere [8–14], we propose the following model for regulation of the ATPase by nucleotide binding. Steps 1 and 2 in the scheme (Fig. 7) indicate that full activation of the latent enzyme is obtained only after illumination for several minutes in the presence of thiol reagents [15–17]. The activation process appears to involve at least two distinct stages: (1) a rapid release of tightly bound ADP and (2) a relatively slow change in the enzyme conformation, which depends on the presence of thiol reagents [10, 15–17] and may involve reduction of disulfide bound(s) [26]. The activated enzyme will slowly decay in the dark to the inactive conformation unless the substrate (ATP) is added [16,17]. We have recently shown [9] that although binding of ADP to the ATPase occurs continuously during the hydrolysis of ATP, it does not lead to inactivation of the enzyme (step 3). Dunham and Selman [13] reached a similar conclusion on the basis of a kinetic analysis.

The free enzyme and the enzyme-ADP complex

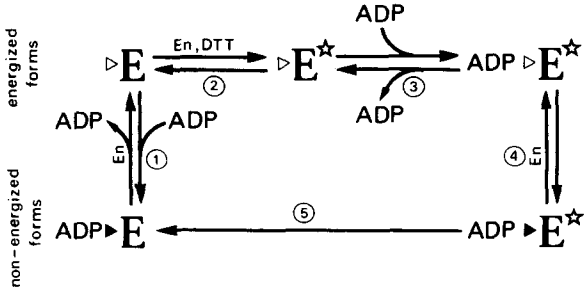

Fig. 7 Proposed scheme for the modulation of the chloroplast ATPase by tight binding of ADP. E and E*, latent and activated ATPase, respectively. Triangles represent the tight nucleotide-binding site(s) on the enzyme. Full symbols are used to indicate the inactive enzyme-ADP complexes. En, energy; DTT, dithiothreitol.

assumed here to be the active enzyme conformations (energized forms) catalyze the P_i-ATP exchange and ATP hydrolysis and are converted to the inactive enzyme-ADP conformation (nonenergized form) only after all the ATP is exhausted (step 4). The inactive enzyme-ADP complex can be reactivated by a short energization period (Table I). Upon addition of ATP to activated chloroplasts, the hydrolysis of ATP will create the necessary ΔpH [27] to provide the energy required to maintain the ATPase in the energized conformations. In the presence of an uncoupler, inactivation by ADP binding occurs before all the ATP is hydrolyzed (Bar-Zvi, D. and Shavit, N., unpublished results). Although in the presence of an uncoupler the initial rate of ATP hydrolysis is enhanced, this hydrolytic activity does not appear to maintain the enzyme-ADP complex in its active conformation.

The decay of the activated enzyme, if nucleotides are not added (Fig. 5A), may occur upon rebinding of ADP (Fig. 7, steps 3 and 4) or by reversal of the activation process (steps 1 and 2). However, reactivation after decay has occurred, in the absence of added ADP [27] or in its presence [8] (Table I), is much faster than activation of the latent enzyme. On this basis we propose that the activated enzyme decays mainly through steps 3 and 4 to form the inactive enzyme-ADP complex.

The ATP analog Ado*PP*[NH]*P* binds to the free enzyme form and inactivates it as does ADP, probably because of its adenine moiety and the inability of the membrane-bound ATPase to hydrolyze its terminal phosphate. Ado*PP*[NH]*P* was shown to bind to soluble [28] and membrane-bound CF_1 [29,30], and to inhibit the binding of ADP and ATP [30].

GDP binds to the thylakoid membranes and appears to compete with ADP for binding to the nucleotide-binding site(s) (Fig. 4 and Table II). However, the tight binding of GDP does not result in the inactivation of the enzyme. On the contrary, GDP protects against ADP inactivation (Fig. 3A and Table I) and decreases the rate of decay of the ATPase (Fig. 5A). The K_d for [^{3}H]GDP binding to activated chloroplast membranes is similar to that obtained in competition studies with nonactivated membranes [31]. We therefore suggest that GDP is bound to the active conformation of the enzyme, but that the resulting CF_1-GDP complex does not undergo the conformational changes needed to yield the inactive enzyme-nucleotide complex (step 4). This conclusion is also supported by the different levels of [^{3}H]GDP and [^{3}H]ADP remaining bound to activated membranes after quenching with ATP (Table II). The difference between activated and nonactivated membranes after quenching with ATP or ADP (Table II) indicates that the hydrolysis of ATP and not its binding causes the release of bound GDP or ADP. Different levels of adenine nucleotides bound to activated membranes, when binding of [^{14}C]ATP was quenched with ATP or ADP, were also reported by Schumann [32]. The partial inactivation by other adenine nucleotide analogs (Fig. 2) can be explained assuming that in their presence a different equilibrium between the active and inactive enzyme-nucleotide complexes is established. Carmeli and Lifshitz [18] reported a higher degree of inhibition of the ATPase by GDP. Since the level of ADP contamination in the GDP samples was not determinated, the inhibition observed may at least be partly due to contaminating ADP. Binding of ADP was also shown to inhibit the soluble mitochondrial F_1 [33]. The lag in ATP hydrolysis observed was suggested to be due to slow replacement of firmly bound ADP by MgATP.

The decrease in the rate of inactivation by ADP, in the presence of PP_i (shown here), may occur if binding of ADP is irreversible and binding of PP_i to the same site is reversible. Reversible binding of PP_i to soluble CF_1 was shown to displace the previously bound nucleotide analog, formycin triphosphate [34]. Binding of ADP to soluble CF_1 [35,36] was also inhibited by PP_i. The effective concentration of PP_i was much lower than that of P_i, resembling the effect we obtained with the membrane-bound enzyme. These findings indicate that PP_i may interact with allosteric or regulatory sites rather than with the catalytic site [35,36].

Girault et al. [37] showed that tripolyphosphate was nearly as effective as PP_i in inhibiting ADP binding to soluble CF_1. In our case, tripolyphosphate did not protect against ADP-induced inactivation of the light triggered P_i-ATP exchange. This difference could be due to the different pre-

parations used, i.e., soluble as opposed to membrane-bound CF_1.

In conclusion, we suggest that two conformations of the enzyme with associated nucleotides exist, which differ in their catalytic and binding properties. Inactivation of the ATPase occurs upon decay of the activated enzyme-nucleotide complex to the inactive conformation. The decay occurs when energy is no longer supplied by illumination or ATP hydrolysis. The bound ADP appears to modulate the enzyme by locking-in the inactive conformation. Its release from this site upon energization is necessary for the subsequent conformational changes leading to the stabilized active enzyme form.

Acknowledgements

We wish to thank Mrs. Z. Conrad for excellent technical assistance. This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft. This work is part of the thesis of D. B.-Z. for a Ph.D. degree (biochemistry) at the Ben Gurion University of the Negev, Beer-Sheva.

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