Biochimica et Biophysica Acta, 1019 (1990) 11–18
Elsevier

BBABIO 43247

Activation and deactivation of the thiol-modulated chloroplast H^+-ATPase during ATP hydrolysis

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(Received 12 January 1990)
(Revised manuscript received 23 April 1990)

Key words: Chloroplast; ATPase, H^+-; Adenine nucleotide; Proton gradient; Enzyme regulation; Thiol modulation

Formation of a transmembrane proton gradient, tight binding of adenine nucleotides and ATPase activity were followed during hydrolysis of a limited amount of ATP by thylakoids preilluminated in the presence of DTT. The following results were obtained: (1) While a micromolar concentration of ADP caused complete deactivation of the ATPase within 15 s, the same concentration of ATP sustained enzyme activity for more than 1 min due to a small proton gradient generated by hydrolysis of the ATP. (2) In the presence of ADP, steady-state ATPase activity and levels of tightly bound nucleotides are inversely influenced by ΔpH. When ADP is replaced by ATP, the activity curve is shifted towards much lower ΔpH values and is not significantly different from the ΔpH curve obtained in the absence of medium nucleotides. (3) At low medium ATP concentration (5 μM), ATP as well as ADP is incorporated into membrane-bound CF_1; the ratio of tightly bound ATP/ADP increases while the sum of ATP plus ADP decreases with increasing ΔpH. An enzyme with a tightly bound ATP can be active or inactive, depending on the actual ΔpH. (4) Hydrolysis of ATP by thylakoids containing a certain percentage of active ATPases, effects extensive reactivation of inactive enzyme molecules due to coupled ΔpH formation. At exhaustion of the substrate, however, the total enzyme population is inactivated within 1 min. Inactivation is a synergic result of the decay of the proton gradient and tight binding of the hydrolysis product ADP.

Introduction

The thylakoid H^+-ATPase is active in illuminated but inactive in dark-adapted chloroplasts. After thiol modulation, however, the enzyme retains catalytic activity for some time in the dark and is capable of hydrolyzing added ATP [1,2]. Thiol modulation includes reduction of a disulfide to a dithiol group in γ subunit of the activated ATPase by thiols like DTT [3,4] or the natural reductant thioredoxin [5]. The respective amino acid segment is present in the γ sequence of chloroplasts [6,7], but not of blue-green algae [8,9].

Activation is triggered by the electrochemical proton gradient and under certain circumstances is accompanied by release of one tightly bound adenine nucleotide per CF_0CF_1 [10–12]. The tightly bound nucleotide was shown to reside in one of the three catalytic centers [13]. Most likely $\Delta\tilde{\mu}_{H^+}$-dependent activation results in the opening of the catalytic sites which are closed in the inactive state. Thereby the two non-occupied sites become accessible to medium substrates and the bound nucleotide at the third site undergoes dissociation [14–16]. The velocity of its dissociation is increased to different extents by binding of medium ADP, ATP or ADP plus P_i to the free sites [14,15].

In contrast to the non-modulated enzyme which is rapidly inactivated with decay of the proton gradient, deactivation of the thiol-modulated ATPase lags behind relaxation of $\Delta\tilde{\mu}_{H^+}$. ATPase activity declines within minutes provided the medium is free from ADP. In the presence of ADP, however, fast deactivation is triggered by tight incorporation of one nucleotide molecule per CF_1 [12,17]. Tight binding of ADP and inactivation of the ATPase is decelerated by inorganic phosphate [2,12,18–20]. When activated thiol-modulated thylakoids are supplied with ATP, the nucleoside triphosphate is hydrolyzed at a constant rate in spite of continuous

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Abbreviations: DTT, dithiothreitol; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; PEP, phospho*enol*pyruvate; P_i, inorganic orthophosphate; PK, pyruvate kinase; PMS, phenazine methosulfate; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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accumulation of the reaction product ADP. Accordingly, the working enzyme seems to be protected from ADP-induced inactivation. Tight binding of ATP in place of ADP was thought to effect stabilization of activity [21]. Actually ADP and ATP were shown to be competitive in tight binding [11]. However, other factors have to be considered, too. As ATP hydrolysis is coupled with generation of a proton gradient [22,23], $\Delta\tilde{\mu}_{H^+}$ may counteract against tight binding of the reaction product ADP. In a previous paper [24] it was shown that the ATPase is inactivated when the proton gradient is decaying as a consequence of exhaustion of substrate ATP. Moreover it is known that the rate of ATP hydrolysis, although being accelerated initially, is rapidly decreasing with time when an uncoupler is added together with substrate ATP [23]. The complex control of the H^+-ATPase during the course of ATP hydrolysis is the issue of this paper. The problem was approached by measurements of enzyme activity, gradient formation and tight nucleotide binding under a variety of experimental conditions.

Methods

Thylakoids were isolated from spinach leaves as in Ref. 11. For measurements of ATP hydrolysis, enzyme activity, nucleotide binding and ΔpH the thylakoids (25 μg Chl/ml) were preilluminated in a medium containing 25 mM Tricine (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT and 50 μM PMS for 2 min with white or red light at 20 ° C. Kinetics of ATP hydrolysis in the following dark were determined after addition of [γ-^{32}P]ATP. Consecutively taken aliquots were deproteinized by $HClO_4$ (final concentration 0.6 M) and analyzed for [^{32}P]P_i [25]. [γ-^{32}P]ATP was prepared as in Ref. 26 and purified as in Ref. 27.

For measurements of actual ATPase activity during the course of ATP hydrolysis, unlabeled ATP was employed as substrate. At the desired time a mixture containing PEP, PK, nigericin (final concentrations and activities, respectively, of 1 mM, 30 U/ml, 0.5 μM) and [^{32}P]ATP (to yield a total concentration of 0.2 mM) was added. The initial rate of ATP hydrolysis was determined by analysis of [^{32}P]P_i in samples taken after 5, 10 and 15 s and treated as described above.

Time-courses of tight nucleotide binding were followed by using ^{14}C-labeled adenine nucleotides. Binding of the radioactive nucleotide was stopped by addition of FCCP (50 μM) plus unlabeled ADP (5 mM final concentrations) [28]. To remove non-bound labeled nucleotides the thylakoids were subsequently washed three times [28]. An aliquot of the resuspended thylakoids was used for chlorophyll determination, another aliquot for measurement of radioactivity in a scintillation cocktail. The actual pattern of bound nucleotides was determined by combining the FCCP/ADP quench with the 'hexokinase accessibility' technique [29]. For this purpose the quench solution was

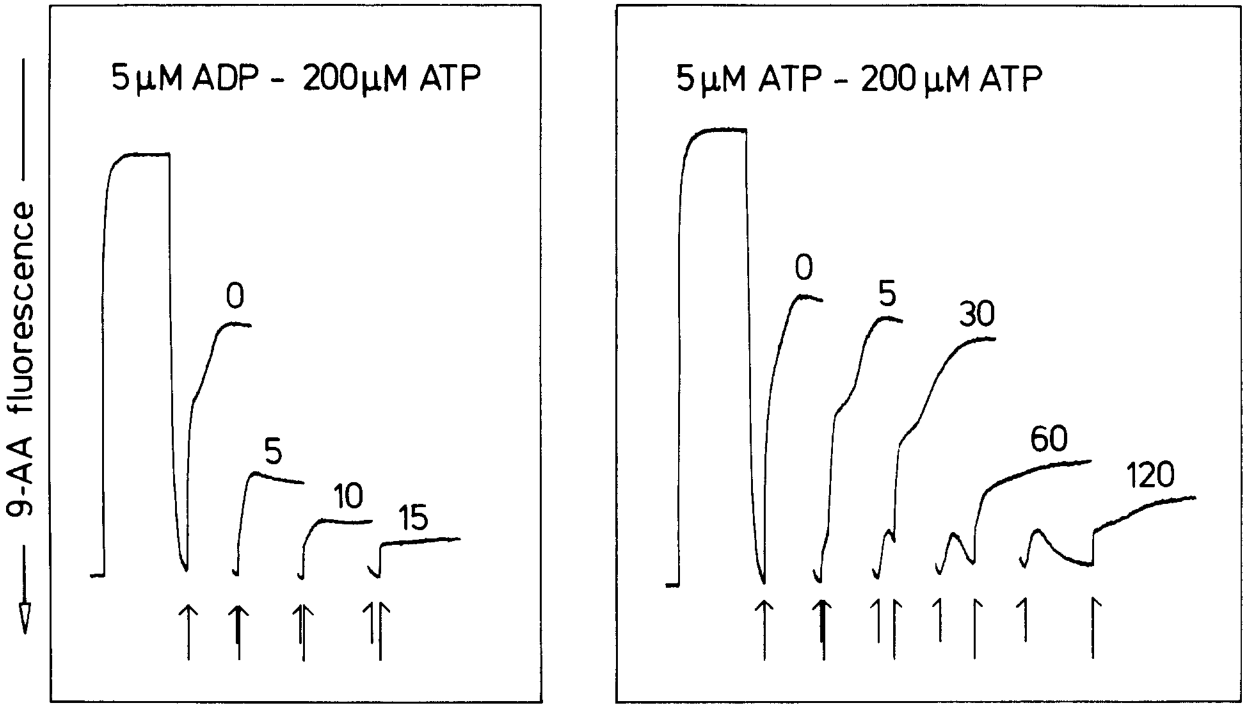

Fig. 1. Formation of a proton gradient coupled with ATP hydrolysis as measured by 9-aminoacridine fluorescence quenching. 45 s after a 2 min preillumination in the presence of DTT, either 5 μM ADP or 5 μM ATP was injected before excess ATP (200 μM) was added at different times. The numbers signify the time (in s) elapsed between the first and second addition. The chlorophyll concentration was 25 μg/ml.

supplemented with 50 mM glucose and 280 U/ml salt-free hexokinase (Sigma) (final concentrations). 5 s after addition of the quench solution, an aliquot was deproteinized by $HClO_4$ (0.6 M final concentration). After neutralization in the extract the labeled nucleotides were separated by TLC on poly(ethyleneimine)-cellulose TLC plates with fluorescence indicator (Macherey and Nagel, Düren). The mobile phase was 0.3 M sodium citrate (pH 4.1). The nucleotide spots were scraped out and counted in scintillation cocktail (Optifluor, Packard Instruments). Transmembrane ΔpH was recorded by the 9-aminoacridine fluorescence technique [30]. Calibration of the fluorescence signal was described in Ref. 31.

Results

In order to induce an active thiol-modulated ATPase, thylakoids were routinely preilluminated for 2 min in the presence of 10 mM DTT. In an experiment shown in Fig. 1, a low concentration (5 μM) of either ATP or ADP was added after a 45 s dark period. At different times afterwards substrate concentrations of ATP (0.2 mM) were supplied. The formation of ΔpH coupled with ATP hydrolysis was followed kinetically by 9-aminoacridine fluorescence. As expected, preincubation with ADP resulted in almost complete inactivation of the ATPase within 15 s (a small instantaneous fluorescence quench observed upon ATP addition is an artifact due to interaction of the nucleotide with the fluorescent dye [31]). Preincubation with 5 μM ATP generated a small transient ΔpH with a maximum (about 2 units) after 20 s. If substrate amounts of ATP were injected less than 60 s after addition of the low ATP concentration, formation of a large ΔpH of almost 3 units was observed. A later supply of substrate amounts of ATP caused progressively lower proton gradients. The kinetics of ATP-induced ΔpH formation showed a rapid initial rise followed by a slower increase. The rapid rise may be attributed to the action of initially active ATPase molecules. The slow phase may indicate proton transport-coupled ATP hydrolysis by enzyme molecules which were reactivated by the initially established ΔpH. Hence, extensive reactivation is possible after a short preincubation with low concentrations of ATP, but not with ADP.

As ATPase reactivation may be related with release of tightly bound nucleotides, we have investigated the changes of the levels of tightly bound nucleotides when during incubation with 5 μM [^{14}C]ADP or [^{14}C]ATP, respectively, substrate concentrations of unlabeled ATP were given. During binding of [^{14}C]ADP, addition of excess ATP led to a stop of further binding [20], but no release of previously bound labeled ADP was observed. In variance, labeled nucleotides incorporated from medium [^{14}C]ATP could be partially liberated when a high concentration of unlabeled ATP was injected (Fig. 2). When the ATP pulse was employed 15 s after the addition of [^{14}C]ATP, about 2/3 of the tightly bound labeled nucleotides were released. However, with progression of [^{14}C]ATP binding, addition of substrate ATP resulted in slower and less complete release. This may be attributed to an increasing deactivation of ATPase molecules. If an uncoupler (nigericin) was supplied together with substrate ATP, liberation of tightly bound labeled nucleotides was completely abolished (not shown), indicating that the generation of a proton gradient was necessary for re-release of tightly bound nucleotide and ATPase reactivation, respectively.

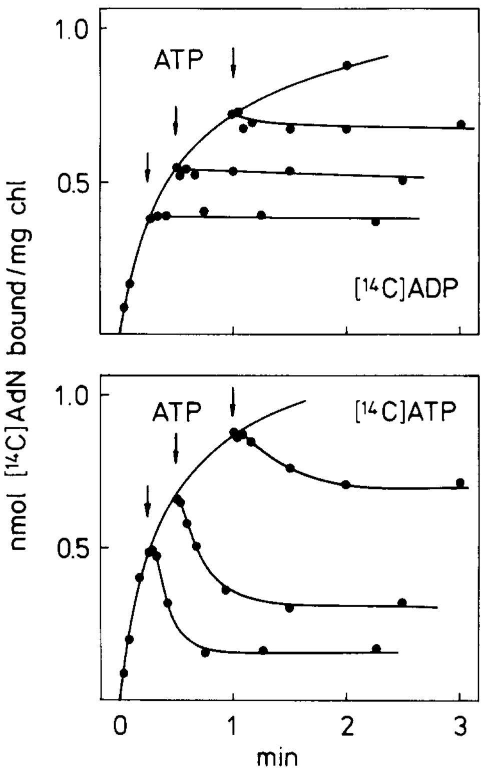

Fig. 2. Time-courses of tight binding of 5 μM [^{14}C]ADP and [^{14}C]ATP as affected by subsequent addition of excess ATP (200 μM). The labeled nucleotides were added 15 s after a 2 min preillumination in the presence of DTT. The chlorophyll concentration was 100 μg/ml. At the indicated times, the reaction was stopped by excess ADP plus FCCP as described in Methods.

If reactivation is excluded by addition of an uncoupler together with substrate ATP, the initial rate of ATP hydrolysis in the presence of an ATP recycling PK system is a measure of the overall enzyme activity of the membrane-bound ATPase [24,31]. This parameter can be used to determine the relative amount of active enzyme molecules as function of different factors. Thus measured ATPase activity as function of ΔpH during incubation of thylakoids in the absence and presence of 10 μM ADP or 10 μM ATP is shown in Fig. 3A. In order to obtain different ΔpH values, the chloroplasts were illuminated at various light intensities. An incuba-

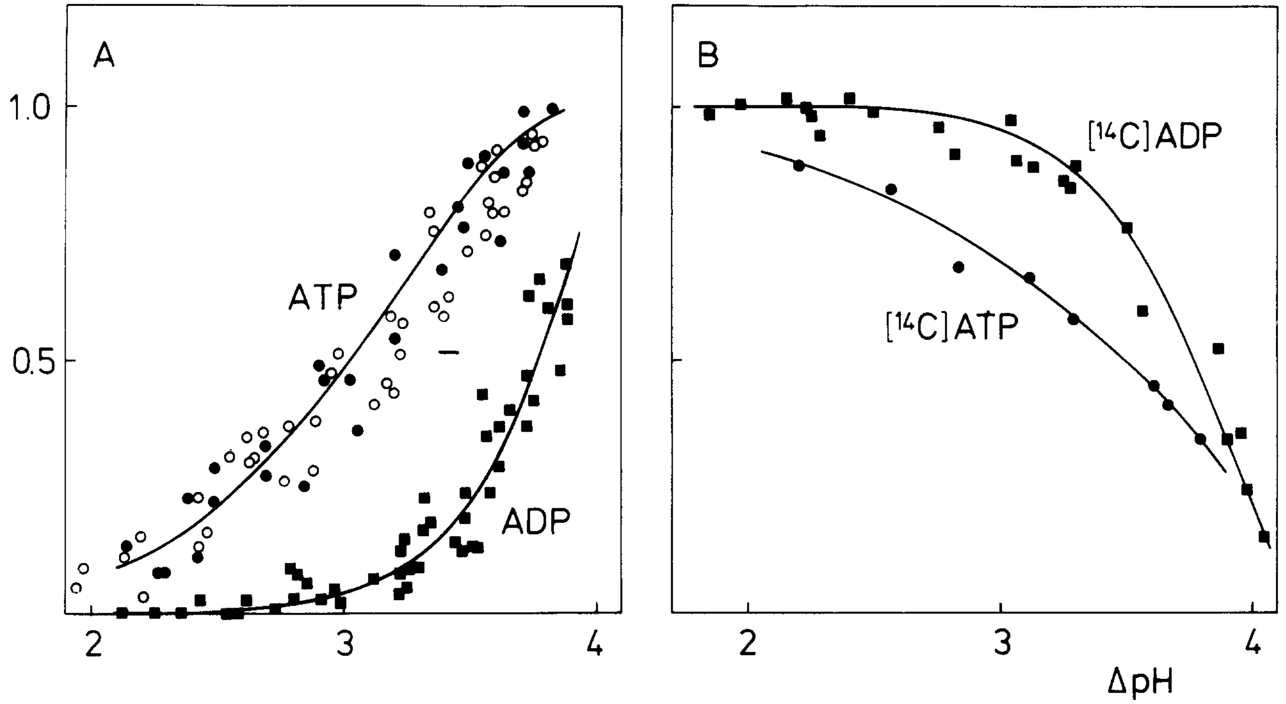

Fig. 3. Steady-state ATPase activity (A) and levels of tightly bound ^{14}C-labeled adenine nucleotides (B) as function of ΔpH presented in relative units. After 2 min preillumination of thylakoids (25 μg chlorophyll/ml), either 10 μM ADP or 10 μM ATP was added; simultaneously light was attenuated to different intensities. The proton gradient was monitored by 9-aminoacridine fluorescence which was calibrated as in Ref. 31. After 3 min equilibration, a mixture of phospho*enol*pyruvate (2 mM), pyruvate kinase (30 U/ml), [γ-^{32}P]ATP (0.2 mM) and nigericin (0.5 μM, final concentrations) was injected. In the variant containing 10 μM ATP, the ATP regenerating enzyme system was present from the beginning. For determination of enzyme activity [^{32}P]P$_i$ was measured in samples taken after 15, 30 and 45 s as described in Methods. The open symbols show control activities obtained in the absence of adenine nucleotides. For determination of tightly bound nucleotides, the reactions were carried out correspondingly, however, with ^{14}C-labeled ADP and ATP. The reaction was stopped after 3 min as in Fig. 2. The data taken from several independent experiments were normalized as described in Ref. 12, Maximal ATPase activities in μmol P$_i$/mg Chl per h: 347 ± 72 (nucleotide-free medium), 289 ± 30 (presence of ATP), 352 ± 81 (presence of ADP). Maximal levels of tightly bound nucleotides in nmol AdN/mg Chl: 1.10 ± 0.12 (presence of ADP), 0.92 ± 0.10 (presence of ATP).

tion time of 3 min was sufficient to achieve steady states under all conditions. The ΔpH/activity relationships found in the presence of ATP were almost identical with the one measured in the complete absence of medium nucleotides. However, the curve was shifted towards much higher ΔpH values when ADP was in the medium. For example at ΔpH 2.5, about 20% of the ATPases were active in the absence or presence of ATP, but no significant activity could be detected in the presence of ADP; at ΔpH 3 nearly half of the enzymes were active with or without ATP in the medium, but only 5% in the presence of ADP. Only at $\Delta\text{pH} > 4$ did the curves approach each other.

Under the same experimental conditions steady-state levels of tightly bound nucleotides were determined by employing ^{14}C-labeled ADP or ATP, respectively (Fig. 3b). While tight binding of medium [^{14}C]ADP exhibited a ΔpH dependency which was more or less inverse to enzyme activity [31], an inverse correlation between enzyme activity and levels of tightly bound nucleotides incorporated from medium [^{14}C]ATP was observed only at $\Delta\text{pH} < 3$. However, at ΔpH 3.5, 70% activity, but 50% of maximal nucleotide binding was attained, and at ΔpH 3.8 enzyme activity was almost 100% whereas 1/3 of the enzyme molecules contained a tightly bound nucleotide. Although the medium concentration of ATP in this experiment was maintained by PEP/PK, not all of the incorporated nucleotides might be [^{14}C]ATP. It is possible that some ATP is hydrolyzed at the site before or after transition to the occluded state. Early studies [32] have shown that after incubation of thylakoids with ATP tightly bound ADP is the predominant species. In those experiments, however, the bound nucleotides were extracted after circumstantial washing of the membranes; therefore the obtained nucleotide pattern possibly was a result of a slow conversion of [^{14}C]ATP within the occluded site. In order to avoid disturbance of the results by secondary reactions as far as possible, a quench method was employed which combines isotope dilution and rapid uncoupling with the 'hexokinase accessibility technique' (see Methods). Upon addition of a mixture containing excess unlabeled ADP, FCCP, glucose and a high activity of hexokinase, all freely accessible [^{14}C]ATP is rapidly converted to [^{14}C]ADP.

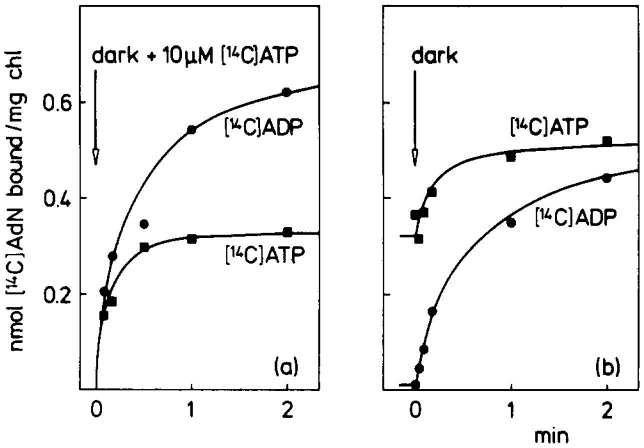

Fig. 4. Changes of the patterns of tightly bound labeled adenine nucleotides with time after addition of 10 μM [^{14}C]ATP either before (b) or at the end of a 2 min preillumination in the presence of DTT (a). The chloroplast concentration was 195 μg/ml. In order to stop the reactions, the quench solution contained glucose and hexokinase in addition to ADP and FCCP (see Methods). 5 s after addition, aliquots of the quenched samples were extracted by $HClO_4$ (0.6 M) and subjected to TLC for determination of hexokinase-inaccessible (= tightly bound) [^{14}C]ATP. The rest was used for the determination of total bound labeled adenine nucleotides.

$HClO_4$ extraction after a few seconds thus extracts the hexokinase inaccessible (= tightly bound) [^{14}C]ATP only and may be separated from [^{14}C]ADP (= tightly bound [^{14}C]ADP plus free [^{14}C]ADP) by chromatography. The tightly bound [^{14}C]ADP can be obtained from the difference between total bound labeled nucleotides and [^{14}C]ATP.

In Fig. 4 the patterns of tightly bound labeled nucleotides were followed as function of time when thylakoids were transferred from light to dark. 10 μM [^{14}C]ATP was either supplied before illumination (2 min) (b) or [^{14}C]ATP was added together with turning off the light (a). During illumination ΔpH was about 3.6 and decreased to zero within about 1 min in the dark. When [^{14}C]ATP was added at light-to-dark transition (Fig. 4a), incorporation of both, [^{14}C]ATP as well as [^{14}C]ADP was observed from the beginning. Binding of [^{14}C]ATP was almost complete after 30 s, whereas binding of [^{14}C]ADP continued. This kinetic difference may be attributed to the fact that virtually all of the [^{14}C]ATP was hydrolyzed after 30 s in the dark (see Fig. 1), so that the nucleotide available afterwards was [^{14}C]ADP. The amount of bound labeled ATP did not decrease during the time of the experiment, thus excluding a rapid subsequent hydrolysis of ATP within the tight site. However, a slow increase of tightly bound ADP at the expense of ATP could be observed in an extended time range. Within 1 h in the dark, the ratio of ADP/ATP increased from 2 to 13 (Table I).

When [^{14}C]ATP was present in the light (Fig. 4b), about 1/3 of the enzyme molecules contained a tightly bound nucleotide at the light-to-dark transition; they consisted almost exclusively of [^{14}C]ATP. From this we may conclude that the nucleotide incorporated from medium ATP at high ΔpH values in Fig. 3b predominantly is tightly bound ATP. Upon turning off the light, additional [^{14}C]ATP and [^{14}C]ADP was bound with similar kinetics but lower extents as in Fig. 4a. After 2 min in the dark the ratio of tightly bound [^{14}C]ATP/[^{14}C]ADP was 0.5 when [^{14}C]ATP was given after preillumination, but a ratio of more than 1 was obtained when [^{14}C]ATP was supplied at the beginning of the light period. Although the electrochemical proton gradient is completely relaxed after 2 min under both conditions, the pattern of occluded nucleotides is not rapidly adapted to the energy state of the membrane.

Fig. 5a shows the time-course of cleavage of 50 μM ATP added 45 s after preillumination. In parallel measurements the change of actual ATPase activity (b), the formation of ΔpH (c) and the kinetics of tight nucleotide binding (d) during the course of ATP hydrolysis were followed. The kinetics of ATP hydrolysis (a) were slightly sigmoidal, indicating self-activation of the ATPase (see Fig. 1). In fact, the actual ATPase activity (b) increased upon substrate addition more than twice. After 30 s a stable activity was observed for 2–3 min. With substrate exhaustion observed after 3 min (a), the enzyme activity decreased to almost zero within 1 min.

At substrate addition a fast rise of ΔpH was observed; after reaching a maximum of about 3 units, ΔpH decreased slowly (c). A faster breakdown between 3 and 4 min coincided with exhaustion of the substrate ATP. The time-course of tight nucleotide binding (d) was complex: upon ATP addition about 50% of the available binding sites were occupied within the first 5 s; after 30 s, a constant level (60% of the sites occupied) was maintained for 2–3 min. With substrate exhaustion the residual free sites were occupied within 1 min. The obvious kinetic correlation suggests that the synergic effects of relaxation of the proton gradient and tight binding of ADP provided complete deactivation of the ATPase.

Different results were obtained when ATP hydrolysis was carried out in the presence of an uncoupler (Fig. 6).

TABLE I

Change of the pattern of tightly bound adenine nucleotides during prolonged dark incubation

Preilluminated thylakoids (50 μg Chl/ml) were supplied with [^{14}C]ATP (5 μM) after 30 s in the dark. At the indicated times aliquots were quenched and analyzed as in Fig. 4.

	Tightly bound (nmol/mg Chl)			
Dark time (min):	5	10	30	60
ADP	0.841	0.949	0.994	1.071
ATP	0.172	0.158	0.139	0.084
Sum	1.013	1.107	1.133	1.155
ADP/ATP	4.9	6.0	7.2	12.8

At the employed concentration of 0.5 μM nigericin added together with ATP, no ATP-induced ΔpH formation could be detected (trace not shown). The time-course of uncoupled ATP hydrolysis revealed a higher initial rate compared with coupled hydrolysis, the velocity, however, decreased from the beginning and ATP hydrolysis stopped when only 80% of the added ATP was cleaved (a). Assay of ATPase activity (b) indicated a continuous deactivation from the beginning, a result which explains the decrease of the rate of ATP hydrolysis with time. The incomplete cleavage of ATP is due to the fact that almost all of the enzyme activity has disappeared after 2 min. Tight nucleotide binding (c) exhibited a biphasic time-course in this case. As in coupled conditions, 50% of the available binding sites were occupied during the first 5 s. In a subsequent slower phase the residual binding sites were filled with a half-time of about 30 s. The phase of slow nucleotide binding resembled the time-course of ATPase deactivation.

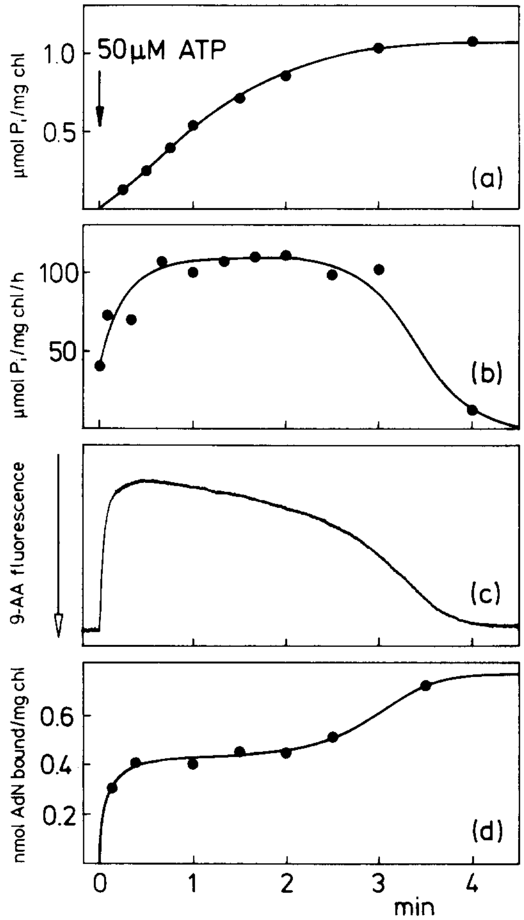

Fig. 5. Kinetics of ATP hydrolysis (a), ATPase activity during the course of the reaction (b), simultaneous formation of a proton gradient (c), and tight binding of adenine nucleotides. Thylakoids were preilluminated for 2 min. ATP (50 μM) was added 45 s after turning off the light. In (a) [γ-^{32}P]ATP, in (d) [8-^{14}C]ATP was employed as substrate. In (c) 5 μM 9-aminoacridine was added 10 s before the addition of ATP. For determination of actual enzyme activity in (b) carrier-free [γ-^{32}P]ATP together with 1 mM PEP, 30 U/ml pyruvate kinase and 0.5 μM nigericin (final concentrations) was injected at the indicated times. Initial rates of [^{32}P]P_i were measured as in Fig. 3. The chlorophyll content during the reaction was 48 μg/ml.

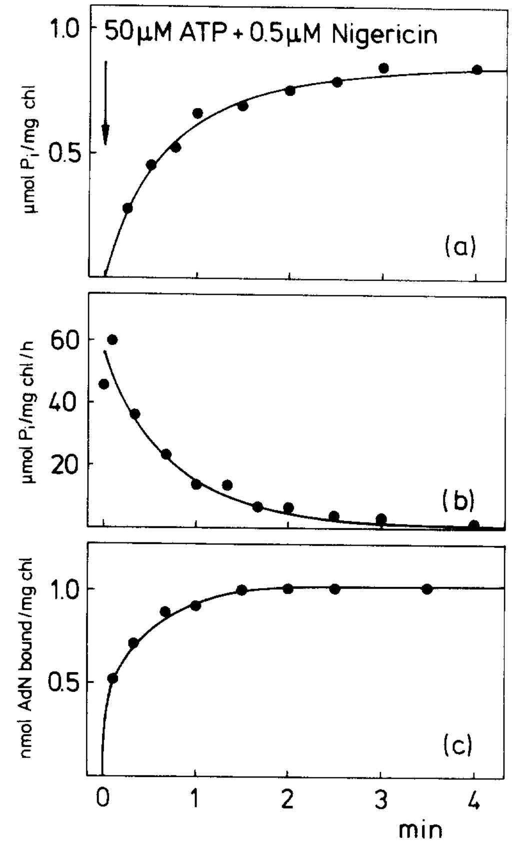

Fig. 6. Kinetics of ATP hydrolysis in the presence of an uncoupler (a), change of ATPase activity during the reaction (b) and tight binding of adenine nucleotides (c). Together with ATP (50 μM) at $t = 0$, 0.5 μM nigericin was supplied. Other conditions as in Fig. 5. The chlorophyll concentration was 44 μg/ml.

Discussion

In the presence of medium ADP, activity of the ATPase is controlled by the antagonistic effects of tight ADP binding leading to inactivation, and $\Delta\tilde{\mu}_{H^+}$ effecting activation and simultaneous release of ADP. According to a report by Zhou et al. [13], a tightly bound ADP is in an occluded catalytic site residing in one of the three β subunits.

In addition to the catalytic sites (presumably three per ATPase), three non-catalytic sites have been identified in the α-β region [33,34]. Determination of nucleotide contents of the membrane-associated CF_1 yielded altogether four tightly bound nucleotides, 2–3 ATP and 1–2 ADP [35]. In contrast to ADP, which was released to a large extent by thylakoid energization, the ATP remained bound [35]. However, upon solubilization of CF_1 the tightly bound ATPs obviously get lost, since only one tightly bound ADP or less is usually detected on isolated CF_1 [34,36,37]. The respective free sites, which were identified as non-catalytic, can be refilled by medium nucleotides. Results reported by Hammes' group showed that the irreversible introduction of one MgATP had no effect on ATPase activity [36]. On the other hand, heat activation of the isolated CF_1 in the presence of ADP, resulting in tight binding of ADP to one of the non-catalytic sites, gave an enzyme form with low GTPase activity. Subsequent filling of the second site with ATP and the third site with MgATP, however, increased the catalytic activity [34]. Such a modulation of activity by non-catalytic binding of ATP occurring in isolated CF_1, is largely excluded in CF_0CF_1 in situ, as the sites in question are permanently occupied by ATP [35]. The result of Fig. 3, indicating that preincubation with ATP in the whole ΔpH range does not change ATPase activity compared with ATP-free controls, strongly supports this view. Nevertheless, some ATP/ATP exchange may take place at non-catalytic sites in illuminated thylakoids [35] without alteration of activity. This could explain the finding that a fully active enzyme population is obtained at high ΔpH with about 1/3 of enzymes containing a tightly bound nucleotide (Fig. 3).

The majority of tightly bound labeled nucleotides incorporated from medium [^{14}C]ATP must be assumed to reside in the same type of sites where [^{14}C]ADP is bound, i.e., in an occluded catalytic site. The reasons are the following:

(1) ATP is known to compete with ADP in the process of tight nucleotide binding [11].

(2) In the tightly bound state most of the ATP is slowly converted to ADP when membranes are kept in the dark for 1 h (Table I), indicating that the site has catalytic activity. Our result confirms an earlier finding by Magnusson and McCarty [32]. The low hydrolytic activity may be explained by the specific conformation or microenvironment of the site in the occluded state. Hence, significant amounts of tightly bound [^{14}C]ATP are detected only when after incorporation the dark time was not too long (cf. Table I and Fig. 4).

(3) This tightly bound ATP, like tightly bound ADP, is released upon reillumination of the thylakoids [15]. As a result of ATP binding and energy-dependent release the steady-state levels increase with decreasing ΔpH and vice versa (Fig. 3). Compared with a nucleotide-free medium, enzyme activity as function of the energy state of the thylakoids is shifted towards higher ΔpH values when the medium is containing ADP (Fig. 3). The process of $\Delta\tilde{\mu}_{H^+}$-dependent activation was considered to include protonation of the enzyme from the intrathylakoidal side, possibly related with simultaneous deprotonations at the medium exposed side [38,39]. An analysis of steady-state activity as function of ΔpH in different media led to the conclusion that the three catalytic entities of the ATPase are sequentially activated leading to a consecutive opening of the three catalytic centers. The involved reversible transitions of enzyme conformation are considered to be rate-limiting in the overall process [31]. In steady state the dynamic equilibrium between forms with zero, one, two or three open sites thus depends on ΔpH. Quantitative evaluation of the results also suggested that enzymes with at least two non-occupied open centers are catalytically active and that opening of a closed site with bound ADP has a higher ΔpH requirement (apparent inner $pK = 4.0$ at external pH = 8.0) than opening of a closed nucleotide-free site (apparent inner $pK = 4.7$) [31].

In contrast to medium ADP, no significant effect of medium ATP is observed on ΔpH dependency of steady-state enzyme activity. This result permits to conclude that under the employed conditions (presence of an ATP recycling system) in the measured ΔpH range (2–4) the incorporated ATP is not hydrolyzed to ADP but remains ATP during its stay in the occluded state and that opening of a site with an occluded ATP has the same ΔpH demand as opening of a closed non-occupied site. In summary: neither an ATP/ATP exchange at non-catalytic sites nor tight binding of ATP to one of the catalytic sites influences the ΔpH-dependent control of activity of the ATPase.

We are faced with a complex situation, however, when preilluminated thylakoids are supplied with substrate amounts of ATP in the dark in the absence of an ATP recycling enzyme (Fig. 5). The conditions are different from those in Fig. 4, where a very low concentration of ATP was employed. Depending on the elapsed dark time after preillumination, a certain proportion between active and inactive enzymes exists, both species being essentially free from nucleotides at catalytic sites due to the pretreatment. The initial increase of enzyme activity observed upon ATP addition can be referred to reactivation of inactive enzymes of the same thylakoid, since a ΔpH (about 3 units) is generated immediately upon addition of ATP. The simultaneous incorporation of labeled nucleotides must be attributed to ATP/ATP exchange at a non-catalytic and tight binding of ATP to catalytic sites (see above) because the initial ATP/ADP ratio is high and ΔpH is relatively large. As discussed above, binding of ATP would not change the activity state of the respective enzyme molecule, therefore the simultaneous nucleotide incorporation and reactivation of other molecules of the enzyme population is not contradictory. Increase of the proportion of active enzymes increases ΔpH and thereby effects further reactivation. However as the reaction proceeds, incorporation of ADP formed by ATP hydrolysis may lead to deactivation of other enzymes. As long as ΔpH is high, the two opposing reactions may be balanced, so that a quasi-steady state of active enzymes is maintained for some time. Only upon exhaustion of

ATP the compulsory decay of the gradient and increase of the ADP/ATP ratio causes the residual active nucleotide-free enzymes to go to an inactive form by rapid uptake of ADP; more slowly the active enzymes containing a tightly bound ATP fall into inactive states, too. Hence, self-support of activity of the population lapses synergically with decay of the proton gradient and increase of the ADP/ATP ratio. This effect plays a significant role in control of ATPase activity at light to dark transition in vivo [40]. Self-support of the system via ΔpH is also demonstrated by the action of an uncoupler (Fig. 6), which effects decrease of enzyme activity from the beginning of the reaction.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 189) and by the Fonds der Chemischen Industrie. The authors thank Miss Renate Thelen for technical help and Mrs. Rita Reidegeld for preparing the manuscript.

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