Biochimica et Biophysica Acta, 934 (1988) 213–219
Elsevier

BBA 42798

Contents of endogenous adenine nucleotides and ATPase activity of isolated whole chloroplasts

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(Received 30 December 1987)

Key words: Intact isolated chloroplast; Regulation of chloroplast ATPase; Endogenous adenine nucleotide

Endogenous adenine nucleotide contents of isolated intact chloroplasts and activities of the H^+-ATPase were measured in parallel under various experimental conditions. In chloroplasts isolated from spinach grown in summer, ATPase activity declines upon light-dark transition in a biphasic manner. Initial rapid inactivation of about 50% of the ATPases corresponds with the rapid increase of the ADP and decrease of the ATP levels. The residual ATPases are slowly deactivated, probably due to the reversal of thiol modulation. In isolated chloroplasts from winter plants the phase of rapid inactivation is largely absent. Those chloroplasts have a low content of endogenous nucleotides. When the ratio of internal ATP/ADP is decreased by the addition of 3-phosphoglycerate or the uncoupler FCCP, the activity of the ATPase decreases. The relationship between activity and ATP/ADP ratio is nearly linear. Inorganic phosphate, although being ineffective in changing the internal nucleotide levels, significantly raises the ATPase activity. The results suggest that in whole chloroplasts the ATPase not only is regulated by the thioredoxin system but also controlled by the internal ATP/ADP ratio. This kind of control is referred to the nucleotide-dependent regulatory mechanism previously demonstrated in isolated thylakoid suspension.

Introduction

The H^+-translocating ATPase of the chloroplast is a regulated enzyme. The physiological significance of regulation lies in the avoidance of ATP breakdown at low protonmotive force. In dark-adapted chloroplasts the enzyme is completely inactive, whereas in the light active forms of the ATPase exist. The main parameter to control activity is the transmembrane electrochemical proton gradient [1–4]. Activation and deactivation follows the formation and decay, respectively, of the proton gradient provided that the ATPase is in a condition which is referred to as 'oxidized' or 'demodulated' state. In the 'reduced' or 'thiol-modulated' state, however, ATPase activity under certain conditions is maintained after relaxation of the gradient, thus enabling hydrolysis of ATP [5–8].

Thiol modulation of isolated washed thylakoids can be achieved by preillumination of the vesicles in the presence of DTT [6]. This treatment causes reduction of a specific disulfide group in γ subunit of CF_1 which is exposed by illumination [9,10]. In intact chloroplasts reduction is mediated by the soluble endogenous thioredoxin system [11] which

Abbreviations: DTT, dithiothreitol; FCCP, carbonylcyanide *p*-fluoromethoxyphenylhydrazone; PGA, 3-phosphoglycerate; GAP, glyceraldehyde 3-phosphate; PGK, phosphoglycerate kinase; GAPDH, glyceraldehydephosphate dehydrogenase; Chl, chlorophyll.

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receives electrons from the photosynthetic electron-transport chain [12]. Reoxidation is caused by internal oxidants which have not yet been characterized. In vitro H_2O_2 can serve as an oxidant [13,14]. Experiments with intact isolated chloroplasts have suggested that 'demodulation' by reoxidation of γ subunit is the way of physiological deactivation of the ATPase when chloroplasts are transferred from light to dark [14,15]. However, the decline of activity was found to take several minutes [14]. Thiol modulation therefore may be a useful means to cut off the ATPase for long periods of darkness, but seems to be unsuitable to avoid ATP losses as a consequence of rapid and large changes of light intensity, events which are entirely normal in the natural habitat of plants.

With DTT-modulated isolated thylakoids deactivation of ATPase in the dark was shown to be strongly accelerated by micromolar concentrations of ADP when added before substrate ATP [16,17]. The ADP is 'tightly' bound to CF_1 [18–20] at a binding site which is located in β subunit [21,22]. Upon membrane energization the bound ADP is released and the enzyme is reactivated [18]. An ATPase molecule containing a tightly bound ADP is catalytically inactive, irrespective of being reduced or oxidized in γ [17]. On the other hand, when ATP is incorporated instead of ADP, the enzyme remains active [23]. Hence in DTT-modulated thylakoids the ratio of concentrations of ATP to ADP and the magnitude of the proton gradient determine the activity of ATPase. Upon turning off the light, the enzyme is immediately deactivated when the ADP/ATP ratio is large, at high excess of ATP over ADP, however, enzyme activity is maintained in the dark [24]. As a consequence of the decreasing gradient, ATP is hydrolyzed. ATP hydrolysis, on the other hand provides generation of a proton potential which facilitates re-release of tightly bound ADP and hence reactivation of the enzyme. As a result, the process of dark inactivation of the ATPase is retarded under these conditions. A cascade-like breakdown of activity is initiated when during the progressing reaction the ADP/ATP ratio exceeds a certain threshold [24].

In this study the relevance of the outlined mechanism of nucleotide-dependent control for regulation of the chloroplast ATPase in situ was investigated. For this purpose ATPase activities were measured as a function of endogenous adenine nucleotides in the intact chloroplast. The ratio of internal ATP/ADP concentrations can be decreased by treatments which either inhibit ATP formation or accelerate ATP consumption. This strategy was employed in order to detect possible effects of ATP/ADP ratio on activity of the ATPase. The results suggest that indeed the intact organelle (and possibly also the intact plant) makes use of the nucleotide-dependent regulatory mechanisms that have been explored in vitro.

Methods

Intact chloroplasts from spinach leaves were isolated as in Ref. 25. Intactness as examined by light microscopy [26] was at least 85%. Experiments were conducted in small thermostated (20 °C) glass vessels which were illuminated (250 $W \cdot m^{-2}$) from the top. The medium usually contained 0.36 M sorbitol, 50 mM tricine buffer and 0.25 mM P_i (pH 8.0) [13], the chlorophyll concentration during incubation was around 100 $\mu g/ml$, the volume was 0.2 ml. For measurement of ATPase activity at a given time, 0.8 ml hypotonic assay medium was added and light was turned off if necessary. The medium consisted of 10 mM tricine buffer, 4 mM $MgCl_2$, 5 μM FCCP and 0.625 mM γ-^{32}P-labeled ATP which was synthesized as in Ref. 27. After 10, 20, 30, 60 s, 0.2 ml samples were deproteinized with 0.075 ml 3 M $HClO_4$. The liberated $^{32}P_i$ was measured as in Ref. 27. For the determination of endogenous adenine nucleotides, 0.4 ml of the reaction mix were deproteinized with 0.075 ml 70% $HClO_4$. ATP, ADP and AMP were determined by the luciferase method as in Ref. 28.

Results

Fig. 1 shows changes of endogenous adenine nucleotide levels of intact chloroplasts in the light and the following dark as well as parallel measurements of ATPase activity. The experiment shown in Fig. 1A was carried out with chloroplasts from spinach grown in early summer. Fig. 1B shows a corresponding experiment conducted with chloroplasts from winter plants. The results of the two

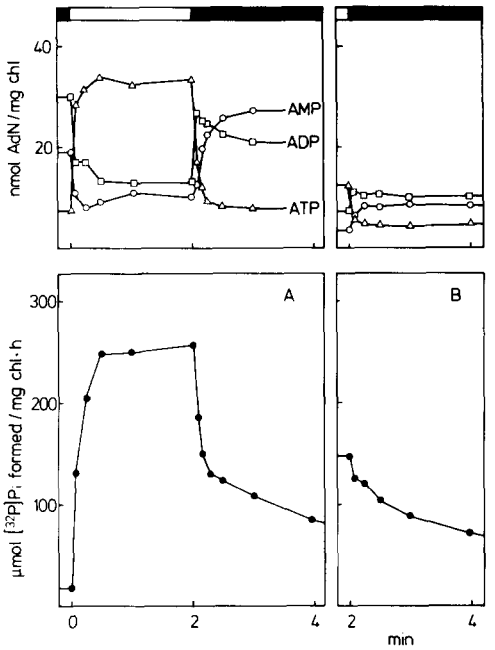

Fig. 1. Changes of the levels of endogenous adenine nucleotides (upper part) and ATPase activity (lower part) of isolated whole chloroplasts in a light-dark transition. The experiment shown in A was conducted on July 8th, 1987 the one shown in B on November 21st, 1986. The experimental conditions were identical (see Methods).

experiments differ in quantitative as well as qualitative respects.

(1) Light-induced increase of the endogenous ATP level at the expense of ADP and AMP as well as the reversal in the dark are qualitatively similar in both types of chloroplast. The reactions were carried out in a medium without added bicarbonate so that essentially no CO_2 fixation took place. Hence the ATP formed by photophosphorylation was almost not consumed by secondary processes. The dark decrease of ATP and increase of ADP and AMP concentrations must therefore be referred to ATP hydrolysis catalyzed by the H^+-ATPase as a consequence of decline of the H^+-gradient. The changes of AMP are obviously due to equilibration by the action of adenylate kinase.

The total content of adenine nucleotides in 'summer chloroplasts' is 56 nmol/mg Chl; that of 'winter chloroplasts', however, is only 23 nmol/mg Chl. Winter plants – like shade plants – may have a higher relative chlorophyll content, which could explain the differences. It is also possible, however, that the envelope membrane of isolated winter chloroplasts is more leaky for low molecular substances including nucleotides, i.e., the low nucleotide content might be an isolation artifact. The lower activity of CO_2 fixation and the longer induction period of CO_2-dependent O_2 evolution observed in winter chloroplasts (not shown) might also speak in favour of increased envelope permeability.

(2) Fig. 1A shows that the process of activation of the ATPase is almost complete after 15 s illumination. The final ATPase activity in the light is higher in summer than in winter chloroplasts. Upon turning off the light, the ATPase activity decays with a half-time of about 2 min in winter chloroplasts. The decay curve is roughly monophasic, but a small initial phase of more rapid deactivation may be present. Similar curves have been obtained in earlier studies. They were interpreted to indicate dark demodulation of the ATPase by slow reoxidation [14,15]. A clear biphasic decline of ATPase activity is observed when summer chloroplasts are transferred from light to dark (Fig. 1A). The rapid initial deactivation coincides with the decrease of the internal ATP and increase of ADP concentration. These results suggest that the activity state of the ATPase might be affected in summer chloroplasts by internal nucleotide concentrations. In order to examine this possibility further, the ratio of internal ATP to ADP was manipulated by different means and the effect on ATPase activity was studied. All these experiments were conducted with chloroplasts from plants harvested between May and September. The internal ATP/ADP ratio can be changed by the addition of 3-phosphoglycerate (PGA) which is brought into the chloroplast via the phosphate translocator [29]. By reduction of PGA to glyceraldehyde 3-phosphate (GAP) catalyzed by the enzyme couple phosphoglycerate kinase (PGK) and glyceraldehydephosphate dehydrogenase (GAPDH), ATP is consumed and ADP is formed. As the reaction also consumes NADPH and the reduction of $NADP^+$ competes with thioredoxin reduction for electrons from the photosynthetic electron-transport chain, PGA was added after 2 min illumination and only 15 s before lysis of the chloroplasts and measurement of

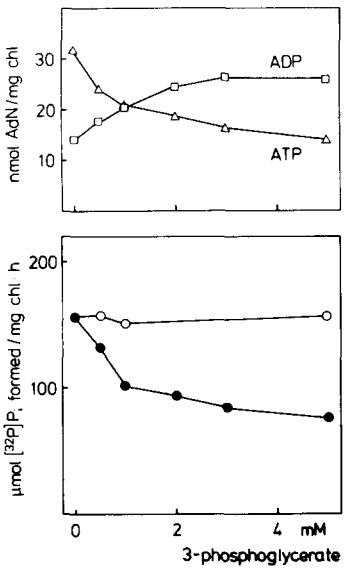

Fig. 2. Effect of increasing concentrations of 3-phosphoglycerate on endogenous ATP and ADP levels and ATPase activity. The chloroplasts were illuminated for 2 min. 3-Phosphoglycerate was added 15 s before measurement of nucleotide contents and ATPase activity (●), respectively. As a control (○) 3-phosphoglycerate was added together with the ATPase assay mix. The total content of adenine nucleotides (ATP + ADP + AMP) was 59 nmol/mg Chl.

ATPase activity. This time is sufficient to decrease the ATP level significantly and to reach a new steady state (not shown). On the other hand, a significant change of the reduction state of thioredoxin during this short time interval is unlikely.

Simultaneously the levels of endonenous adenine nucleotides and ATPase activity were determined as a function of PGA concentration (Fig. 2). The congruence of the results is striking. About 3 mM PGA decreased the internal ATP concentration by 50% and raised the ADP concentration to a similar extent. By the same PGA concentration the ATPase activity was also decreased by about 50%. No decrease of activity was observed when PGA was added together with the ATPase assay mix. The addition of PGA did not reduce the 9-aminoacridine fluorescence signal significantly (not shown), indicating that the electrochemical proton gradient was nearly unaffected.

The internal ATP/ADP ratio can be shifted towards lower values by decreasing the light intensity or by adding varying concentrations of uncoupler. By uncoupling, the proton gradient is degraded but reduction of thioredoxin by the electron transport chain should not be influenced. As in the former experiment, FCCP was added briefly before chloroplast lysis and activity measurement.

TABLE I

EFFECT OF FCCP ON ENDOGENOUS ADENINE NUCLEOTIDE LEVELS AND ATPase ACTIVITY

Conditions as in Fig. 2 FCCP at the indicated concentration was added 15 s before measurement of nucleotide contents and ATPase activity, respectively. During the ATPase reaction the FCCP concentration was 5 µM.

FCCP (µM)	ATP (nmol/mg Chl)	ADP	AMP	ATPase activity (µmol P_i/mg Chl per h)
0	25.1	16.3	15.6	220
1	13.2	17.3	26.5	165
2.5	8.0	20.4	28.6	76
5	5.9	21.9	29.2	56

In suspensions of DTT-modulated isolated thylakoids, a sudden degradation of the proton gradient does not affect the activity of the ATPase significantly within a time span of 15 s provided that the medium is free from ADP [16] or the ratio of ADP/ATP is low [24]. A rapid deactivation, however, is observed when micromolar concentrations of ADP are present or the ADP/ATP ratio is high at the transfer from high- to low-energy state [24,28]. Table I shows that the addition of FCCP to intact chloroplasts effects increase of internal ADP/ATP ratio and in a similar dependence on FCCP concentration lowers the activity of ATPase. Hence in view of the in vitro results we have to conclude that modulation of ATPase activity in chloroplasts by FCCP as by PGA is mediated by the internal ADP/ATP ratio.

ATPase activities as function of the ATP/ADP ratio are plotted in Fig. 3. The data were taken from Fig. 2 and Table I. Based on the results obtained from in vitro experiments, this evaluation may be well justified. In those studies it was demonstrated that ADP and ATP exhibit the same affinity to the regulatory 'tight' nucleotide binding site of CF_1 [20,30]. Since the enzyme molecules with a bound ADP are inactive, whereas those containing ATP remain active [11], the ratio of ATP/ADP in the surroundings may give a mea-

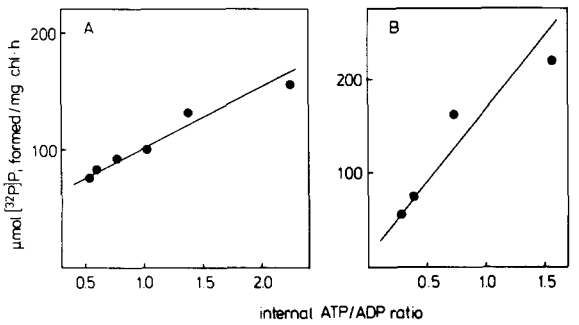

Fig. 3. ATPase activity altered by the addition of PGA (A) or FCCP (B) as a function of the internal ATP/ADP ratio. The data were taken from Fig. 2 and Table I.

sure of the activation equilibrium of the ATPase. Indeed the results of Fig. 3 indicate a nearly linear relationship between the two parameters in whole chloroplasts.

In isolated thylakoid suspensions inorganic phosphate is known to stabilize ATPase activity in the dark, when the CF_0-CF_1 complex was previously brought into the thiol-modulated state [16]. This stabilization is effected by phosphate-dependent inhibition of tight binding of ADP to β subunit of CF_1 [17,31,32]. As a consequence, ADP-dependent inactivation is decelerated. In an experiment shown in Fig. 4, the effect of added phosphate on internal nucleotide levels and on ATPase activity was investigated. Like PGA, inorganic phosphate is transported across the envelope membrane via the phosphate translocator [29]. The results show that the internal contents of ADP and ATP are only slightly changed by increasing the phosphate concentration. The activity of ATPase, however, is increased. No change of activity is observed when phosphate is added together with the ATPase assay mix. The phosphate concentration producing half-maximal stimulation of ATPase activity is in the range of 1 mM. In isolated thylakoids phosphate acts as a non-competitive inhibitor of tight binding of ADP with a K_i of 0.6 mM [32].

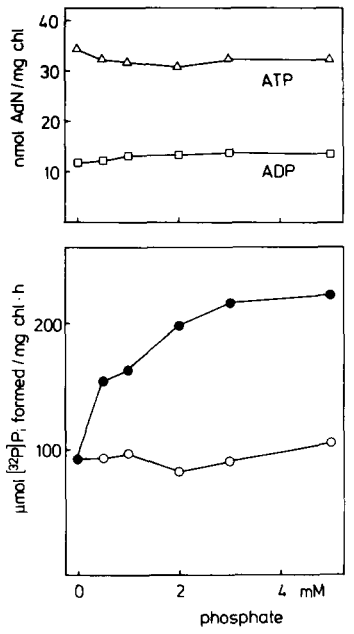

Fig. 4. Effect of increasing concentration of inorganic phosphate on internal ATP and ADP levels and ATPase activity. Conditions as in Fig. 2. Controls of ATPase activity (○) were obtained by addition of P_i to the ATPase assay medium. The total adenine nucleotide content was 55 nmol/mg Chl.

Discussion

In thylakoid suspensions preilluminated in the presence of DTT, a slow dark decline of ATPase activity is observed [8,16]. The half-time is in the range of minutes, and it is somewhat diminished by Mg^{2+} [8,22]. In contrast, the activity of the non-modulated enzyme declines immediately with relaxation of the proton gradient. This is the reason for the lack of dark ATP hydrolysis by broken chloroplasts containing CF_0CF_1 in its oxidized form. Dark decline of ATPase activity of thiol-modulated thylakoids is strongly enhanced by ADP [16]. This deactivation which is complete within seconds, is caused by tight binding of ADP to CF_1 [17,31]. The results of this paper suggest a similar kind of inactivation in isolated whole chloroplasts which are characterized by high ATPase activity, high stability and high contents of endogenous adenine nucleotides ('summer chloroplasts'). Upon darkening about 50% of the ATPases are switched off within 15 s, the remaining 50% are inactivated with a half time of about 2 min. In 'winter chloroplasts' the slow inactivation predominates, a reaction which has been referred to enzyme reoxidation [14,15]. However, Mills and Mitchell [14] already pointed out that dark thiol demodulation in whole chloroplasts lags behind the decline of ATPase activity and they concluded

that the decay of activity may also be determined by other factors. A fast dark inactivation of the ATPase has also been concluded from the fact that under conditions of blocked CO_2 fixation a substantial amount of free endogenous ATP remains in the dark [35]. On the other hand, a slow dark deactivation was observed when ATPase activities of thylakoids isolated from preilluminated leaves were determined [36,37].

It is tempting to assume a relationship between the deactivation kinetics and the contents of internal nucleotides. Seemingly, even the low content of adenine nucleotides in winter chloroplasts still signifies rather high internal nucleotide concentrations. Assuming a chloroplast volume of 100 μl/mg Chl – which would certainly be the upper limit [35] – 23 nmol nucleotides/mg Chl might yield an internal concentration of more than 200 μM. Such a calculation, however, is rather irrelevant, since we do not know the portion of internally bound nucleotides. Inoue et al. [34] found that 4 nmol/mg Chl of the endogenous ATP may be bound. Assuming similar amounts of absorbed ADP and AMP, we may conclude that in winter chloroplasts less than 50% of the internal nucleotides are freely available. Hence the actual amount of free ADP in those chloroplasts indeed might be too low to exert full control of the ATPase. Moreover – in particular at low ADP concentration – dark deactivation by tight ADP binding would be hindered by phosphate [32]. On the other hand, this protective effect of phosphate may be overcome when the ADP concentration is sufficiently high as in summer chloroplasts.

The results of Fig. 2 and Table I as summarized in Fig. 3 suggest that the control of ATPase activity at a constant phosphate concentration (here 0.25 mM) is mainly caused by the internal ATP/ADP ratio. The adaptation of enzyme activity to the ATP/ADP ratio probably has a physiological significance during short term changes of energy input. Under natural conditions, alterations of light intensity which may amount to one order of magnitude or more, are frequent events due to clouding or leaf self-shading. As a result, parallel changes of the proton gradient have to be assumed. A rapid transient decline of ATPase activity could minimize ATP losses when $\Delta\tilde{\mu}_{H^+}$ drops below the threshold which is capable of driving ATP synthesis. If the time of depression of the gradient is short, the enzyme may remain in its reduced form. Upon re-increase of the gradient, ATP synthesis may start with a higher efficiency since the thiol-modulated ATPase needs to overcome a lower activation barrier than the oxidized enzyme [38–40].

Acknowledgement

This work was supported by Deutsche Forschungsgemeinschaft.

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