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Equilibration of the ATPase reaction of chloroplasts at transition from strong light to weak light

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Broken chloroplasts activated by preillumination in the presence of dithiothreitol were supplied with phosphate and with a limited concentration of ADP. On re-illumination, ATP was formed until a steady state was attained. If after reaching the steady state light intensity was reduced to 20–50 $W \cdot m^{-2}$, net ATP hydrolysis took place, but after some time in weak light the level of ATP re-increased. Similarly, a drop of transmembrane ΔpH followed by a slow recovery was observed. Further data indicate that the reversible changes of ATP level and ΔpH are the result of partial uncoupling induced by ATP during the preceding strong light period and of restoration of coupling within a few minutes in weak light. Since similar changes of endogenous ATP level were found when intact chloroplasts were subjected to a strong-light / weak-light transition, it is proposed that ATP-induced partial uncoupling may play a role in regulation of photosynthetic energy conservation as a means to dissipate abundant transmembrane electrochemical energy and to permit flexibility of the stoichiometry of ATP-to-NADPH production.

Introduction

Activation of a reversible proton-translocating ATPase in chloroplast thylakoids requires membrane energization and protein modification by thiols [1–4]. Modification includes reduction of a disulfide bond in the γ subunit of CF_1 which is exposed by membrane energization [5–7]. The activity of the reduced enzyme is maintained over several minutes in the dark so that ATP added after preillumination is effectively hydrolyzed. In contrast, the oxidized enzyme, which is capable of catalyzing ATP formation in the light, loses its catalytic activity as soon as the electrochemical proton gradient relaxes. In intact chloroplasts reduction of the disulfide bond is accomplished by the endogenous thioredoxin system [8–10]. Hence, with regard to the functional state of the ATPase broken chloroplasts preilluminated in the presence of thiols like dithiothreitol resemble preilluminated intact chloroplasts.

The efficiency of phosphorylation at low proton-motive force is higher in dithiothreitol-preactivated than in non-treated thylakoids [6,11,12], because no energy barrier for enzyme activation has to be exceeded. In untreated thylakoids the rate of ATP synthesis as a function of $\Delta\tilde{\mu}_{H^+}$ represents the energy requirement of the activation process whereas in thiol-pretreated thylakoids this curve which is shifted towards lower $\Delta\tilde{\mu}_{H^+}$ values indicates the thermodynamic energy requirement for the phosphorylation reaction itself [12].

Abbreviations: Chl, chlorophyll; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMS, phenazine methosulfate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; P_i, inorganic phosphate.

Thiol modification and demodification by reoxidation of the ATPase is one important principle of regulation of energy transduction, but additional control factors are involved. It has been demonstrated that the reduced activated ATPase is readily deactivated by ADP [13]. ADP is bound in the dark to a site on the β subunit [14] which was depleted during previous light activation [15–18]. Rebinding which results in the formation of 'tightly bound ADP' converts the active ATPase to a reduced, but inactive form [19–21]. Hence the enzyme – irrespective of its oxidized or reduced state – may be switched off upon membrane deenergization anyhow, if the ADP/ATP ratio of the medium is high enough. A different regulatory mechanism is elicited at low ADP/ATP ratio. Recently, it was reported that illumination of thiol-activated chloroplasts in the presence of ATP results in transitory stimulation of ATP hydrolysis in the subsequent dark. This effect is caused by partial disconnection of energy coupling between the ATPase reaction and the transmembrane flux of protons [22]. Since uncoupling is triggered by conditions which may occur naturally (strong light and high endogenous ATP/ADP ratio) and is reverted by normalization of the conditions, this reaction could play a role in regulation, e.g., as a means to dissipate abundant energy.

Plants at their natural habitat have to sustain large changes of light intensity which causes fluctuations of $\Delta\tilde{\mu}_{H^+}$. Such changes of energy flux demand respective adaptions of the energy-transducing apparatus. We have observed unexpected transient changes of the phosphate potential and of ΔpH when chloroplasts kept in strong light were suddenly subjected to low light. In the present paper these effects are studied and explained in the framework of the regulatory principles discussed above.

Methods

Broken chloroplasts from spinach leaves were prepared as in Ref. 18, chloroplasts with an intact envelope were isolated according to Ref. 10. Reactions with broken chloroplasts were carried out in test tubes which were inserted in a temperature-controlled water bath. The reaction medium initially contained 25 mM Tricine buffer (pH 8)/50 mM KCl/5 mM $MgCl_2$/10 mM dithiothreitol/50 μM PMS. After addition of chloroplasts (approx. 25 μg/ml final chlorophyll concentration) the stirred mix was illuminated for 2 min with white light (250 $W \cdot m^{-2}$). After 15 s in the dark, the substrates ADP and [^{32}P]P_i were added at concentrations indicated in the legends. After additional 15 s in the dark, light was turned on again. The formation of [^{32}P]ATP and its possible hydrolysis after changing the light intensity was followed by transferring 0.2 ml aliquots into 0.05 ml 3 M $HClO_4$ at the indicated times. In the extracts ^{32}P-labelled organic phosphate was analyzed according to Ref. 23. Variations of the experimental procedure are explained in the legends. If rates of [^{32}P]ATP hydrolysis were measured, [^{32}P]P_i was analyzed as in Ref. 24. 9-Aminoacridine fluorescence was measured as in Ref. 22.

Incubations for measurement of endogenous adenine nucleotides of intact chloroplasts were carried out in a syringe with stirring device and temperature-controlled water jacket. The medium contained 0.36 M sorbitol, 50 mM Tricine buffer (pH 8.0) and 0.25 mM P_i. The chlorophyll concentration was 100 μg/ml. At the indicated times 450 μl samples were injected into 75 μl 70% $HClO_4$. Three 100 μl aliquots of the neutralized extracts were incubated for 1 h at room temperature with 25 μl of a solution containing (a) 17.5 mM Hepes buffer (pH 7.75)/37.5 mM $MgSO_4$/135 mM KCl/1.05 mM phosphoenol pyruvate, (b) the same components plus pyruvate kinase (2.5 U), and (c) plus pyruvate kinase (2.5 U) and adenylate kinase (3.15 U). The reactions were stopped by addition of 25 μl 3 M $HClO_4$. After centrifugation the neutralized supernatants were used for determination of the adenine nucleotides in a luciferase assay, yielding ATP alone in (a), ATP + ADP in (b) and ATP + ADP + AMP in (c). The monitoring assay contained 600 μl of a medium containing 100 mM Tris buffer (pH 7.75)/10 mM K_2SO_4/5 mM $MgSO_4$/2 mM EDTA/60 μl monitoring reagent (1243-200 LKB)/10 μl of the extracts. Bioluminescence was calibrated by addition of 10 μl 0.5 μM ATP as an internal standard. The measurements were conducted with Luminometer 1250 (LKB).

Results

In order to activate and modulate the membrane-bound ATPase, broken chloroplasts were routinely preilluminated for 2 min at 250 $W \cdot m^{-2}$ in a medium containing 10 mM dithiothreitol, before the substrates ADP + P_i were added in an intermediate dark period of 30 s. In an experiment shown in Fig. 1 the initial concentrations of ADP and [^{32}P]P_i were 50 μM and 2.5 mM, respectively. Afterwards the chloroplasts were illuminated again with either 20 $W \cdot m^{-2}$ or 250 $W \cdot m^{-2}$. After about 1 min at 250 $W \cdot m^{-2}$ a steady state was reached indicating that 96% of the added ADP was converted to [^{32}P]ATP. The calculated steady state phosphate potential was 55 $kJ \cdot mol^{-1}$. With 20 $W \cdot m^{-2}$ no steady state was attained within 5 min. Moreover, in the course of strong light irradiation, light intensity was attenuated to 20 $W \cdot m^{-2}$ at different times. When the change was performed after 15 s (2/3 of the added ADP converted to ATP), phosphorylation was found to continue, but at a lower rate. When the light was changed after 1 or 2 min, however, net hydrolysis of [^{32}P]ATP was observed and on continuation of weak light irradiation hydrolysis was followed by net resynthesis of [^{32}P]ATP. The initial velocity of ATP hydrolysis, the location and depth of the ATP minimum and the rate of ATP resynthesis were different after 1 or 2 min, respectively, of strong light pretreatment.

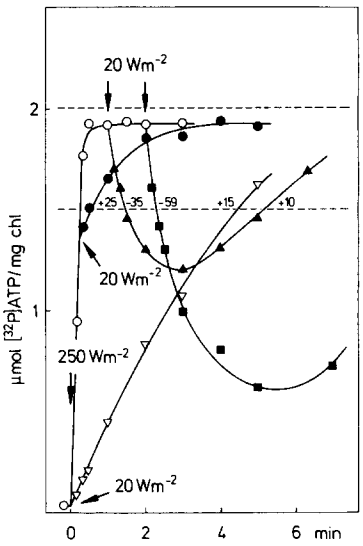

Fig. 1. [^{32}P]ATP formation in strong and weak light and changes of the [^{32}P]ATP level upon strong-to-weak light transition. The initial concentrations of ADP and [^{32}P]P_i were 50.25 μM and 2.5 mM, respectively, the chlorophyll concentration was 25.05 μg/ml. The temperature was 20°C. The upper dashed line shows the maximal ATP level (calculated from the added ADP concentration), the lower indicates an ATP level corresponding to a phosphate potential of 44.5 $kJ \cdot mol^{-1}$. ΔG_p was computed according to:

$$\Delta G_p \ (kJ \cdot mol^{-1}) = 32.5 + \frac{8.314 \cdot 293}{1000} \cdot \ln \frac{c_{ATP}}{c_{ADP}\, c_{P_i}}$$

The numbers indicate the rates of ATP formation (positive sign) or ATP hydrolysis (negative sign) determined at ΔG_p = 44.5 $kJ \cdot mol^{-1}$ by interpolation.

Since adenylate kinase activity of the employed thylakoid preparations was very low (less than 2 μmol per mg Chl per h), the phosphate potential $\Delta P = [ATP]/[ADP]\ [P_i]$ was nearly identical in any point parallel to the time axis. A phosphate potential corresponding to a free-energy change of 44.5 $kJ \cdot mol^{-1}$ is marked by a dashed line in Fig. 1. It is quite evident that at the same phosphate potential and the same light intensity of 20 $W \cdot m^{-2}$ the ATPase reaction proceeds either in forward or back direction, depending on the conditions of pretreatment and on time in low light. The direction of the coupled ATPase reaction is determined by the sign of the total free-energy change according to

$$\Delta G = \Delta G_P - n\Delta G_{H^+}$$

(ΔG_p, ΔG_{H^+}: changes of free enthalpy of ATPase reaction and of the electrochemical proton gradient, respectively; n: H^+/ATP stoichiometry).

Hence, the results of Fig. 1 permit the conclusion that the proton-motive force generated by the same low light intensity likewise varies depending on the mode of pretreatment and the time in low light. This was verified by traces of 9-aminoacridine fluorescence as measures of transmembrane ΔpH (Fig. 2). The experiment was conducted under essentially the same conditions as the one shown in Fig. 1. On irradiation in the presence of 50 μM ADP and 2.5 mM P_i with either 250 or 25 $W \cdot m^{-2}$, ΔpH rises to a transitory steady-state level (which may be assigned to coupled net photophosphorylation) and then increases to a final steady-state level (indicating equilibrium). Probably because of the higher rate of phosphorylation

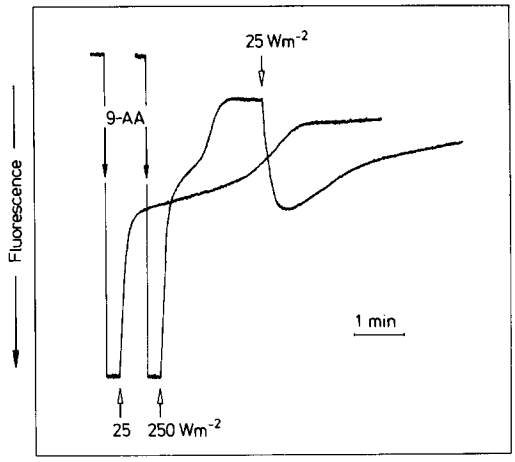

Fig. 2. 9-Aminoacridine fluorescence traces of chloroplast suspensions irradiated with weak and strong light followed by weak light. The medium contained 50 μM ADP and 2.5 mM P_i, the chlorophyll concentration was 25 μg/ml, the 9-aminoacridine concentration 5 μM. The actinic light was filtered through a Schott RG 630 Filter. The temperature was 20°C.

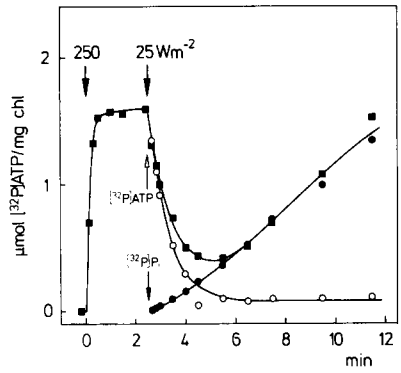

Fig. 3. Gross [^{32}P]ATP synthesis and hydrolysis at 25 W · m^{-2} following ATP synthesis at 250 W · m^{-2}. The strong light illumination was performed with unlabeled P_i and ADP. Together with transition to the low light, either [^{32}P]P_i or [^{32}P]ATP (both carrier-free) was added. In a control curve, the net changes were measured as for example in Fig. 1. From the ATP concentration attained in the controls the specific activities of [^{32}P]ATP and [^{32}P]P_i for the measurements of the gross reactions were calculated. The initial concentrations of ADP, P_i and chlorophyll were 49.2 μM, 1 mM and 24.7 μg/ml, the temperature was 21°C.

which provides a faster consumption of the added ADP, the equilibrium state was attained much faster in strong light than in weak light. When strong light was changed to weak light, ΔpH dropped far below the low light steady state of the control, but increased again after some time. Kinetic comparison of the results with those of Fig. 1 shows that the ΔpH minimum precedes the ATP minimum. This appears reasonable because the rate of ATP hydrolysis should be maximal when ΔpH attains a minimum. In a series of experiments the conditions for the occurrence of reversible net ATP hydrolysis in a light-intensity change were optimized. The higher the light intensity of the previous light, the larger was the drop of ATP level. On variation of light intensity of the second irradiation period, ATP hydrolysis followed by ATP resynthesis was observed at 25 W · m^{-2} and to a lesser extent also at 50 W · m^{-3}. ATP hydrolysis, but no recovery of ATP synthesis was detected at 10 W · m^{-2}. Reversible net ATP hydrolysis was found at 10, 15 and 20°C. At 30°C almost all of the previously formed ATP was hydrolyzed during the weak light period and recovery of ATP synthesis was extremely weak.

The temporary drop of the ATP level may be the result of changing rates of simultaneous ATP hydrolysis and ATP synthesis. In order to prove this, the two gross reactions were measured independently in parallel series. Chloroplasts were allowed to form unlabeled ATP from ADP and P_i during a strong light period. Together with the transition to 25 W · m^{-2} either carrier-free [^{32}P]-ATP or [^{32}P]P_i were added and the kinetics of cleavage of [^{32}P]ATP and formation of [^{32}P]ATP were followed (Fig. 3). It is evident that the two opposing processes take place simultaneously from the beginning, but the rates are changing with time. The sum of the measured gross reactions is in agreement with the obtained reaction. The rates of the gross reactions are affected by the actual concentrations of the substrates and products which are varying with the reaction time. In order to exclude these variable parameters, carrier-free [^{32}P]ATP or [^{32}P]P_i, respectively, was supplied together with an ATP- or ADP-regenerating enzyme system at different times during the reaction in low light. The regenerating systems provide constant conditions with regard to the concentrations of ATP or ADP within a few seconds. Therefore the rates determined in the course of the reaction represent activities of ATP hydrolysis and ATP formation under comparable experimental conditions. The activities were found to vary in an

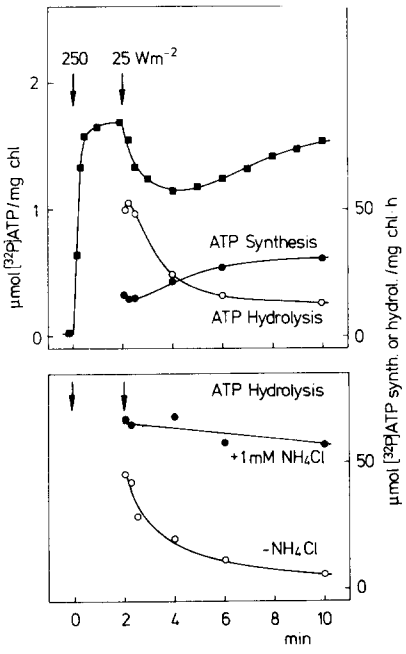

Fig. 4. Activities of [^{32}P]ATP synthesis and hydrolysis at 20 $W \cdot m^{-2}$ following ATP synthesis at 250 $W \cdot m^{-2}$. ATP formation in strong light was conducted with unlabeled P_i (2.5 mM) and ADP (50 µM). (a) At the indicated times in low light, mixtures of carrier-free [^{32}P]P_i plus hexokinase (final concentration, 115 units/ml) and glucose (20 mM) or carrier-free [^{32}P]ATP plus pyruvate kinase (80 units/ml) and phospho*enol*pyruvate (1 mM) were added and the rates of [^{32}P]ATP formation or hydrolysis, respectively, were determined by analyzing aliquots taken after 10, 20 and 30 s. The chlorophyll concentration was 24.5 µg/ml. In a parallel experiment the net changes of [^{32}P]ATP were measured as in Fig. 1. (b) The pulse mixture added in weak light contained [^{32}P]ATP, pyruvate kinase and phospho*enol*pyruvate with or without NH_4Cl (1 mM). The chlorophyll concentration was 24.5 µg/ml.

inverse manner depending on the time in weak light (Fig. 4). Starting with a high rate, ATP hydrolysis decreased with time in low light and, on the other hand, the rate of ATP formation increased. Similarly, activities of uncoupled ATP hydrolysis were determined by adding ammonium chloride together with the assay mix. Under these conditions essentially the same rates were obtained throughout the weak light irradiation period, demonstrating that the total enzyme activity did not significantly change (Fig. 4).

As shown in Figs. 1 and 2, strong light preilluminations is a necessary prerequisite to trigger the reversible drop of ATP level and ΔpH in

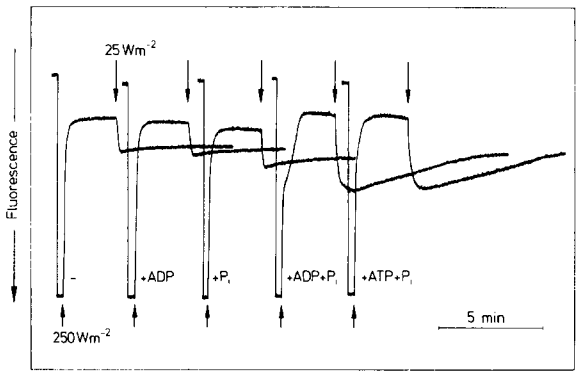

Fig. 5. 9-Aminoacridine fluorescence changes at 250 $W \cdot m^{-2}$ followed by 25 $W \cdot m^{-2}$ in different media. The final concentrations of the added substrates were 50 µM ADP or ATP and 1 mM P_i. Other conditions as in Fig. 2.

subsequent weak light. Another essential factor is the presence of ATP during pretreatment. Fig. 5 shows the effect of strong/weak light transition on the 9-aminoacridine traces in different media. An immediate decrease to the stationary low light ΔpH was observed if the medium contained ADP or P_i alone or none of the two substrates. A transitory depression followed by recovery of ΔpH as in Fig. 2 took place if either ADP + P_i or ATP + P_i was present during the strong light period. This result suggests that ATP which is

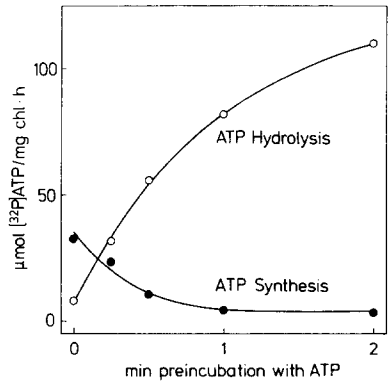

Fig. 6. Rates of [^{32}P]ATP formation and hydrolysis at 25 $W \cdot m^{-2}$ as a function of preincubation time with 50 µM ATP in the previous strong light (250 $W \cdot m^{-2}$). The ATP was added at different times during the strong light period of 2 min. Together with the light intensity change either carrier-free [^{32}P]P_i with hexokinase system or carrier-free [^{32}P]ATP with pyruvate kinase system was added and the activities were measured as in Fig. 4. The chlorophyll concentration was 26 µg/ml.

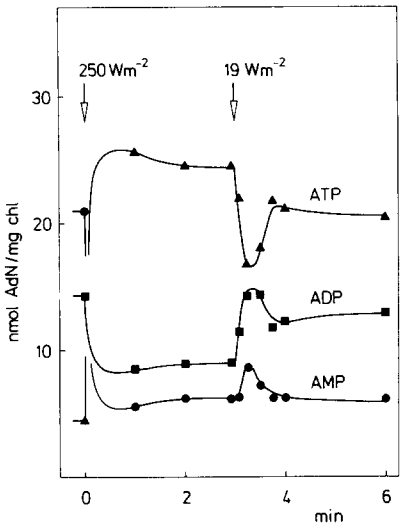

Fig. 7. Changes of endogenous concentrations of ATP, ADP and AMP of intact chloroplasts upon transition of light intensity from 250 to 19 W·m^{-2}. The portion of intact chloroplasts as checked by the method given in Ref. 27 was 85%. Experimental details are presented in Methods.

either synthesized by the system or added, may be the critical agent which together with strong light produces the observed effect.

In the experiment shown in Fig. 6, unlabeled ATP was added at different times during a strong light period of 2 min. At the transition to weak light, carrier-free [^{32}P]ATP with pyruvate kinase system or carrier-free [^{32}P]P_i with hexokinase system was added and the rates of [^{32}P]ATP hydrolysis and synthesis were measured. The results indicate that it is not the time of strong light irradiation itself (which was the same), but the time of ATP incubation during strong light which determines the rates of ATP hydrolysis and ATP synthesis in the subsequent low light.

Fig. 7 shows changes of the endogenous concentrations of ATP, ADP and AMP of isolated intact chloroplasts in a strong light to weak light transition. As with isolated thylakoids, a transitory drop of the ATP level and a corresponding increase of the ADP and AMP levels was observed. The chloroplasts were not supplied with bicarbonate. Small quantities of CO_2 present in the medium may be consumed during the strong-light period, so that secondary dark reactions may be largely excluded when the chloroplasts were subjected to low-light conditions. The observed effect therefore may be also explained by a transient rearrangement of the coupled ATPase reaction.

Discussion

It is reasonable to assume that the decrease followed by a re-increase of phosphate potential at a strong-light/weak-light change is a consequence of a transitory depression of the proton gradient, which may be traced back either to a rearrangement of electron flow or a disturbance of proton coupling of the ATPase reaction. In a recent paper [22] it was reported that the initial rate of light-triggered dark ATP hydrolysis was stimulated when ATP was present in the light instead of being added after illumination. This stimulation was shown to be caused by an increase of membrane permeability for protons induced by ATP binding to the ATPase in light. It was proposed that ATP binding may establish a conformation of the ATPase complex that permits part of the protons of the proton gradient to by-pass the coupled route. The partial disconnection of coupling was self-repaired within about 1 min in the dark. There is no doubt that this effect and the one reported here are related with each other and have a common reason: both of them need the presence of ATP during pre-illumination, the same preincubation time with ATP (about 1 min half-time), they are abolished by ADP, and they are reversible after some time in the dark or at low light intensity, respectively. Hence it is rather obvious that the drop of $\Delta\tilde{\mu}_{H^+}$ observed in a strong-light-to-weak-light transition is likewise caused by ATP-induced partial uncoupling. The decrease of $\Delta\tilde{\mu}_{H^+}$ becomes apparent only a limiting rates of H^+ influx, i.e., if the rate of electron transport is suddenly reduced by attenuation of light intensity. Recovery of coupling may be explained by the increase of the ADP to ATP ratio during the phase of net ATP hydrolysis in weak light, thus enabling replacement of bound ATP by ADP [22].

The fact that this type of uncoupling is reversible and is induced by conditions (high-light intensity and high ATP/ADP ratio) which under certain circumstances may occur in vivo gives rise to speculate on its physiological significance. Partial disconnection of the coupling device may be a

means to maintain electron flow even at high phosphate potential and strong light. One might imagine metabolic situations where more NADPH than ATP is required; this could be provided by such a mechanism. On the other hand, induced uncoupling could prevent over-acidification of the intrathylakoid space, a precaution which may be important to exclude irreversible membrane damage. It is known that the rate of electron transport is controlled by the internal pH [25] so that the pH inside the thylakoid may be self-regulated by feedback. Induced uncoupling could be an alternative or additional way which leads to the same goal.

The observed changes of endogenous adenine nucleotides in intact chloroplasts (Fig. 7) suggest that disconnection of coupling is indeed a relevant phenomenon in vivo. Heber [26] found a large decrease of the ATP level when intact CO_2 fixating chloroplasts were transferred from strong to weak light. This cannot be simply explained by the reduction of the rate of ATP formation at a maintained rate of ATP consumption by the Calvin cycle, since almost no decrease of the NADPH level was observed; rather, these results may indicate a change of the stoichiometry of ATP-to-NADPH formation in weak light, which again may refer to an alteration of coupling between the two reactions caused by the previous strong-light treatment.

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