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CORRELATION BETWEEN THE ACTIVITY OF MEMBRANE-BOUND ATPase AND THE DECAY RATE OF FLASH-INDUCED 515-nm ABSORBANCE CHANGE IN CHLOROPLASTS IN INTACT LEAVES, ASSAYED BY MEANS OF RAPID ISOLATION OF CHLOROPLASTS

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(1) The relationship between activation of the membrane-bound ATPase and the stimulation of dissipation of the flash-induced membrane potential by preillumination was studied in intact spinach leaves by measuring the ATPase activity of rapidly isolated chloroplasts and the decay of the flash-induced 515-nm absorbance change (ΔA_{515}) in intact leaves. (2) The decay of ΔA_{515} was accelerated by preillumination. The ΔA_{515} decay in leaves treated with *N,N'*-dicyclohexylcarbodiimide (DCCD) became slower and was not accelerated by preillumination. However, treatment with DCCD did not lower the intensity of delayed fluorescence. (3) Membrane-bound ATPase of chloroplasts which were rapidly isolated from the preilluminated leaves (90 s preparation time) showed a higher activity (over 200 μmol P_i/mg chlorophyll per h in the case of 2-min preillumination) than that of chloroplasts isolated from dark-adapted leaves. (4) The acceleration of ΔA_{515} decay and the activation of ATPase showed similar dependences on illumination time in intact leaves. 3-(3',4'-Dichlorophenyl)-1,1-dimethylurea, carbonyl cyanide *p*-chlorophenylhydrazone and DCCD inhibited the activation of ATPase and the acceleration of the ΔA_{515} decay by preillumination. (5) The ATPase activity of chloroplasts isolated from illuminated leaves showed a single exponential decay ('dark inactivation in vitro'). The ATPase activity induced by illuminating the leaves became lower as the dark interval between illumination and the isolation of chloroplasts was increased ('dark inactivation in vivo'). The time course of the decay of activity had a lag and showed a sigmoidal curve when plotted semilogarithmically. The decay had an apparent half-time of 25 min. (6) The recovery of the accelerated ΔA_{515} decay in preilluminated leaves to the original slow rate showed a sigmoidal decay similar to that of the activity of ATPase in intact leaves with a half-time of about 23 min in the dark. (7) It was concluded that the decay rate of ΔA_{515} reflected the chloroplast ATPase activity in intact leaves and that the ion conductance of thylakoid membrane was mainly determined by the H^+ flux through the ATPase, the activity of which was increased after the formation of the high-energy state.

Introduction

In a previous paper [1], we showed that preillumination accelerated the dark decay of the flash-induced 515-nm absorbance change (ΔA_{515}) in intact leaves of *Zea mays*, as has been reported in algal cells and isolated chloroplasts [2,3]. There was a correlation between this acceleration of ΔA_{515} decay by preillumination and the formation of a high-energy state during the illumination monitored by the millisecond-range delayed fluorescence of chlorophyll. The acceleration was not observed when no high-energy state was formed by the illumination. As

Abbreviations: CCCP, carbonyl cyanide *p*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)methylglycine.

ΔA_{515} reflects the membrane potential generated across the thylakoid membrane [4], it was concluded that the high-energy state formed by illumination increased the permeability of the thylakoid membrane to ions, especially to H^+, and accelerated the dissipation of the flash-induced membrane potential.

Dark-adapted isolated thylakoid membranes exhibit only a very low rate of ATP hydrolysis [5–7]. Illumination of chloroplasts activates the Mg^{2+}-dependent ATPase activity of CF_1 bound to the membranes [5–7]. The light-induced electrochemical potential difference of H^+ ($\Delta\tilde{\mu}_{H^+}$) across the thylakoid membrane activates the ATPase activity of the membrane-bound CF_1-CF_0 complex [8–11]. This ATPase activity is coupled to the proton translocation across the thylakoid membrane [11,12]. Therefore, the change in the ATPase activity would regulate the permeability of thylakoid membrane to H^+. Thus, the acceleration of ΔA_{515} decay by preillumination was interpreted to result from the light-induced activation of membrane-bound ATPase [1]. The correlation between the ΔA_{515} decay and the activity of membrane-bound ATPase in intact leaves was investigated in the present study by measuring the ATPase activity after rapid isolation of chloroplasts from illuminated leaves. Chloroplasts with high ATPase activity were obtained by rapid isolation of chloroplasts from illuminated leaves [13,14]. It was indicated that the decay rate of ΔA_{515} in intact leaves reflected the activity of the membrane-bound ATPase. It was also shown that the dark inactivation of the ATPase was accelerated when chloroplasts were isolated from the leaves after illumination. Dark inactivation of the light-activated ATPase in intact leaves seems to be suppressed by factors or conditions which are lost during isolation of chloroplasts.

Materials and Methods

Chloroplast isolation and ATPase assay. Leaf segments of market spinach were preilluminated by light from a 650 W slide projector through a Hoya HA-50 heat-absorbing filter (light intensity, $1.8 \cdot 10^5$ erg $\cdot$ $cm^{-2} \cdot s^{-1}$) at room temperature (11–13°C), and then chloroplasts were rapidly isolated at 4°C by the following procedure: leaf segments were rapidly homogenized by a Biotron homogenizer for 3 s in 9 ml of a medium containing 0.4 M sorbitol, 5 mM $MgCl_2$ and 25 mM Tricine-KOH (pH 8), and filtered through eight layers of cheesecloth. 1.6 ml of the filtrate were centrifuged at $5000 \times g$ for 20 s in an Eppendorf Centrifuge 5412. The pellet obtained was suspended in 0.3 ml of the same solution. For the assay of ATPase activity, 0.1 ml of the chloroplast suspension (containing 20–60 μg chlorophyll) was added to 0.9 ml of medium containing 2 mM $MgCl_2$, 1.1 mM NH_4Cl, 2.2 mM ATP and 15 mM Tricine-KOH (pH 8) in a centrifuge tube in a shaking water bath maintained at 28°C. After 4 min incubation in the dark, ATPase activity was stopped by adding 1 ml of ice-cold 20% trichloroacetic acid solution. The precipitate was removed by centrifugation [7] and 1 ml of the supernatant was used for the determination of P_i [15]. The time from the end of illumination of leaves to the beginning of the incubation of chloroplasts in the ATPase assay system was 90 s in every case. All isolation procedures were done under dim green light. Halves of leaves, which had been cut into two parts along the main vein, were used as the zero-time control of in vivo illumination experiments (Fig. 5).

Measurements of dark-decay time courses of the flash-induced absorbance change at 515 nm in spinach leaves were performed as reported in Ref. 1. Time courses of the change in the intensity of delayed fluorescence (emitted between 0.5 and 12.5 ms after 15 ms light excitation) were measured by a phosphoroscope [1]. Preillumination and dark incubation of leaves and chloroplasts, and measurements of absorbance change and delayed fluorescence were carried out at 11–13°C.

Results

We reported previously [1] that the decay of the flash-induced 515-nm absorbance change (ΔA_{515}) in intact *Zea mays* leaves was accelerated by preillumination only when the high-energy state, which can be monitored by the measurement of the millisecond delayed fluorescence, was formed. In spinach leaves the decay of ΔA_{515} was also accelerated by 1 s preillumination (Fig. 1a and b). Typical half-decay times were about 333 and 88 ms after a long dark adaptation and a 1 s preillumination, respectively. When the leaves were pretreated with 1 mM DCCD, which is an inhibitor of energy transfer at the level of the cou-

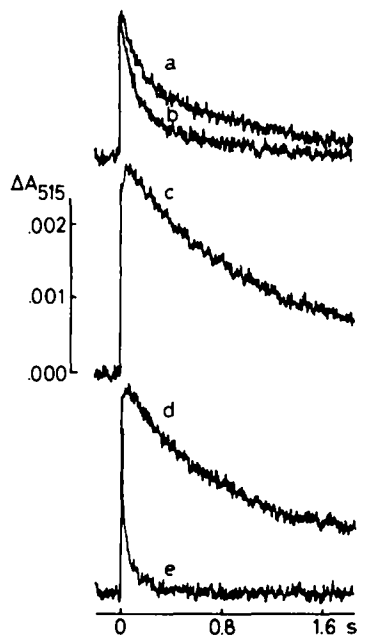

Fig. 1. Time courses of the flash-induced 515-nm absorbance change in intact leaves. Conditions of leaves before the measurements were: a, dark adapted for 2 h; b, dark adapted for 2 h and preilluminated for 1 s; c, treated for 15 min with 1 mM DCCD in the dark; d, the leaf in c was preilluminated for 1 s after the DCCD treatment; e, dark adapted for 25 min after adding 10 μM CCCP to the leaf in d. In preillumination experiments, the flash-induced absorbance change was measured between 90 and 170 s after the preillumination. Traces are averages of eight measurements (0.1 Hz excitation rate).

pling factor and decreases the H^+ permeability of thylakoid membranes under phosphorylating conditions [16,17], the decay of ΔA_{515} became slower (with a half-time of 800 ms (Fig. 1c)) than that in dark-adapted leaves and was not accelerated by the preillumination any more (Fig. 1d). Treatment with 3 μM CCCP accelerated the decay of ΔA_{515} in a leaf pretreated with DCCD. DCCD increased the initial extent of ΔA_{515}. Fig. 2 shows the time courses of millisecond delayed fluorescence under conditions similar to those in Fig. 1. DCCD did not suppress the increase in delayed fluorescence which reflects the formation of the high-energy state [18,19], indicating that the electron transport and formation of the high-energy state were not inhibited by DCCD treatment. Addition of 3 μM CCCP decreased the delayed fluorescence significantly, probably by increasing the permeability of the membrane to H^+.

The results, mentioned above and reported in the previous report [1], suggest that the acceleration of the decay of ΔA_{515} by preillumination resulted from the increased H^+ permeability through the membrane-bound ATPase, which changed its conformation upon the energization of the chloroplast membrane. We measured the ATPase activity of chloroplasts rapidly isolated from preilluminated leaves. Preillumination of leaves increased the ATPase activity of the chloroplasts (Figs. 3 and 4). The effects of various inhibitors of photosynthesis on the light-induced ATPase

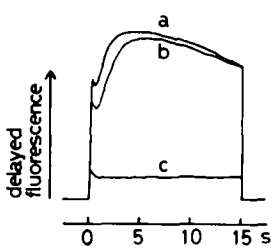

Fig. 2. Time courses of delayed fluorescence in intact spinach leaf. a, dark adapted for 2 h; b, dark adapted for 20 min after addition of 1 mM DCCD; c, dark adapted for 30 min after addition of 3 μM CCCP to the leaf in b.

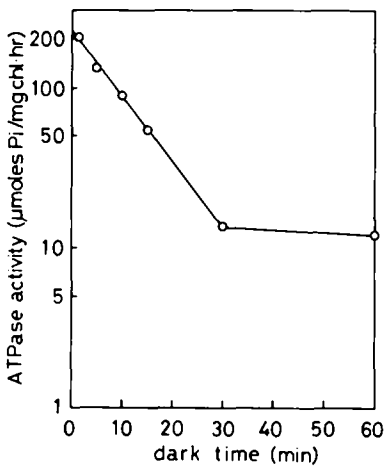

Fig. 3. Dark inactivation of light-activated ATPase activity after isolation of chloroplasts. After a preillumination for 2 min, leaves were homogenized and chloroplasts were isolated in 90 s under dim green light. ATPase activity was assayed after dark incubation of isolated chloroplasts in an isolation medium containing 0.4 M sorbitol, 25 mM Tricine-KOH (pH 8) and 5 mM $MgCl_2$ for various periods at 11°C.

TABLE I

EFFECTS OF PHOTOSYNTHETIC INHIBITORS ON LIGHT ACTIVATION OF CHLOROPLASTS ATPase

DCMU (100 μM), CCCP (10 μM), DCCD (1 mM) were added by incubating the lower-epidermis-stripped leaves in aqueous solutions for 20–50 min in the dark, except for DCCD added at assay stage (last line). Activities are expressed in μmol/mg chlorophyll per h.

Treatment	Activity	Control	Inhibition (%)
DCMU	82	153	46
CCCP	134	171	22
DCCD (activation stage)	52	179	71
DCCD (assay stage)	18	171	89

activity were tested (Table I). DCMU (100 μM) inhibited the light activation of the ATPase by 54% and CCCP (10 μM) by 22%. Addition of DCCD before the preillumination inhibited the activity by 70%. When DCCD was added at the assay stage after isolation of chloroplasts, 90% inhibition was observed.

The preillumination-induced ATPase activity of chloroplasts showed a single-exponential decay with a half-time of about 8 min when the isolated chloroplasts were dark incubated at 11°C and the ATPase activity was measured at various times after the isolation from the 2-min-preilluminated leaves (dark inactivation in vitro). It seemed that the assay of the ATPase activity, which started 90 s after the homogenization of leaves, represented the activity in intact leaves adequately. Extrapolation of the dark-decay curve indicated that the activity at 90 s corresponded to 86% of the value at zero time, under the present experimental conditions. Fig. 4 shows the ATPase activity of chloroplasts isolated from leaves preilluminated for various times. Half-maximal activation of ATPase was achieved by 10 s preillumination

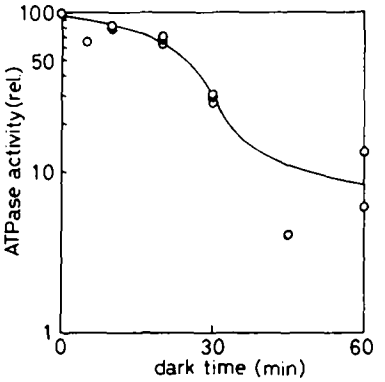

Fig. 5. Dark decay, in vivo, of light-activated ATPase in intact leaves. ATPase activity of chloroplasts isolated from intact leaves dark incubated for various periods at 11°C after 2 min preillumination was assayed. The activity measurement started 90 s after rupturing the dark-incubated leaves.

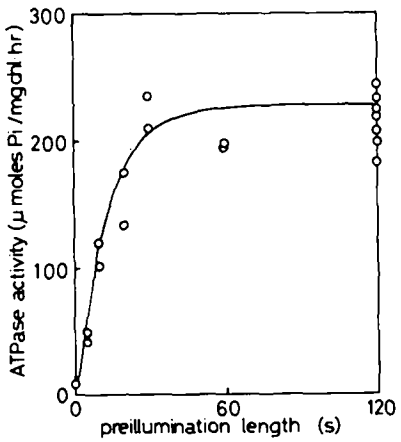

Fig. 4. Dependence of ATPase activity on illumination length. ATPase activity of chloroplasts isolated from leaves preilluminated for various periods was assayed. Time from the rupturing of leaves to the beginning of the ATPase assay was 90 s in every case.

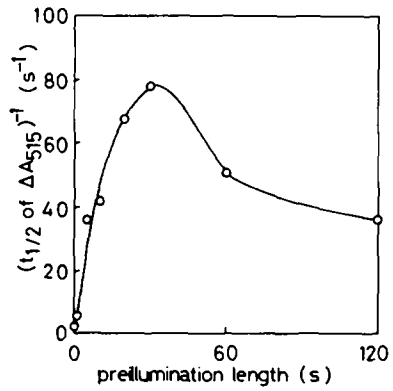

Fig. 6. Dependence of the apparent decay constant of ΔA_{515} on illumination length. Preilluminated for various lengths before measurements. Other conditions were the same as in Fig. 1.

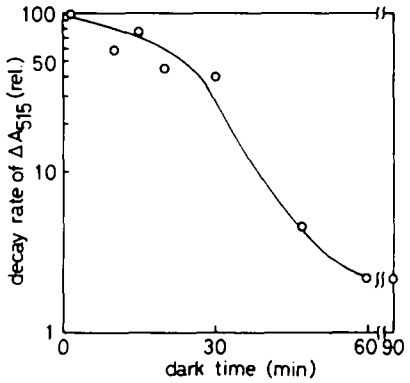

Fig. 7. Time course of the dark recovery of the preillumination-induced acceleration of ΔA_{515} decay in intact leaf. The sample was preilluminated for 2 min. Other conditions were the same as in Fig. 1.

under the present conditions. Fig. 5 shows the change in the ATPase activity of chloroplasts isolated from leaves which were kept in the dark at 11°C for various times after 2-min preillumination (dark inactivation in vivo). The dark inactivation of the ATPase in vivo had a lag and showed a sigmoidal curve when plotted semilogarithmically, with an apparent half-time of 25 min.

The apparent rate constants of decay of ΔA_{515} in a leaf preilluminated for various times are compared in Fig. 6. The half-maximal acceleration of the decay was observed with a preillumination for about 10 s. Fig. 7 shows the dark recovery of the preillumination-accelerated decay of ΔA_{515}. The dark recovery had a lag and showed a sigmoidal curve when plotted semilogarithmically as in the in vivo dark inactivation of ATPase activity. The recovery had an apparent half-time of 23 min.

Discussion

Chloroplast ATPase is known to be activated by illumination in isolated intact chloroplasts. Activation of the ATP-hydrolysis activity was found to be closely related to the activity of ATP synthesis [9] in isolated chloroplasts. In the present study, it was shown that the chloroplast ATPase was activated more than 20-fold by preillumination of intact leaves. Deactivation of the ATPase activity during isolation of chloroplasts after the light activation was estimated by assaying the activity at various times after the homogenization of leaves. It can be concluded that the procedure used in the present study (assay of ATPase began at 90 s after rupture of leaves) can follow the change in the ATPase activity in intact leaves, although the activity measured may be somewhat lower than the true in vivo value (14% lower under the present conditions). The kinetics of the dark inactivation of chloroplast ATPase in intact leaves (Fig. 6) were very similar to those of the dark recovery of the preillumination-induced acceleration of the ΔA_{515} decay in intact leaves (Fig. 7). Both kinetics had a lag in recovery to the dark level and showed sigmoidal time courses when plotted semilogarithmically. Both had apparent half-times of about 25 min. Moreover, the activation of chloroplast ATPase and acceleration of the decay of ΔA_{515} by preillumination showed similar dependences on illumination time, except for the decrease in the decay rate of ΔA_{515} after the preillumination longer than 1 min. As longer preillumination made the initial extent of ΔA_{515} smaller, the rate of H^+ translocation through the ATPase could be regulated by some factors other than the ATPase activity, e.g., by adenylate level [20]. Treatment of leaves with DCCD, an inhibitor of the membrane-bound ATPase, suppressed the acceleration of the decay of ΔA_{515} by preillumination, but not the formation of the high-energy state by preillumination. CCCP, an uncoupler of photophosphorylation, accelerated the decay of ΔA_{515} and suppressed the formation of the high-energy state. DCMU, an electron-transport inhibitor, also suppressed the acceleration of ΔA_{515} decay (not shown). The activation of chloroplast ATPase by illumination was also inhibited by DCMU, CCCP and DCCD. These results indicate that the ATPase is activated by the high-energy state [1,8,9]. That the addition of DCCD at assay stage of ATPase activity significantly inhibited the hydrolysis of ATP indicates that the ATPase was tightly coupled to the H^+ translocation across the thylakoid membrane. It can be concluded that the change in the decay rate of ΔA_{515} in intact leaves mainly reflects the change in activity of chloroplast ATPase. It seems that when the ATPase is in the activated state, the flash-induced membrane potential is more rapidly dissipated by the accelerated movement of protons through the H^+ channel of the ATPase complex coupled to the ATP synthesis.

Dark inactivation of ATPase in vitro showed a single-exponential decay with a half-time of 8 min, while that in vivo showed a sigmoidal decay with a long half-time of 23 min. This suggests that the inactivation of the ATPase activity in vivo is regulated by some factors (or conditions) which are lost during isolation. These may include the intactness of the membrane system and the levels of adenylates in chloroplasts. Inoue et al. [21] analyzed the change in the levels of ATP in intact chloroplasts under low- and high-frequency saturating flashes. They interpreted the data in terms of the light-induced activation and dark inactivation of the ATP-synthesizing and -hydrolyzing activities of the membrane-bound ATPase. They presumed a fast decay (with $t_{1/2}$ of the order of seconds) of ATPase after illumination. On the other hand, Mills and Hind [6] reported that the light-activated ATPase in intact chloroplasts was inactivated over a period of several minutes. Our result in Fig. 3 is consistent with their results. It can be concluded that the photophosphorylation is tightly coupled to H^+ movement across the thylakoid membrane in chloroplasts in intact leaves and that the rate of H^+ movement and therefore, the ATPase activity can be directly monitored by the decay rate of the flash-induced 515-nm absorbance change. The ATPase in intact leaves is rapidly activated by illumination but seems to decay slowly in the dark. The inactivation process changes under appropriate conditions. The ATPase in intact leaves is rapidly activated by illumination but seems to decay slowly in the dark. The inactivation process depended on the energy level of chloroplasts. The anaerobic incubation of intact leaves gave the lowest decay rate of ΔA_{515} [1] which was comparable to that observed in the presence of DCCD in the present study. Dunham and Selman [23] recently reported on the regulation of light-activated ATPase of thylakoid membrane by adenylates [22]. It seems likely that the energy level of chloroplasts in cells is interrelated with that of cytosol which is dependent mainly on respiration in mitochondria [24]. In isolated chloroplasts, a correlation between ATP synthesis and ΔA_{515} decay was reported and interpreted in terms of an ATPase-coupled H^+ current [4,25]. In chromatophores from *Rhodopseudomonas capsulata*, a similar correlation between the decay rate of carotenoid shift and ATP synthesis was reported [26].

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