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FLASH-INDUCED PHOTOPHOSPHORYLATION IN CHLOROPLASTS WITH ACTIVATED ATPase

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Chloroplasts which were rapidly isolated from illuminated leaves showed activity of ATP hydrolysis at a level much higher than that of the dark control. Under the high-intensity illumination or under repetitive flash excitation, the activated chloroplasts synthesized more ATP than those with a low ATP hydrolysis activity. $\Delta\tilde{\mu}_{H^+}$ formed under repetitive flashes was smaller in the activated chloroplasts than in the inactive chloroplasts. The inhibition of ATP yield per flash by valinomycin or nigericin in the presence of K^+ was stronger in the inactive chloroplasts than in the activated chloroplast. ATP synthesis in the activated chloroplasts seems to have a lower $\Delta\tilde{\mu}_{H^+}$ threshold.

Introduction

According to the chemiosmotic theory of Mitchell [1], ATP synthesis is coupled to the passive H^+ flux down the electrochemical potential gradient of H^+ ($\Delta\tilde{\mu}_{H^+}$) through the reversible ATPase on chloroplast thylakoid membranes. Gräber and Witt [2] and Ho et al. [3] reported that the rate of ATP synthesis depended equally on $\Delta\psi$ and ΔpH, and that there existed a threshold of $\Delta\tilde{\mu}_{H^+}$ for ATP synthesis. The membrane-bound ATPase of chloroplast has a latent ATP hydrolysis activity, which is activated under illumination by ΔpH imposition across the membrane [4–6], by the thiol modulation of the ATPase itself [6,7], and by the release of ADP from the ATPase [8,9]. Deactivation processes of the activated ATPase include the rebinding of ADP [8,9], and the demodulation of thiol groups by the thioredoxin system [10,11]. The activation of ATP hydrolysis activity of the ATPase in intact leaves or intact chloroplasts by illumination accompanied the acceleration of the decay of the field-indicating absorbance change at 515 nm (ΔA_{515}) [12–14]. It seems that the acceleration of ΔA_{515} reflects the faster H^+ efflux through the activated ATPase complex coupled to ATP synthesis [14,15]. It was concluded that the activation of the ATPase reduced the activation energy for ATP synthesis [14]. Mills and Mitchell [15] recently reported that the threshold of $\Delta\tilde{\mu}_{H^+}$ for ATP synthesis was lower in the activated chloroplasts than in the inactive chloroplasts in the pH-transition-induced ATP formation.

We report that ATP was formed with higher yield in the activated chloroplasts than in the inactive chloroplasts under repetitive flash excitation, though the level of $\Delta\tilde{\mu}_{H^+}$ formed in the former was lower.

Materials and Methods

Chloroplasts were isolated rapidly from spinach leaves which had been kept in the dark (typically

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; Tricine, *N*-tris(hydroxymethyl)methylglycine; Chl, chlorophyll.

for 4 h) or illuminated for 2 min after the dark incubation. The procedure for rapid isolation of chloroplasts was as follows: 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. This rapid isolation (within 90 s) made it possible to observe the activity close to that in vivo, since the ATPase activity decreased little within this period [5]. When the activities of dark chloroplasts and light chloroplasts were compared, chloroplasts were prepared from the dark-adapted and preilluminated halves of the same single leaf. ATPase activity was assayed by the measurement of P_i released as previously reported [5]. 0.1 ml of the chloroplast suspension (containing 20–60 μg Chl) 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 at 28°C. After reaction for 4 min, ice-cold trichloroacetic acid was added and P_i in the reaction mixture was determined.

Synthesis of ATP was assayed by the method of Nishimura et al. [16] measuring the pH change of the medium at 25°C. The reaction mixture consisted of 1 mM P_i, 10 mM $MgCl_2$, 0.38 mM ADP, 23 μM methyl viologen and chloroplasts equivalent to 20—30 μg Chl/ml (pH 8). Continuous illumination was provided by a combination of a tungsten lamp and a Toshiba R-69 glass filter. Intensity was changed by inserting appropriate neutral density filters. A combination of a xenon flash (Sugawara Laboratories, model MF-1500-U3) and a Toshiba R-68 glass filter gave repetitive flash illumination (flash duration 10 μs, repeated at 2 Hz). Flash-induced absorbance changes of chloroplasts at 515 nm (ΔA_{515}) were measured under conditions similar to those for synthesis of ATP. ΔpH was measured by the quenching of 9-aminoacridine fluorescence [17] in a reaction mixture containing 1 mM P_i, 10 mM $MgCl_2$, 0.9 μM 9-aminoacridine, 19 μM methyl viologen, 9 mM KCl, 0.37 mM ADP and chloroplasts equivalent to 20–35 μg Chl/ml. Conditions for flash illumination were the same as for ATP synthesis. 2-Hz illumination was given until a steady state was reached (usually 2–3 min).

Results and Discussion

As reported previously [16], the chloroplasts isolated from the preilluminated leaves (light chloroplasts) had an activity of ATP hydrolysis much higher than those from leaves kept in the dark (dark chloroplasts) (Table I). The result indicates that the inactive ATPase of the dark chloroplasts was activated by illumination of leaves. The activation was sensitive to the presence of DCCD in the medium used for infiltrating the leaves. ATP-forming activities in dark and light chloroplasts during continuous illumination (Fig. 1) were measured by alkalinization of the medium [15]. This method of measuring phosphorylation was used to measure directly the phosphorylation rate during illumination. The rate of ATP hydrolysis observed after the illumination period was added to the phosphorylation rate as a correction factor. At relatively low illumination intensities (less than $3 \cdot 10^4$ erg/cm^2 per s), at which the rate of electron transport is limiting the rate of net ATP synthesis, ATP-formation rates of both dark and light chloroplasts were almost the same. However, at a high light intensity, the rate of ATP formation in light chloroplasts was higher by about 20% than

TABLE I

ACTIVATION OF MEMBRANE-BOUND CHLOROPLAST ATPase AND PHOSPHORYLATION BY PREILLUMINATION OF LEAVES

Activities of ATPase and phosphorylation were measured as indicated in Materials and Methods. In the case of DCCD-treated light chloroplasts, the chloroplasts were rapidly isolated from the illuminated leaves infiltrated with DCCD. Photophosphorylation was measured under saturating continuous illumination. The rates are expressed in μmol P_i liberated or ATP formed/mg Chl per h. The ranges of standard deivation are also shown.

	ATP hydrolysis	Photophosphorylation
Dark chloroplasts	8 ± 2	175 ± 18
Light chloroplasts	205 ± 22	246 ± 16
DCCD-treated light chloroplasts	56	

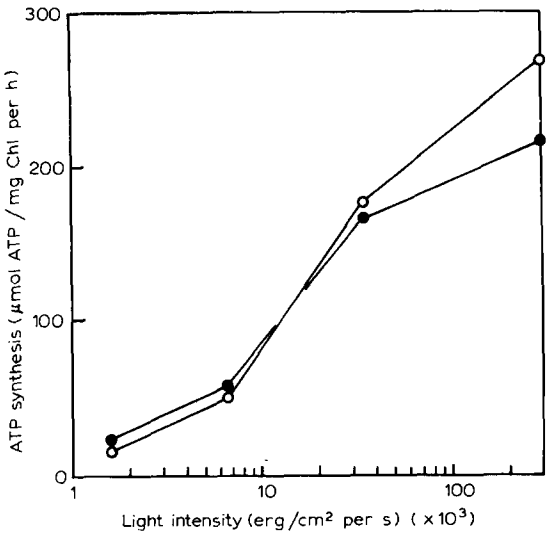

Fig. 1. Effect of intensity of continuous illuminations on the rate of ATP formation. (● and ○) Dark and light chloroplasts, respectively. Reaction mixture contained 3 mM P_i, 10 mM $MgCl_2$, 0.37 mM ADP, 22 μM methyl viologen and was adjusted to ph 8.0 by adding 0.1 M NaOH. Chl content was 20–24 μg per ml.

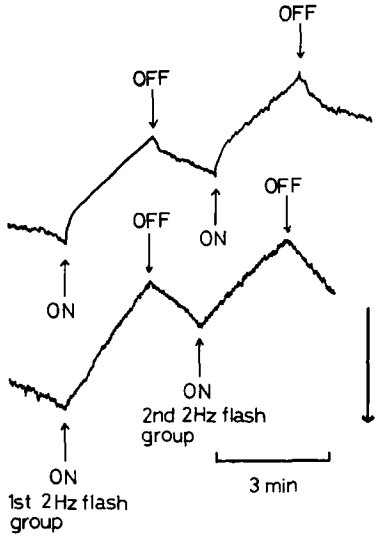

Fig. 2. pH change of the medium upon repetitive flash excitation (2 Hz) of chloroplast suspension under phosphorylating conditions. Medium pH was measured with a glass electrode. Upper trace, dark chloroplasts. Lower trace, light chloroplasts. The arrow on the right-hand side indicates the direction of acidification. Other conditions were the same as in Fig. 1.

that of dark chloroplasts (Fig. 1 and Table I). The difference indicates that the turnover of the ATP synthase, which was the rate-limiting factor at the high illumination intensity, was higher in the light-activated chloroplasts than in the dark chloroplasts.

Traces of the pH change in chloroplast suspension due to phosphorylation of ADP under repetitive 2-Hz flashes are shown in Fig. 2. In the case of dark chloroplasts, a rapid alkalinization during initial several-flash illuminations was followed by a relatively slow steady rate which reflected the ATP synthesis. On the other hand, a higher steady rate of medium alkalinization was observed in light chloroplasts, which indicated a faster ATP synthesis. The phase of the initial rapid alkalinization of the medium was lacking. Under nonphosphorylating conditions in the absence of ADP the initial rates of the rapid alkalinization were the same in light and dark chloroplasts. Therefore, the absence of the rapid alkalinization in light chloroplasts under phosphorylating conditions suggests that a smaller ΔpH was built up in light chloroplasts under repetitive flash illumination.

Table II shows the yield of ATP under repetitive flash illumination, expressed as the number of ATP molecules formed per electron-transfer chain (600 molecules of chlorophyll) per flash. The effects of valinomycin $+K^+$ and nigericin $+K^+$ on the yield are also indicated. The ATP yield was higher in light chloroplasts. Ionophores suppressed ATP synthesis more effectively in dark chloro-

TABLE II

ATP YIELD PER FLASH (NUMBER OF MOLECULES OF ATP FORMED PER 600 MOLECULES OF CHLOROPHYLL PER FLASH) IN DARK AND LIGHT CHLOROPLASTS AND EFFECTS OF IONOPHORES

Reaction mixture contained 1 mM P_i, 10 mM $MgCl_2$, 0.9 μM 9-aminoacridine, 19 μM methyl viologen, 9 mM KCl, 0.37 mM ADP (pH 8.0) and about 30 μg Chl per ml. Concentrations of ionophores were 50 nM. Values in parentheses indicate values relative to the control (no ionophore).

	No ionophore	Valinomycin	Nigericin
Dark chloroplasts	0.64	0.46 (72%)	
	0.64		0.16 (24%)
Light chloroplasts	0.76	0.59 (80%)	
	0.82		0.40 (49%)

plasts than in light chloroplasts, especially under flash illumination with longer flash intervals (data not shown). The ATP yield can be assumed to depend to the H^+ efflux through the ATPase complexes (J_{CF}). The changes in the passive H^+ flux across the lipid region of thylakoid membrane (J_{lipid}) and the fluxes of other ions should affect J_{CF}. If the active ATPase complexes have a higher rate of H^+ efflux through them, i.e., a larger J_{CF}, the ratio of J_{CF} to (J_{lipid} plus J_{CF}) will be less affected by the increase of J_{lipid} by the ionophores. Therefore, the ATP synthesis in light chloroplasts with activated ATPase is expected to be less affected by the ionophores, as indeed was the case (Table II).

Fig. 3 shows the 515-nm absorbance change (ΔA_{515}) by the repetitive 2-Hz flashes in dark and light chloroplasts under phosphorylating conditions. ΔA_{515} is an indicator of membrane potential

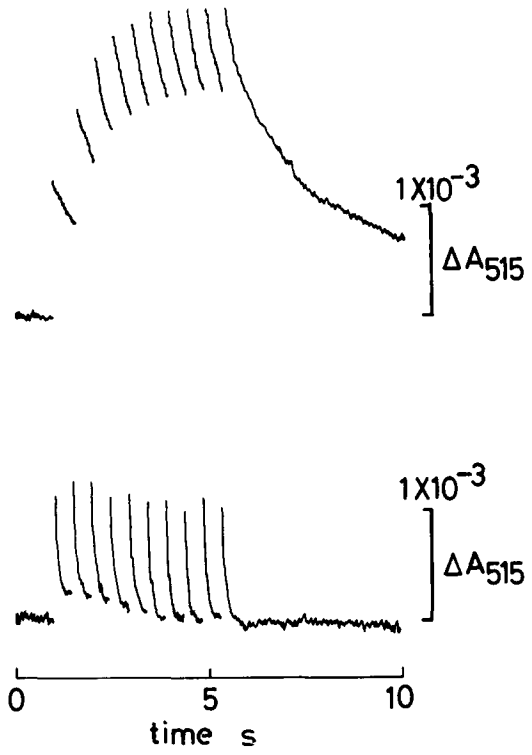

Fig. 3. 515-nm absorbance change induced by 2-Hz flash illumination. Upper and lower traces are the time courses in dark and light chloroplasts, respectively. Reaction mixture contained 3 mM P_i, 10 mM $MgCl_2$, 0.9 μM 9-aminoacridine, 19 μM methyl viologen, 9 mM KCl, 0.15 mM ADP and chloroplasts equivalent to about 35 μg Chl per ml.

formed in chloroplasts [18]. The decay of membrane potential generated by flashes was slower in dark chloroplasts. This led to the formation of a larger membrane potential during repetitive flashes in dark chloroplasts. As the membrane potential built up during the repeating 2-Hz flashes, the values of 110 and 50 mV were obtained in dark and light chloroplasts, respectively. These values were calibrated according to the method of Gräber and Witt [2]. These levels of the membrane potential ($\Delta\psi$) in the 'pseudo-steady state' were attained after several flashes in a train of repetitive flash illumination. The pH difference across the vesicle membranes, ΔpH, under the same conditions was measured by 9-aminoacridine fluorescence quenching. As shown in Table III, values of 2.5 and 2.8 were observed for the ΔpH in dark and light chloroplasts, respectively, under phosphorylating conditions. From these values of $\Delta\psi$ and ΔpH, the electrochemical potential differences, $\Delta\tilde{\mu}_{H^+}$, of 19.1 and 26.6 kJ/mol (corresponding to 200 and 280 mV) were calculated under phosphorylating conditions under 2-Hz flash illumination in light and dark chloroplasts, respectively. As expected, $\Delta\tilde{\mu}_{H^+}$ in light chloroplasts was smaller. A small additional $\Delta\psi$ might have been produced before flash illumination in light chloroplasts due to the hydrolysis of a small amount of preformed ATP [13]. This result confirms that the efflux of H^+ through the ATPase complexes is faster in light chloroplasts which have the 'fully active ATPase' complexes. Gräber and Witt [2] reported that the ATP yield per flash became higher at

TABLE III

ΔpH, $\Delta\psi$ AND $\Delta\tilde{\mu}_{H^+}$ GENERATED IN DARK AND LIGHT CHLOROPLASTS UNDER PHOSPHORYLATING CONDITIONS DURING THE PSEUDO-STEADY STATE MAINTAINED UNDER 2-Hz FLASH ILLUMINATION

ΔpH was measured by 9-aminoacridine fluorescence quenching. $\Delta\psi$ was calibrated from the absorbance change at 515 nm induced by flash illumination.

	ΔpH (inside acidic)	$\Delta\psi$ (inside positive)	$\Delta\tilde{\mu}_{H^+}$ (kJ/mol)
Dark chloroplasts	2.8	110 mV	26.6 (280 mV)
Light chloroplasts	2.5	50 mV	19.1 (200 mV)

larger $\Delta\tilde{\mu}_{H^+}$. A higher ATP yield at a larger $\Delta\tilde{\mu}_{H^+}$ indicates different dependences of J_{CF} and J_{lipid} on $\Delta\tilde{\mu}_{H^+}$; for example, there is a possibility that when $\Delta\tilde{\mu}_{H^+}$ increases F_{CF} increases more dramatically than the increase in J_{lipid}, as suggested in previous studies [2,3]. In these papers, chloroplasts with rather inactive ATPase were used. In our experiments, we used light chloroplasts with activated ATPase. It is suggested that J_{CF} in chloroplasts with fully active ATPase has a less marked dependence on the extent of $\Delta\tilde{\mu}_{H^+}$, although there is a limitation by the thermodynamic constraint for the conversion of free energy stored at a given level of $\Delta\tilde{\mu}_{H^+}$ to the bond energy of ATP. It seems likely that the ATP synthase with low ATP hydrolysis activity requires a certain level of $\Delta\tilde{\mu}_{H^+}$ to be activated to a state with a lower threshold value of $\Delta\tilde{\mu}_{H^+}$ for ATP synthesis. It is important to study the mechanism of the change of the threshold of $\Delta\tilde{\mu}_{H^+}$ in the activated ATP-synthesizing system.

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