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Light Activation of NADP-Malate Dehydrogenase in Guard Cell Protoplasts from *Vicia faba* L.[1]

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ABSTRACT

Light-induced swelling of guard cell protoplasts (GCP) from *Vicia faba* was accompanied by increases in content of K^+ and malate. DCMU inhibited the increase of K^+ and malate, and consequently swelling.

Effect of light on the activity of selected enzymes that take part in malate formation was studied. When isolated GCP were illuminated, NADP-malate dehydrogenase (NADP-MDH) was activated, and the activity reached a maximum within 5 minutes. The enzyme activity underwent 5- to 6-fold increase in the light. Upon turning off the light, the enzyme was inactivated in 5 minutes NAD-MDH and phosphoenolpyruvate carboxylase (PEPC) were not influenced by light. The rapid light activation of NADP-MDH was inhibited by DCMU, suggesting that the enzyme was activated by reductants from the linear electron transport in chloroplasts. An enzyme localization study by differential centrifugation indicates that NADP-MDH is located in the chloroplasts, NAD-MDH in the cytosol and mitochondria, and PEPC in the cytosol. After light activation, the activity of NADP-MDH in guard cells was 10 times that in mesophyll cells on a chlorophyll basis. The physiological significance of light-dependent activation of NADP-MDH in guard cells is discussed in relation to stomatal movement.

Light is one of the environmental factors that affect stomatal aperture. It also causes volume increase of guard cell protoplasts from *Allium cepa* (30) and *Vicia faba* (6). The results demonstrate that the photoreceptor is within the guard cell itself. Guard cells have chloroplasts, which are thought to be one of the primary sites of light action. There is no doubt about the existence of PSII in guard cells of *Chlorophytum comsum* (28) and *V. faba* (15, 18, 24). Linear electron transport is now considered a common feature of GCP[2] (12). There are reports that PSII activity is involved in stomatal aperture regulation (22, 23) and guard cell volume regulation (8).

High rates of photosynthetic O_2 evolution in GCP (8) imply that both ATP and NADPH generated by linear electron transport are utilized in GCP. Linear electron transport in photosynthesis is essential for supplying ATP and NADPH needed for CO_2 assimilation. Guard cell chloroplasts, however, have anomalous properties compared to mesophyll chloroplasts. They lack some key enzymes in photosynthetic carbon reduction pathway (11, 17), so that photosynthetic carbon reduction pathway is currently thought to be absent from guard cells. Elucidation of the role of linear electron transport in guard cell chloroplasts is an urgent problem in stomatal physiology. Possible functions of linear electron transport include ion transport (19) and enzyme activation (4). We report here PAR-dependent activation of NADP-MDH in guard cells, and discuss its significance in stomatal movement.

MATERIALS AND METHODS

Isolation of Guard Cell Protoplasts and Mesophyll Cell Protoplasts. GCP were isolated from lower epidermis of *Vicia faba* leaves as reported previously (8). MCP were isolated from *Vicia* leaf segments freed of lower epidermis by enzymic digestion. The enzyme solution for isolation of MCP consisted of 0.01% Pectolyase Y-23 (Seishin Pharmaceutical Industry Co., Japan), 1% Cellulase Onozuka RS (Yakult Pharmaceutical Industry Co., Japan), 0.2% BSA, 1 μg ml^{-1} Pepstatin A (Protein Research Foundation, Japan), 0.6 M mannitol, and 1 mM $CaCl_2$, at pH 5.5 adjusted with HCl. The leaf segments were infiltrated with the enzyme solution, and were incubated for 5 min at 27°C. The used enzyme solution was discarded. The leaf segments were further incubated for 1 to 2 h with the newly prepared enzyme solution. Isolated MCP were purified by low-speed centrifugation (110*g*), resuspended in 0.6 M mannitol and 1 mM $CaCl_2$, and kept on ice.

Volume and K^+ Determination. GCP volume and K^+ content were determined by a previously described method (6).

Determination of Organic Acids. GCP were collected by silicone oil centrifugation as described previously (6), except that the bottom layer was replaced by 20 μl of 3.5 M ammonium acetate. The bottom layer was cut and dissolved in water. After sonication (Sonifier cell disrupter 185E, Branson, England) for 30 s, the suspension was incubated with 4 volumes of ethanol at 80°C for 30 min, and then dried. The dried sample was dissolved in 250 μl of H_2O and filtered through 0.45 μm Millipore filter (HV, Nihon Millipore Kogyo, Japan). Organic acids were separated and determined by HPLC using a JASCO Trirotor SR-2 (Japan Spectroscopic Co.) in an anion exchange column, Shodex C-811 (500 × 8 mm ϕ) with a mobile phase of 2.5 mM $HClO_4$ and a flow rate of 0.8 ml min^{-1} at 40°C. The mobile phase passed through the column, was mixed with a pH reagent solution which consisted of 3.5 mM Na_2HPO_4 and 0.2 mM bromocresol purple, and flowed at 1.0 ml min^{-1}. The amount of organic acids in the extracts were estimated from the absorbance change at

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[2] Abbreviations: GCP, guard cell protoplast(s); NADP-MDH, NADP-malate dehydrogenase; NAD-MDH, NAD-malate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; MCP, mesophyll cell protoplast(s); OAA, oxaloacetate; PEP, phosphoenolpyruvate.

430 nm.

Illumination of GCP. GCP suspended in 0.4 M mannitol, 10 mM KCl, 1 mM $CaCl_2$, and 5 mM Mes-Tris buffer (pH 6.5) were illuminated with a white light from 300-W slide projector (Elmo Co., Japan) through a 50-mm water layer at 300 $\mu E\ m^{-2}\ s^{-1}$.

Enzyme Assay. Enzyme activities were measured by rapidly transferring 20 μl aliquots of GCP suspension from the incubation medium to an assay mixture and immediately starting the assay. The assay mixture for NADP-MDH (EC 1.1.1.82) consisted of 0.5 mM OAA, 0.2 mM NADPH, 1 mM EDTA, 15 mM 2-mercaptoethanol, 0.05% Triton X-100, and 100 mM Tris-HCl (pH 7.8) (10) in a total volume of 0.5 ml. The activity was followed at 25°C as the decrease in *A* at 340 nm. For the assay of NAD-MDH (EC 1.1.1.37), NADPH was replaced by NADH. The activity of PEPC (EC 4.1.1.31) was determined by the oxidation rates of NADH coupled with MDH according to the methods of Maruyama *et al.* (13) in a medium containing 0.05% Triton X-100 in a total volume of 0.5 ml. Since the reaction could be catalyzed by PEP carboxykinase as well as by PEPC, the dependency of the oxidation rates on GDP and ADP was examined. The activity was expressed on a Chl basis. Chl content of the GCP was determined as described previously (8).

Isolation of Organelles. GCP were ruptured by 4 to 6 passages through a 10-μm nylon mesh mounted on the open end of a 1-ml syringe. Separation of chloroplasts, mitochondria, and the cytosol was carried out according to the method of Moore *et al.* (14).

RESULTS

The effects of DCMU on the GCP volume and on the K^+ and malate contents of GCP are given in Table I. GCP showed light-induced swelling during the first 30 min of illumination, and a further swelling during an additional 30-min period of illumination. The K^+ and malate contents increased in parallel with the volume increase. DCMU added at the beginning of preincubation inhibited accumulation of both K^+ and malate, and consequently the swelling. When it was added after 30 min of incubation, it caused volume decrease. DCMU decreased K^+ content and inhibited the increases in malate content.

As shown in Figure 1, the NADP-MDH was activated by light, and the activity reached a maximum within 5 min of illumination. The enzyme was activated up to 5- to 6-fold by light. The activity decreased to the previous level 5 min after turning off the light. This activation/inactivation cycle was repeatable. The light activation of NADP-MDH in GCP was completely inhibited by DCMU (Table II). The apparent K_m(OAA) of NADP-MDH was 40 μM.

Table I. *Effects of DCMU on Light-Induced Changes in Volume and K^+ and Malate Contents of Guard Cell Protoplasts from V. faba L.*

GCP (7.2×10^5 GCP ml^{-1}) were preincubated in 0.4 M mannitol solution containing 10 mM KCl, 1 mM $CaCl_2$, and 5 mM Mes-Tris (pH 6.5) in the dark at 25°C for 5 min, followed by incubation in the light or in the dark. DCMU (5 μM) was added at the onset of preincubation, or 30 min after the light was turned on. Each value for GCP volume is the mean of 90 GCP with the SE. K^+ and malate contents were determined using duplicate samples.

Treatment	Protoplast Volume	K^+	Malate
	pl	*pmol/GCP*	
Initial	3.67 ± 0.09	0.306	0.054
Light, 30 min	4.02 ± 0.11	0.358	0.085
+DCMU	3.72 ± 0.09	0.297	0.058
Light, 60 min	4.65 ± 0.14	0.425	0.139
+DCMU (30–60 min)	3.86 ± 0.09	0.310	0.089
Dark, 60 min	3.46 ± 0.08	0.216	0.052

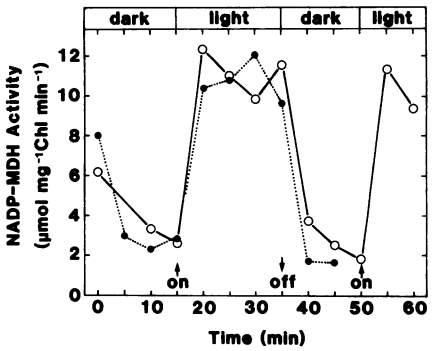

FIG. 1. Light activation and dark inactivation of NADP-MDH in GCP. GCP (O——O, 2.1 μg Chl; ●- - -● 5.9 μg Chl) were incubated in 1.0 ml of 0.4 M mannitol, 10 mM KCl, 1 mM $CaCl_2$, and 5 mM Mes-Tris (pH 6.5) at 25°C. The activity at time 0 was that in GCP kept on ice after isolation until experiment. The activity was measured at a time interval of 5 min as described in "Materials and Methods".

Table II. *Effect of DCMU on Light Activation of NADP-Malate Dehydrogenase in Guard Cell Protoplasts from V. faba L.*

GCP (3.6 μg Chl ml^{-1}) were incubated in 0.4 M mannitol, 10 mM KCl, 1 mM $CaCl_2$, 5 mM Mes-Tris (pH 6.5) in the absence or presence of 5 μM DCMU in the dark for 15 min at 25°C, and the activity of NADP-malate dehydrogenase was measured. Then the GCP were illuminated for 5 min and the activity was measured again.

Treatment	Dark	Light	Light/Dark
	μmol mg⁻¹ Chl min⁻¹		*ratio*
None	1.8	10.6	5.6
DCMU	1.9	1.9	1.0

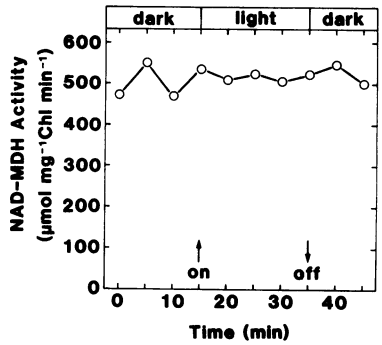

FIG. 2. Effect of light illumination on the activity of NAD-MDH. GCP (1.7 μg Chl) were incubated in 1.0 ml of 0.4 M mannitol, 10 mM KCl, 1 mM $CaCl_2$, and 5 mM Mes-Tris (pH 6.5) at 25°C. The activity was measured as in Figure 1.

The activity of NAD-MDH was not influenced by illumination (Fig. 2). The apparent K_m(OAA) of NAD-MDH was 180 μM.

The activity of PEPC was not influenced by illumination (Fig. 3). Neither 5 mM ADP nor 5 mM GDP enhanced the oxidation rates of NADH in the assay mixture for PEPC. The results show that there is little activity of PEP carboxykinase (GTP, EC 4.1.1.32; ATP, EC 4.1.1.49) in guard cells, and that the oxidation of NADH was due to the activity of PEPC.

Intracellular distribution of the three enzymes in guard cells was studied by differential centrifugation (Table III). The results indicated that most of NADP-MDH was in the chloroplasts. NAD-MDH was mostly cytosolic and was partly located in the mitochondria. PEPC was located predominantly in the cytosol.

The activity of NADP-MDH in GCP was compared with that

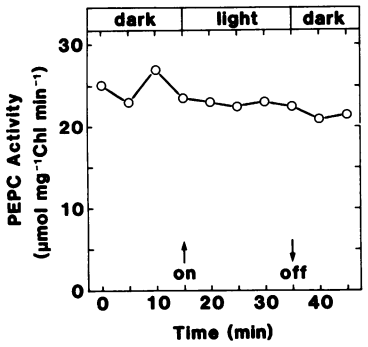

FIG. 3. Effect of light illumination on the activity of PEPC. GCP (2.4 μg Chl) were incubated in 1.0 ml of 0.4 M mannitol, 10 mM KCl, 1 mM $CaCl_2$, and 5 mM Mes-Tris (pH 6.5) at 25°C. The activity was measured as in Figure 1.

Table III. *Intracellular Distribution of Enzyme Activities in V. faba Guard Cells as Determined Using Isolated Protoplasts*

The protoplasts were ruptured, fractionated by differential centrifugation, and each fraction was subjected to enzyme analysis according to Moore *et al.* (14).

Enzyme	Distribution			Recovery
	Chloroplasts	Cytosol	Mitochondria	
		%		
NADP-MDH	76	17	7	88
NAD-MDH	14	62	24	125
PEPC	5	90	5	97
Cyt *c* oxidase	13	0	87	64
Pyruvate kinase	20	80	0	100

Table IV. *NADP-Malate Dehydrogenase Activities in Guard and Mesophyll Cell Protoplasts from V. faba L.*

GCP (2.4 μg Chl ml^{-1}) were suspended in 0.4 M mannitol, 10 mM KCl, 1 mM $CaCl_2$, and 5 mM Mes-Tris (pH 6.5). The activity in the dark was measured after 15 min preincubation in the dark at 25°C. The activity in the light was measured after 5-min illumination. MCP (60 μg Chl ml^{-1}) were suspended in 0.6 M mannitol, 1 mM KCl, 1 mM $CaCl_2$, and 5 mM Mes-Tris (pH 7.5). The activity in MCP was measured as in GCP.

	Dark	Light	Light/Dark
	μmol mg^{-1} Chl min^{-1}		*ratio*
GCP	2.4	10.7	4.5
MCP	0.2	1.1	5.5

in MCP (Table IV). In both protoplast types, the enzyme was activated up to 5-fold by light. The activity of NADP-MDH in the light showed 10.7 μmol mg^{-1} Chl min^{-1} and 1.1 μmol mg^{-1} Chl min^{-1} in GCP and in MCP, respectively. The activity of NADP-MDH in GCP was 10 times higher than that in MCP on a Chl basis.

DISCUSSION

Light-induced swelling of GCP was accompanied by accumulation of K^+ and malate, and was inhibited by DCMU (Table I). DCMU also inhibited the enhanced $^{14}CO_2$ fixation by GCP in the light (7). These results imply that photosynthetic processes are involved in the light-induced swelling of GCP. There are several lines of evidence that malate is synthesized in guard cells. The accumulation of malate was observed in experimental systems in which only guard cells are living cells, such as in rolled epidermal strips (1), in sonicated epidermal strips (16), and in GCP (21). These results are consistent with our results (Table I). $^{14}CO_2$ fixation results in [^{14}C]malate formation in stomatal systems (17). Guard cells show high levels of PEPC activity (17). These results clearly indicate that malate is synthesized in guard cells by CO_2 fixation (25), and that it acts as a dominant counterion for K^+ during stomatal opening (29).

Malate formation in guard cells of *Vicia faba* was observed in the dark (17), and was enhanced by light (16). Blue light at high intensities was more effective than red light in malate formation, but at low intensities it was ineffective without background red light (16). We focused our attention on enzyme reactions which participate in malate synthesis by CO_2 fixation. The first step is OAA formation from PEP by CO_2 fixation which is catalyzed by PEPC. The second step is malate formation from OAA which is catalyzed by two types of MDH specific either to NADPH or NADH. There is a report (20) on light modulation of enzymes in the epidermal strips from *Pisum sativum:* the activities of NADP-MDH and PEPC were increased 3.8- and 2.2-fold, respectively, by illumination of epidermal strips for 2 h, which is rather too long. In our study, no light activation of PEPC was observed at least during 20 min of illumination. The activity of NAD-MDH was also unaffected by light. The present study shows that only NADP-MDH was activated by light. The light-dependent activation of NADP-MDH was inhibited by DCMU, suggesting that the enzyme was activated by the reductants from linear electron transport in chloroplasts. Enzyme distribution studies located NADP-MDH in the chloroplasts, NAD-MDH in the cytosol and mitochondria, and PEPC in the cytosol. The distribution pattern is similar to that in other cells (14). Thus, NADP-MDH can be a marker enzyme of guard cell chloroplasts. The enzyme is a light-activated chloroplast enzyme that is widely distributed in green plants, and is activated by thioredoxin *m* (4). This seems also the case with guard cell chloroplasts.

The activity of NADP-MDH in guard cells was higher than that in mesophyll cells on a Chl basis. The enzyme, therefore, seems to play a more important role in guard cell chloroplasts than in mesophyll cell chloroplasts. Generally, the activity of NADP-MDH on a Chl basis is higher in C_4 plants than in C_3 plants. The activity in guard cell chloroplasts is comparable to the reported activity in C_4 mesophyll cells (14). It is generally assumed that OAA is reduced by NADP-MDH present in the mesophyll chloroplasts of C_4 plants (5). It is probable that light-enhanced malate formation is catalyzed by NADP-MDH as in C_4 mesophyll cells. The activity of NAD-MDH is much higher than that of NADP-MDH under the present assay conditions (Figs. 1 and 2). All plant tissues contain high levels of the NAD-specific enzyme, although NAD-MDH has not been implicated in CO_2 fixation in C_4 photosynthesis (5).

The present study shows that the accumulation of malate was induced by light and inhibited by DCMU. Red light greatly enhances malate formation under low intensities of blue light (16). These results imply that PAR-dependent processes are involved in malate formation. The malate formation from PEP needs reducing power in the second step. The production of reducing equivalents on the reducing side of PSI was indicated by fluorescence transient (24). Linear electron transport plays a role in both the activation of the enzyme and the supply of reducing power. Thus, it is likely that malate is synthesized from OAA with NADPH by NADP-MDH in guard cell chloroplasts.

Malate synthesis via CO_2 fixation seems to be regulated by several factors. It is considered that PEPC plays an anaplerotic role in carbon metabolism in guard cells (26). The light-stimulation of malate synthesis is speculated to be triggered by increased carboxylation by PEPC in the alkalinized cytoplasm that is expected to be caused by light-driven H^+ pump on the plasmalemma (27, 29). The present study reveals that a primary

action of light on guard cells is to activate the NADP-MDH and that the spectrum of light here is that of PAR. Presumably, this PAR-activation of NADP-MDH constitutes a regulatory step in malate synthesis.

The enzyme compartmentalization in the synthesis of malate from PEP, however, throws complexity into the carbon metabolism in guard cells. Prior to the reduction of OAA to malate in chloroplasts, OAA should be synthesized by the carboxylation of PEP in the cytoplasm and then transported into chloroplasts as is known to occur in C_4 mesophyll cells such as maize (3). The production route of PEP in guard cells may be different from that in C_4 mesophyll cells. It is postulated that in guard cells PEP is formed by the breakdown of starch localized in chloroplasts (16). In addition, sucrose found in GCP (unpublished results) can be a candidate for PEP formation. Further studies are needed on metabolite movements in guard cells.

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