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Light and Stomatal Metabolism[1]

I. POSSIBLE INVOLVEMENT OF LIGHT MODULATION OF ENZYMES IN STOMATAL MOVEMENT

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I. MADHUSUDANA RAO[2] AND LOUISE E. ANDERSON[3]
Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois 60680

ABSTRACT

New evidence is provided regarding the direct effect of light on stomatal opening in the epidermis of the pea (*Pisum sativum* L. var Little Marvel) leaf. Light modulates the activity of a number of key enzymes involved in stomatal metabolism. When isolated epidermal strips are illuminated, phosphoenolpyruvate carboxylase, NADP-malate dehydrogenase, and NADP-isocitrate dehydrogenase are activated; and aspartate aminotransferase is inactivated. Sulfhydryl compounds, dithiothreitol and glutathione, enhance stomatal opening in epidermal strips both in light or darkness while the sulfhydryl reagent *N*-ethylmaleimide inhibits, indicating the possible involvement of sulfhydryl groups in stomatal movements. Further, light treatment increases measureable thiol levels in the epidermis about 3-fold. These results suggest that light modulation of enzymes in the epidermis may play a significant role in the mechanism of stomatal movement.

Stomatal movements are caused by changes in guard cell turgor arising from the movement of K^+ and H^+ (11, 13) with electroneutrality being maintained by movement of Cl^- or internal production of malate (1, 16). The occurrence of wider stomatal opening with increasing light intensity and of smaller apertures in the dark than in the light has been known for some time (4). Sharkey and Raschke (19) measured the magnitude of the direct and indirect effects of light on stomatal movement. The stomatal response to light was determined to be a direct response to light and, to a small extent only, a response to changes in intercellular CO_2 concentration.

The presence of both photosystems in guard cell chloroplasts was shown by the elegant experiments of Outlaw (13). Because the reductive step enzyme of the photosynthetic carbon reduction cycle (NADP-linked glyceraldehyde-3-P dehydrogenase) is absent in guard cells, Outlaw (13) suggested that noncyclic photosynthetic electron flow is an environmental sensor which causes stomata to remain open in light. It was further pointed out that one of the important physiological changes associated with H_2O oxidation by photosynthetic electron transport might be the modulation of enzyme activity in the guard cells.

Light modulation of the activity of the enzymes of photosynthetic carbon metabolism has been shown to occur in C_3, C_4, and CAM plants (2). The enzymic equipment for malic acid carboxylation and decarboxylation as well as its compartmentation in guard cells shows many similarities with those of C_4 and CAM plants (5, 6, 13, 17, 24, 27). The synthesis of malic acid and its catabolism in the starch-containing system of guard cells has been demonstrated to be a response to K^+ induced H^+ extrusion (16).

The purpose of the present study was to investigate the possible involvement of light modulation of enzyme activity in light-mediated stomatal opening in the epidermis of pea leaves.

MATERIALS AND METHODS

Plant Material. Fully expanded first pair of leaves from 10- to 12-d-old pea (*Pisum sativum* L. var Little Marvel) plants grown in vermiculite in a greenhouse during autumn and winter were used for the studies.

Preparation of Epidermal Strips. The lower epidermis from 50 to 60 leaves was carefully peeled from either side of the main vein and floated cuticle up on distilled H_2O until sufficient material had been collected. Epidermal strips were removed at right angles to the lamina to optimize epidermal cell viability and minimize mesophyll contamination (25). This technique allowed us to obtain approximately 50% viable epidermal cells with no signs of damage to guard cells as revealed by neutral red uptake. Epidermal strips were carefully screened by microscopic examination for freedom from mesophyll tissue. When stained with neutral red, the epidermal strips showed no mesophyll contamination as seen under a research microscope. The strips were cut into 5- × 5-mm pieces and were rinsed with ice-cold distilled H_2O two or three times and were again rinsed for about 7 to 8 min in four changes of 0.1 mM $CaCl_2$ to remove solutes from the broken epidermal cells. The strips were then incubated in distilled H_2O at room temperature (20°C) in darkness for 1 h. This treatment resulted in consistent and large stomatal response to illumination.

Incubation of Epidermal Strips. The epidermal strips were transferred to small Petri dishes (5 cm) containing 10 ml of treatment solution. The solutions contained 10 mM Pipes-KOH buffer (pH 6.8), 50 mM KCl, 0.1 mM $CaCl_2$, and varying amounts of DTT, GSH, or *N*-ethylmaleimide. We chose 50 mM K^+ since preliminary experiments indicated that light-dependent stomatal opening in pea leaf epidermal strips was greatest at this level. The strips were incubated at 25°C in light or in darkness in a water bath. The light intensity was 140 $w \cdot m^{-2}$ (Yellow Springs Instruments Kettering model 65 Radiometer) provided by two General Electric 120-v, 150-w reflector flood lamps. Stomatal aperture was measured under a microscope (× 1000) using a precalibrated ocular micrometer. At least 10 strips were chosen from each treatment, and the widths of five stomata selected at random from each strip were recorded. The experiments were repeated three times and the mean values are reported here.

Preparation of Epidermal Tissue Extracts. Epidermal strips were incubated in 10 ml of 10 mM Pipes-KOH buffer (pH 6.8) with 50 mM KCl, 0.1 mM $CaCl_2$ at 20°C in light (140 $w \cdot m^{-2}$) or darkness for 2 h. Epidermal tissue was then homogenized in a precooled tissue homogenizer with approximately 5 to 7 ml of 50 mM Tris-HCl (pH 8.3), 1 mM EDTA, and 5 mM $MgCl_2$ buffer.

[1] Supported by Department of Energy Contract EP 78-S-02 1961.

[2] Present address: Department of Botany, University of Illinois at Urbana-Champaign, Urbana, IL 61801.
[3] Address reprint requests to this author.

The homogenate was centrifuged at 1000*g* for 10 min, and the supernatant was retained for assay of enzyme activity and protein.

Preparation of Whole Leaf Extracts. Detached whole leaves were incubated on 50 ml of distilled H_2O at 25°C in light (140 w·m^{-2}) or in darkness for 60 min. The leaf tissue was then homogenized in darkness in a precooled mortar and pestle in the same extraction medium used for epidermal extracts. The homogenate was centrifuged at 1000*g* for 20 min, and the supernatant was retained for enzyme and protein assay.

Protein Assay. A small aliquot of the extract after centrifugation was taken and was mixed with an equal volume of 10% (w/v) TCA to precipitate protein. The protein was spun down by centrifugation at 2500*g* for 20 min and was measured by the method of Lowry *et al.* (10).

Enzyme Assays. The activity of all the enzymes was assayed at 22°C. PEP[4] carboxylase (EC 4.1.1.31) was assayed in a coupled enzyme assay according to the method developed by Donkin and Martin (6) except that DTT was not included in the assay mixture. $NADP^+$ specific malate dehydrogenase (EC 1.1.1.37) was measured as described by Willmer *et al.* (27). $NADP^+$ specific isocitrate dehydrogenase (EC 1.1.1.42) was assayed according to the method of Ochoa (12). L-Aspartate:α-ketoglutarate aminotransferase (EC 2.6.1.1) was measured by coupling to NAD-malate dehydrogenase (18). Change in *A* was followed on Cary 219 or 210 recording spectrophotometers.

Potassium Determination. K^+ content was estimated by a slight modification of the methods described by Fischer (7). The stain for potassium was prepared by dissolving 10 g of sodium cobaltinitrite in 25 ml of 7% glacial acetic acid. After the incubation period, the strips were floated on distilled H_2O. All of the above steps were carried out in an ice bath. The strips were then placed in a 1:1 mixture of ammonium sulfide and 50% glycerin for 1 min, washed in distilled H_2O twice, and mounted on a glass slide in glycerin. The strips were observed microscopically and scored for the degree of the black cobaltous sulfide precipitate formed within the guard cells.

SH Determination. The free thiol content was estimated according to the method of Asahi (3). Freshly prepared DTNB (5,5′-dithiobis[2-nitrobenzoic acid]) (40 μg dissolved in 50 mM phosphate buffer, pH 7.0) was added to the reaction mixture (0.6 ml) and 300 μl of epidermal extract. Absorption of the nitrothiophenolate anion at 412 nm was measured.

Reagents and Seed Material. All biochemicals were obtained from Sigma. Other chemicals were analytical reagent grade. Pea seeds were obtained from Northrup and King, Chicago.

RESULTS

The time course of stomatal opening in light or darkness in the epidermal strips showed that the stimulation of stomatal opening was greater in the light than in darkness (Fig. 1, A–C). There was more stimulation of stomatal opening at 3 h after incubation than at 2 h. Sulfhydryl compounds such as DTT and GSH stimulated the stomatal opening process appreciably after 3 h of incubation in light or darkness. Conversely, the sulfhydryl reagent *N*-ethylmaleimide inhibited the stomatal opening process in light or darkness, but the inhibition was greater in the light (Fig. 1, A–C). Histochemical estimation of potassium indicated higher potassium levels in the guard cells of the epidermal strips incubated in the light than in darkness (Fig. 1, D and E). Incubation of epidermis with sulfhydryl compounds enhanced potassium uptake into the guard cells while sulfhydryl reagents inhibited potassium uptake (Fig. 1, D and E).

There was a marked increase (up to 329%) in the free thiol content when the epidermis was illuminated (Table I).

Light effects on the enzyme activities of epidermis and whole leaf extracts are detailed in Table II. Enzyme activities are expressed on a buffer-soluble protein basis. On the basis of the enzyme activities, epidermal tissue can be distinguished from whole leaf tissue as being enriched in the enzymes involved in the metabolism of malate and aspartate. PEP carboxylase and NADP-malate dehydrogenase showed higher activities in the epidermal tissue than in whole leaf extracts. Incubation of the epidermal strips in light for 2 h increased the activity of PEP carboxylase, NADP-malate dehydrogenase, and NADP-isocitrate dehydrogenase, and decreased the activity of aspartate aminotransferase. Light activation ranged from 2.2- to 3.8-fold and inactivation was 1.6-fold. Similar effects of light on the activity of these same enzymes in whole leaves were observed, except that, in whole leaves, PEP carboxylase was not affected by light treatment (Table II).

The time course of light activation of PEP carboxylase in attached epidermis of detached leaves incubated on distilled H_2O in light showed an increase in the enzyme activity in relation to the increased exposure to illumination up to 2 h (Fig. 2). Short-term exposure (5 and 10 min) of epidermis to illumination resulted in a small increase in the enzyme activity. It can be seen that the light activation of PEP carboxylase is reversible. When the epidermal strips were transferred back to darkness, the enzyme activity was decreased.

Effects of sulfhydryl compounds and a sulfhydryl reagent on the light modulation of PEP carboxylase in epidermis are shown in Table III. PEP carboxylase activity is increased to 2.0-fold by DTT and 1.6-fold by glutathione in darkness, indicating that these two sulfhydryl compounds in darkness mimic the light effect. In light, the enzyme activity was increased 1.3-fold by DTT and 2.0-fold by GSH. *N*-Ethylmaleimide decreased the enzyme activity more in light than in darkness, indicating that the light modulation is very sensitive to this sulfhydryl reagent.

DISCUSSION

Light modulated the activity of a number of key enzymes in the pea leaf epidermis. We report for the first time the light modulation of NADP-isocitrate dehydrogenase and aspartate aminotransferase in epidermis and whole leaf extracts of *Pisum sativum*. It is interesting to note that PEP carboxylase is light modulated in epidermis but not in the whole leaf. The light activation of the enzymes involved in malate formation and metabolism, PEP carboxylase and NADP-malate dehydrogenase, could explain the 1.2- and 1.4-fold enhancement of CO_2 assimilation into organic acids in light in the epidermis of *Commelina diffusa* (26) and *Vicia faba* (16). Higher levels of malic, citric, and isocitric acids were observed in open stomata than in the closed stomata (13). The light activation of PEP carboxylase, NADP-malate dehydrogenase, and NADP-isocitrate dehydrogenase might result in the higher levels of these organic acids in opened stomata.

The activity of NADP-malate dehydrogenase in pea leaf epidermal tissue reported in the present study is markedly lower than the activity observed in *Commelina* sps. (5, 24, 27). The low activity probably reflects differences between species. From the kinetics of the activation of PEP carboxylase, it would appear that light modulation of enzyme activity is rather sluggish in epidermal tissue in comparison with mesophyll (2).

Long-term illumination (8 to 12 h) of C_4 plants has been shown to increase the activity of PEP carboxylase in whole leaves (8, 21). Gavalas *et al.* (8), based on their preliminary experiments, suggested that the activity of PEP carboxylase in C_4 plants might be regulated *in vivo* by a reversible oxidation-reduction of sulfhydryl groups. The epidermal malate metabolism in C_3 species shows many similarities with that of C_4 and CAM leaves (5, 6, 13, 17, 24, 27). The observed restriction of light modulation of PEP carboxylase to epidermal tissue in C_3 plants and the apparent activation

[4] Abbreviation: PEP, phosphoenolpyruvate.

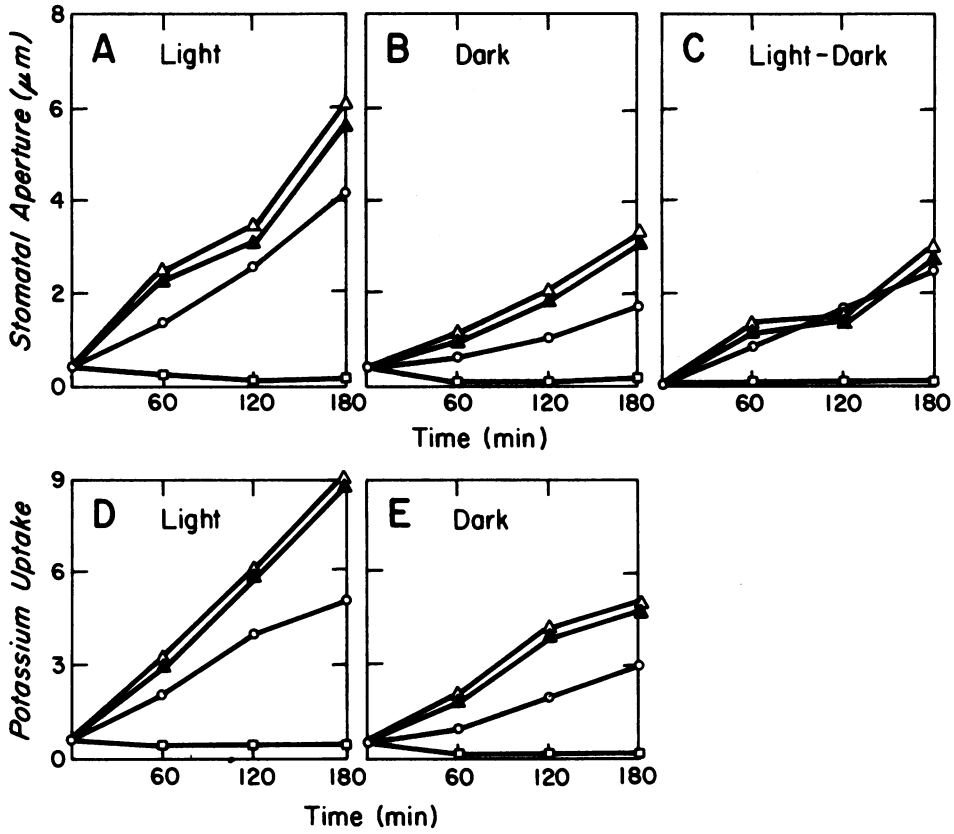

FIG. 1. Influence of sulfhydryl compounds and sulfhydryl reagents on the time course of stomatal opening and potassium accumulation in isolated epidermal strips of *P. sativum* leaves. Epidermal strips were placed in 10 ml of 10 mM Pipes-KOH buffer, 50 mM KCl, 0.1 mM $CaCl_2$ at pH 6.8 with different test compounds in light or darkness. Air was bubbled through the incubation medium at 100 cm^3 min^{-1}. After incubation at 25°C under 140 $w \cdot m^{-2}$ irradiance, the width of the stomatal aperture was measured at regular intervals. A, Light effect; B, dark effect; C, light minus dark effect (*i.e.* A minus B). D and E, Potassium content of the guard cells was visually scored on a 0 to 9 point scale (0, no accumulation; 5, good accumulation; 9, very high accumulation) after incubation of epidermis in test compounds. (○), control; (△), DTT (10 mM); (▲), GSH (10 mM); (□), *N*-ethylmaleimide (1.0 mM). Similar results were obtained in duplicate experiments.

Table I. *Effect of Light on the SH Levels in the Epidermis of P. sativum Leaves*

Epidermal strips were incubated in 10 mM Pipes-KOH, 50 mM KCl, and 0.1 mM $CaCl_2$ at pH 6.8 for 2 h in light or darkness. Normal air was passed through the incubation medium. Similar results were obtained in five additional experiments.

Exp.	SH Content		
	Dark	Light	Increase in Light
	nmol·mg⁻¹ protein		%
1	16.0	28.0	175
2	15.0	32.0	213
3	11.8	38.9	329

in mesophyll of C_4 plants is consistent with the similarities in malate metabolism in C_3 epidermis and C_4 and CAM leaves (5, 6, 13, 17, 24, 27).

Light modulation of enzyme activity involves the generation of membrane bound vicinal dithiol groups within the chloroplasts, apparently by reduction of disulfide bonds (2). The effect of light can be mimicked by DTT (2). If DTT can mimic the effect of light, we would expect a good stimulation of stomatal opening in darkness by DTT. We did observe a marked stimulation of stomatal opening by DTT and by GSH in darkness. Further, the membrane-permeable *N*-ethylmaleimide (9) inhibited stomatal opening. Stimulation of stomatal opening and the increase of PEP carboxylase activity in darkness by DTT is consistent with the effect of light being via light modulation of enzyme activity. In the case of the epidermis, GSH also seems to have a definite effect on stomatal opening and light modulation of enzymes. It is possible that there may be additional thiol involvement in stomatal movement. When we estimated the free thiols in the illuminated and darkened epidermis, the SH content of the epidermis was increased about 3-fold in light (Table I). Slepchenko (22) determined the content of sulfhydryl groups in guard cells by using various histochemical methods and speculated that labile sulfhydryl groups might be involved in stomatal function. The present investigation clearly demonstrates the involvement of free sulfhydryl groups in the mechanism of stomatal movement. Very recently, Slovacek and Vaughan (23) have shown that there is a marked increase in stromal thiol groups in irradiated intact chloroplasts.

The unexpected finding that the Calvin-Benson Cycle is absent in guard cells (13) caused researchers in stomatal metabolism to question the photochemistry of the guard cells. Guard cell chloroplasts possess both photosystems (13) and are capable of linear light-driven electron transport (28). This makes guard cell chloroplasts the only chloroplast-type known which has both PSII and PSI while lacking NADP-glyceraldehyde-P dehydrogenase. The photosystems might provide guard cells with a sensitive measure of continued presence of PAR, and this signal might be processed by maintainance of light-sensitive membrane hyperpolarization or by activation of a key enzyme (13). We present here experimental evidence for the involvement of light modulation of enzyme activity in stomatal opening.

Raschke (14) proposed that the malate level in the cytoplasm of

Table II. *Effect of Light on the Enzyme Activity Levels in Isolated Epidermal Strips and Leaf Extracts of P. sativum*

The epidermis was incubated for 2 h in light (140 $w \cdot m^{-2}$) or darkness at 25°C in 7 ml of 10 mM Pipes-KOH buffer, 50 mM KCl, and 0.1 mM $CaCl_2$ buffer at pH 6.8. Air was bubbled through the incubation medium at 100 $cm^3 \cdot min^{-1}$. Detached leaves were incubated on distilled H_2O in light (140 $w \cdot m^{-2}$) or in darkness for 60 min. After incubation, the epidermis and leaves were extracted separately in 7 ml of 50 mM Tris-HCl (pH 8.3) with 10 mM $MgCl_2$ and 1.0 mM EDTA. Similar results were obtained in each case in five additional experiments.

Enzyme	Tissue	Activity			Light Modulation	
		Initial[a]	Dark	Light	Activation	Inactivation
		μmol·mg⁻¹ protein·min⁻¹			*-fold*	
PEP carboxylase	Epidermis	0.049	0.030	0.067	2.2	
	Leaf	0.007	0.008	0.008		
NADP-malate dehydrogenase	Epidermis	0.008	0.005	0.019	3.8	
	Leaf	0.004	0.003	0.008	2.7	
NADP-isocitrate dehydrogenase	Epidermis	0.014	0.011	0.034	3.1	
	Leaf	0.037	0.035	0.043	1.2	
Aspartate aminotransferase	Epidermis	3.21	3.55	2.17		1.6
	Leaf	3.3	3.7	2.27		1.6

[a] At the start of the experiment.

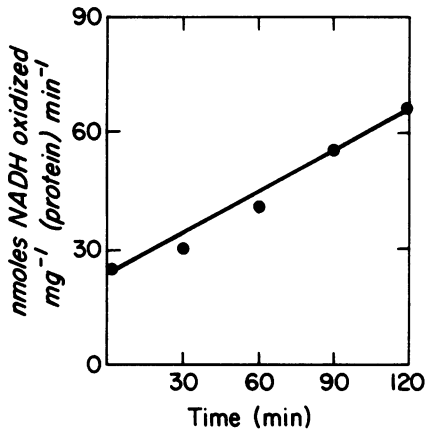

FIG. 2. Time course of light activation of PEP carboxylase of intact epidermis. Detached leaves were incubated in light (140 $w \cdot m^{-2}$) on distilled H_2O and, at regular intervals, epidermis was isolated and extracted for the enzyme assay. The light was turned off at 120 min and, after an additional 2 h, the activity was equal to the initial dark level (data not shown). Similar results were obtained in duplicate experiments.

guard cells reflects the level of CO_2 in the environment of these cells and that a high level of malate in the cytoplasm causes ion leakage from the vacuoles by reduction of the semipermeability of tonoplast and plasmalemma. The malate level in the cytoplasm reflects the balance between malate formation, malate removal into the vacuole, and deacidification. CO_2 assimilation into organic acids in epidermis was enhanced between 1.2- and 1.4-fold in starch-containing *Commelina diffusa* (26) and *Vicia faba* (16), and 2.6-fold in *Tulipa gesneriana* (15, 20). The light activation of PEP carboxylase (2.2-fold) and NADP-malate dehydrogenase (3.8-fold) observed in the present investigation provides the basis for the enhanced carbon assimilation in the light observed in the above starch-containing species. The light activation of PEP carboxylase and NADP-malate dehydrogenase results in the enhanced production of malic acid in light. The increased level of malic acid in the light might be the effect of an increased availability of reducing equivalents.

Table III. *Effects of SH Compounds and SH Reagents on Light Modulation of PEP Carboxylase in Isolated Epidermal Strips of Pea Leaves*

Epidermal strips were placed in 10 ml of 10 mM Pipes-KOH buffer, 50 mM KCl, 0.1 mM $CaCl_2$ at pH 6.8 with different test compounds in light (140 $w \cdot m^{-2}$) or darkness for 2 h. Air was bubbled through the incubation medium. Initial activity of PEP carboxylase was 49.0 $nmol \cdot mg^{-1}$ protein $\cdot min^{-1}$. The extraction of epidermis was carried out as described in Table II. Similar results were obtained in duplicate experiments.

Treatment	Activity	
	Dark	Light
	% of control	
Control	100	100
DTT, 10 mM	200	129
Glutathione, 10 mM	160	205
N-Ethylmaleimide, 10 mM	57	17

Concluding Remarks. The present investigation demonstrates that light-dark modulation of the activity of enzymes does occur in the epidermis and probably plays a significant role in stomatal movements. Further, we provide new evidence about the direct effect of light on the stomatal opening process. Our experiments and those of Sharkey and Raschke (19) and Outlaw (13) suggest that light absorbed by the guard cells itself adds to the stomatal opening stimulus. Inasmuch as photosynthetic electron transport occurs in guard cell chloroplasts (13, 28), it supplies reducing equivalents for the modulation of malate production through light activation of the key enzymes in guard cells.

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