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Light Activation of Pyruvate,Pi Dikinase and NADP-Malate Dehydrogenase in Mesophyll Protoplasts of Maize[1]

EFFECT OF DCMU, ANTIMYCIN A, CCCP, AND PHLORIZIN

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ABSTRACT

Pyruvate,Pi dikinase (PPDK, EC 2.7.9.1) and NADP-malate dehydrogenase (MDH, EC 1.1.1.82) were activated in the light and inactivated following a dark treatment in mesophyll protoplasts of maize. DCMU (up to 33 micromolar), an inhibitor of noncyclic electron transport, inhibited activation of MDH much more strongly than it did PPDK. Antimycin A (6.6-33 micromolar), an inhibitor of cyclic photophosphorylation, inhibited the activation of PPDK (up to 61%), but had little or no effect on activation of MDH. Carbonyl cyanide *m*-chlorophenylhydrazone (0.2-2 micromolar) and nigericin (0.4 micromolar), uncouplers of photophosphorylation, inhibited activation of PPDK while stimulating the activation of MDH. Phlorizin (0.33-1.7 millimolar), an inhibitor of the coupling factor for ATP synthesis, strongly inhibited activation of PPDK but only slightly effected light activation of MDH. These results suggest that noncyclic electron flow is required for activation of NADP-MDH and that photophosphorylation is required for activation of PPDK.

In C_4 plants, two enzymes of the C_4 cycle are known to be light activated: NADP-MDH[2] and PPDK (3). Although both enzymes are located in the mesophyll chloroplasts of C_4 plants, studies with the isolated enzymes have shown their mechanisms of activation/inactivation are quite different. MDH is activated via reduction of disulfide groups on the protein by thioredoxin mediated electron transfer with DTT. *In situ*, the electron donor is presumed to come from noncyclic electron flow in the chloroplast. In the dark, the enzyme may be reoxidized by donating electrons to O_2 via thioredoxin (3). Recently, the mechanism of interconversion of PPDK between the active and inactive form has been intensively studied. The activation/inactivation takes place by Pi dependent dephosphorylation and ADP dependent phosphorylation of threonine groups on the enzyme (2, 3). Previously, we showed with mesophyll chloroplast extracts from maize that the activation/inactivation of PPDK is controlled by adenylate energy charge, and further regulated by pyruvate and its analogs oxamate and oxalate (8). However, the way in which the activation/inactivation is regulated *in situ* is unknown. In the present study, we have investigated the influence of inhibitors of electron transport and phosphorylation on the light activation of PPDK and MDH in intact protoplasts of maize to determine the relative importance of noncyclic and cyclic electron flow and photophosphorylation to the activation of these enzymes *in situ*.

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[2] Abbreviations: MDH, NADP-malate dehydrogenase; PPDK, pyruvate,Pi dikinase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CF_1, ATP synthesizing component of coupling factor.

MATERIALS AND METHODS

Plant Material and Reagents. Maize (*Zea mays*, hybrid sweet corn Royal Crest 37009-12002, Sun Seeds, Inc. Bloomington, MN) was grown in a commercial premix (55% peat moss, 30% pumice, and 15% sand) in a controlled environment with a day/night temperature regime of 23/17°C with a light (800 $\mu E\ m^{-2}\ s^{-1}$)/dark period of 13/11 h. Light was provided by a combination of 400 W metal halide and sodium vapor lamps giving a photosynthetic photon flux density of approximately 800 $\mu E\ m^{-2}\ s^{-1}$. Plants were fertilized every other day with Peter's fertilizer (W. R. Grace and Co., Fogelsville, PA). The most fully expanded

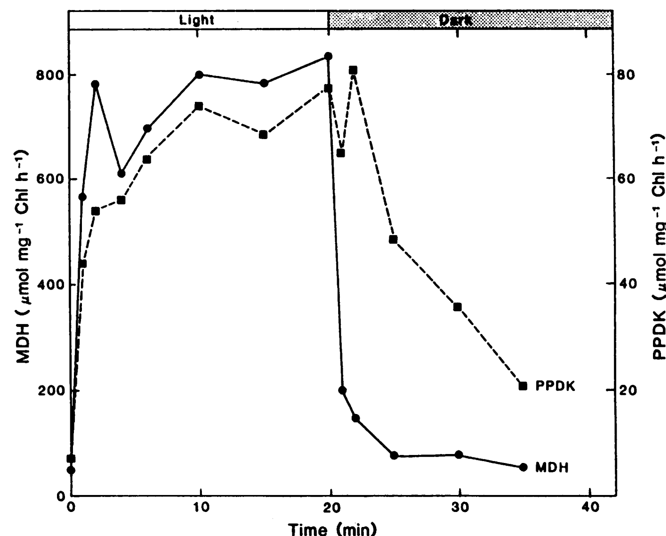

FIG. 1. Time course of the activation of MDH and PPDK in maize mesophyll protoplasts.

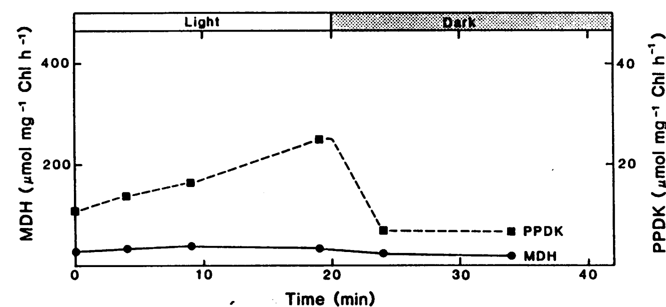

FIG. 2. Time course of the activation of MDH and PPDK in maize mesophyll protoplasts in the presence of 10 μM DCMU.

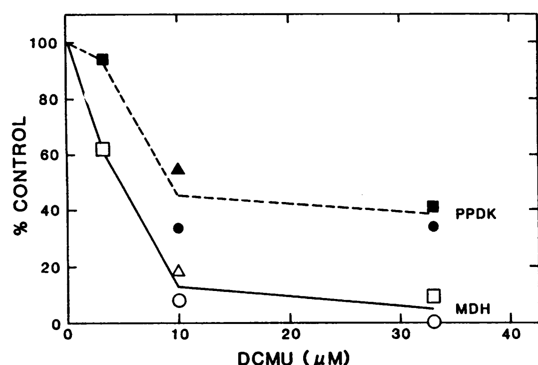

FIG. 3. Effect of various concentrations of DCMU on the light activation of MDH and PPDK in maize mesophyll protoplasts. Duplicate samples were prepared for each treatment and one illuminated and the other kept in the dark. After incubation for 10 min the light dependent activity was determined and the activity expressed as a percentage of control (without DCMU). The control activities were 31 and 475 μmol mg Chl^{-1} h^{-1} for PPDK and MDH, respectively. Open symbols indicate MDH activities and closed symbols indicate PPDK activities. The three different symbols represent data from three different experiments (○,● at 22°C, △,▲, and □,■ at 30°C).

leaves were used from plants 12 d old. Onozuka R-10 Cellulase and Macerozyme R-10 were from Yakult Pharmaceutical Industry Co., Japan. All other reagents were of analytical grade.

Protoplast Isolation. Isolation of mesophyll protoplasts was carried out according to Aoyagi and Nakamoto (1) with the following modifications in the media. The digestion medium consisted of 2% (w/v) Onozuka R-10 Cellulase, 0.2% (w/v) Macerozyme R-10, 0.2% (w/v) BSA, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1 mM $MgCl_2$, 0.5 M sorbitol, and 10 mM Mes-KOH buffer (pH 5.5). Solution A (used in protoplast purification) contained 0.6 M sucrose, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1 mM $MgCl_2$, and 10 mM Hepes-KOH (pH 7.8). Solution B (used in protoplast purification and resuspension) contained 0.6 M sorbitol, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1 mM $MgCl_2$, and 10 mM Hepes-KOH (pH 7.8). After isolation, protoplasts were kept in darkness for 1 to 1.5 h at 22°C to inactivate MDH and PPDK. The resuspension and assay medium contained 0.6 M sorbitol, 0.2 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1 mM $MgCl_2$, and 10 mM Hepes-KOH (pH 7.8). The concentrations of Chl in the assays were from 300 to 450 μg/ml. Illumination was provided by a 150 W low temperature flood lamp at 1500 μE m^{-2} s^{-1}. The assay temperature was 30°C unless otherwise mentioned.

Assay Procedures. To examine effects of various compounds, protoplasts were preincubated in each treatment in the darkness

Table I. *Effect of DCMU and Antimycin A on the Light Activation of MDH and PPDK in Maize Mesophyll Protoplasts*

Protoplasts were incubated in the light or dark at 22°C for 10 min under the treatments indicated. The percentage values indicate the light dependent activity as a percentage of the control (minus DCMU, minus antimycin A).

Treatments		NADP-Malate Dehydrogenase				Pyruvate,Pi Dikinase			
DCMU	Antimycin A	Light	Dark	Light−Dark		Light	Dark	Light−Dark	
μM		*μmol mg⁻¹ Chl h⁻¹*			%	*μmol mg⁻¹ Chl h⁻¹*			%
0	0	360	51	309	100	38.4	3.0	35.4	100
0	6.6	360	53	307	99	17.1	3.4	13.7	39
0	33	347	69	278	90	18.8	3.4	15.4	44
10	0	96	70	26	8	14.5	2.6	11.9	34
10	6.6	128	49	80	26	18.8	3.4	15.4	44
10	33	107	53	56	18	4.3	3.8	0.5	1.4

Table II. *Effect of CCCP and Phlorizin on the Light Activation of MDH and PPDK in Mesophyll Protoplasts of Maize*

Protoplasts were incubated in the light or dark for 10 min under the treatments indicated. The percentage values indicate the light dependent activity as a percentage of the control (without CCCP or phlorizin). Experiments 1 and 2 were with two separate protoplast preparations.

Treatments	NADP-Malate Dehydrogenase				Pyruvate,Pi Dikinase			
	Light	Dark	Light−Dark		Light	Dark	Light−Dark	
	μmol mg⁻¹ Chl h⁻¹			%	*μmol mg⁻¹ Chl h⁻¹*			%
Experiment 1								
Without CCCP	523	41	482	100	43.1	7.6	35.5	100
CCCP:								
0.2 μM	552	57	495	103	32.9	6.3	26.6	75
2 μM	685	48	637	132	19.0	4.4	14.6	41
Experiment 2								
Without phlorizin	576				55.5			
Phlorizin:								
0.33 mM	447				20.2			
1.67 mM	492				8.1			

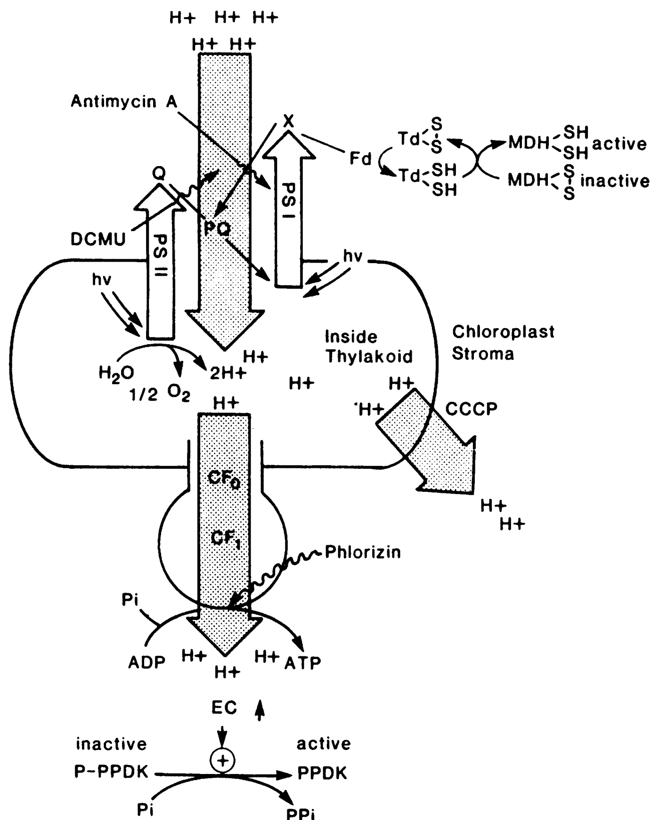

FIG. 4. A scheme illustrating the light dependent activation of NADP-MDH and PPDK and sites of action of inhibitors. As shown, MDH is activated via reduction of disulfide groups on the protein by thioredoxin mediated electron transfer through the noncyclic electron transport system which is inhibited by DCMU. Cyclic (sensitive to antimycin A) and noncyclic electron transport generate a proton gradient across the thylakoid membrane. This transthylakoid proton gradient can be dissipated by transport through the coupling factor which mediates ATP synthesis (inhibited by phlorizin) or by uncouplers like CCCP. An increase in the ATP level in the light will lead to an increase in the adenylate energy charge and activation of PPDK. Abbreviations are as follows: Q, the primary acceptor of PSII; PQ, plastoquinone; X, the primary acceptor of PSI; Td, thioredoxin; CF_0, hydrophobic membrane protein attached to CF_1; EC, adenylate energy charge; P-PPDK, inactive dikinase phosphorylated at threonine residue; PPDK, active dikinase with free threonine residue. Other abbreviations are as indicated in the text.

for 4 min (Fig. 3, Table II, experiment 1), 6 min (Fig. 2), 10 min (Fig, 3, Table I) or 15 min (Table II, experiment 2) prior to illumination. Phlorizin was dissolved in solution B (see above) and other compounds (DCMU, antimycin A, CCCP, and nigericin) were dissolved in 100% ethanol and the amount of ethanol in the assay was 0.1% (v/v) (Fig. 2, Fig. 3, and Table II, experiment 1) or 0.133% (v/v) (Fig. 3 and Table I).

For experiments shown in Figures 1 and 2, the volume of the assay was one ml and 0.4 ml, respectively; in all other experiments the volume of the assay was 60 μl. To stop the reaction, aliquots from the assay (70 μl for Fig. 1 and 50 μl for Fig. 2) or the whole assay medium (Fig. 3, Tables I and II) were immediately placed in liquid N_2. After thawing at room temperature, the enzymes (10 μl for MDH assay, 25 μl for PPDK assay) were assayed immediately as described previously (9). The reactions were started by the addition of enzymes.

RESULTS AND DISCUSSION

Time Courses of Light Activation/Dark Inactivation of MDH and PPDK in Mesophyll Protoplasts from Maize. The time courses of the light activation of MDH and PPDK were very similar with the half time for activation, less than 1 min at 30°C (Fig. 1). However, the kinetics of the dark inactivation were quite different between the two enzymes. The half-time for inactivation of MDH was less than 1 min, whereas the inactivation of PPDK was much slower, with a half-time of approximately 10 min. This pattern of activation/inactivation of the enzymes in protoplasts is similar to that obtained in maize leaves (9–11). Also, the half-times for activation of the two enzymes are comparable to those of several Calvin cycle enzymes in C_3 plants (12).

Influence of DCMU and Antimycin A on the Light Activation of MDH and PPDK. DCMU, an inhibitor of noncyclic electron flow, at a concentration of 10 μM strongly inhibited activation of MDH, while PPDK was still activated to some extent (Fig. 2). However, the rate of activation of PPDK in the presence of DCMU (Fig. 2) was much slower than in the absence of the inhibitor (Fig. 1). The influence of different concentrations of DCMU on the light activation of the two enzymes after 10 min illumination was examined (Fig. 3). At 3.3 μM DCMU, the light activation of MDH was inhibited by almost 40%, while there was little effect on the light activation of PPDK. At 10 μM DCMU, the activation of MDH was almost completely inhibited, suggesting this level of inhibitor is sufficient to prevent noncyclic electron flow. However, the activation of PPDK was only partially inhibited (by approximately 60%) at either 10 or 33 μM DCMU (Fig. 3). These results with protoplasts suggest activation of MDH is dependent on noncyclic electron flow in accordance with previous studies with isolated chloroplasts and leaves of maize (see Ref. 3) and that activation of PPDK is only partially dependent on noncyclic electron flow. Previously we found with mesophyll chloroplast extracts of maize that the activation of PPDK was controlled by the adenylate energy charge, and suggested that it may control activation of the enzyme in the chloroplast stroma *in vivo* (8). Since DCMU does not completely inhibit the activation of PPDK, it is possible that a degree of activation of the enzyme occurs as a result of cyclic photophosphorylation. In studies with the C_4 mesophyll chloroplasts of *Digitaria sanguinalis* there is evidence that a low concentration of DCMU, which will largely inhibit noncyclic electron flow, stimulates cyclic photophosphorylation (5, 6). This may have occurred in the present study with the mesophyll protoplasts of maize under 3.3 μM DCMU (Fig. 3).

To examine whether cyclic photophosphorylation may be a factor in light activation of these enzymes, the influence of antimycin A, which previously was used to inhibit cyclic photophosphorylation in intact C_4 mesophyll chloroplasts (5, 6), was examined. As shown in Table I, in the absence of DCMU there was a strong inhibition (about 60%) of the light activation of PPDK by 6.6 and 33 μM antimycin A, while there was little or no effect on the activation of MDH. Further evidence that both noncyclic and cyclic electron flow contribute to the light activation of PPDK comes from the fact that 10 μM DCMU + 33 μM antimycin A completely inhibits activation of the enzyme. The inhibition of PPDK by 10 μM DCMU and 6.6 μM antimycin A alone was not additive when both inhibitors were added together in the assay. Previously, it was found that antimycin A was less effective as an inhibitor of cyclic photophosphorylation in the presence of DCMU due to a greater sensitivity of cyclic photophosphorylation to antimycin A under reducing conditions (6). Therefore, higher concentrations (33 μM) of antimycin A are required to inhibit light activation of PPDK in the presence of DCMU than in its absence. Also, there was some stimulation of the light activation of MDH with addition of 6.6 μM antimycin

A in the presence of 10 μM DCMU. This may suggest that partial inhibition of cyclic electron flow by antimycin A causes an increase in noncyclic electron flow in the presence of DCMU. It is interesting to compare these results with a previous study in which the effect of Antimycin A on the state of light activation of fructose 1,6-bisphosphatase in intact chloroplasts of wheat (C_3 plant) was examined (7). In the presence of DCMU at a concentration which inhibited photosynthesis by 50%, there was an increase in the state of light activation of the enzyme by the addition of antimycin A. It was suggested that diversion of electrons from ferredoxin through cyclic electron flow was prevented by antimycin A. In summary, our results with antimycin A indicate that cyclic electron flow is not required for activation of MDH while it contributes to the light activation of PPDK.

Previously, we showed that in the absence of CO_2 anaerobic conditions strongly inhibited the degree of activation of PPDK in maize leaves under low or high light (9). We suggested that O_2 might be required for maximum activation of PPDK through allowing pseudocyclic photophosphorylation to occur or by providing a proper poising of the electron transport system for cyclic photophosphorylation to function. It is possible that *in vivo* pseudocyclic photophosphorylation is also involved in light activation of PPDK.

Influence of Inhibitors of Photophosphorylation on the Light Activation of MDH and PPDK. CCCP, an uncoupler of photophosphorylation which diminishes the transthylakoid proton gradient, inhibited activation of PPDK; whereas it either had no effect on, or stimulated activation of MDH (Table II). Nigericin (0.4 μM), another uncoupler, gave similar results to CCCP (not shown). These results indicate that development of a transthylakoid proton gradient with accompanying photophosphorylation, are required for activation of PPDK, but not for activation of MDH. The stimulation of activation of MDH by CCCP may occur because of increased noncyclic electron flow due to uncoupling of photophosphorylation from electron transport.

Direct evidence that photophosphorylation is required for light activation of PPDK was obtained with phlorizin, which inhibits ATP synthesis by binding to CF_1 without influencing Δ pH. Phlorizin strongly inhibited the light activation of PPDK but only slightly influenced the activation of MDH (Table II).

These results show *in situ* that MDH and PPDK are light activated by different means in C_4 plants. Noncyclic electron flow is required for activation of MDH, which is consistent with evidence that the isolated enzyme is activated by reduction of disulfide groups. Photophosphorylation is required for activation of PPDK in chloroplasts. Recent studies with isolated PPDK show it is activated by Pi dependent dephosphorylation and inactivated by ADP dependent phosphorylation (2, 3) and a high adenylate energy charge is required for activation (8). The light modulation of these two enzymes and sites of action of various inhibitors are summarized in Figure 4. It has also been suggested that pyruvate, a substrate for PPDK, might help maintain a high state of activation by inhibiting the ADP dependent inactivation (8). The uptake of pyruvate by maize mesophyll chloroplasts is light dependent and sensitive to CCCP (4). Therefore, inhibition of uptake of pyruvate in chloroplasts by CCCP might cause a further reduction in the activation of PPDK. Measurements of adenylate energy charge and pyruvate levels in mesophyll chloroplasts isolated from leaves after light/dark transitions will be required to confirm current evidence that these factors control the state of activation of PPDK *in vivo*.

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