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Pea leaf mitochondrial pyruvate dehydrogenase complex is inactivated *in vivo* in a light-dependent manner

(photorespiration/photosynthesis/phosphorylation)

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ABSTRACT **We examined the effect of light on the activity of the mitochondrial pyruvate dehydrogenase complex (mtPDC) by using intact green pea (*Pisum sativum*) seedlings. Upon illumination there is an initial drop in mtPDC activity followed by oscillations that dampen during the initial period of photosynthesis to a steady-state level of one-fourth or less of the mtPDC activity measured in the dark. The initial light-dependent decrease in mtPDC activity is inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (an inhibitor of photosystem II of photosynthesis) and does not occur in etiolated seedlings. Therefore, the effect of light is indirect and most likely associated with photosynthesis and/or photorespiration. Conditions that would be unfavorable for photorespiration also inhibited the light-dependent decrease in mtPDC activity.**

The mitochondrial pyruvate dehydrogenase complex (mtPDC), which catalyzes the oxidative decarboxylation of pyruvate to acetyl CoA, is the primary entry point of carbohydrates into the Krebs cycle. This laboratory has determined that the pea leaf mtPDC is regulated by product inhibition and reversible phosphorylation *in vitro* (1, 2). *In situ* studies with purified mitochondria from green pea seedlings have shown that the mtPDC activity is a function of the steady-state phosphorylation level of the complex (3). The *in situ* steady-state level of phosphorylation is regulated by pyruvate, ATP, and the particular substrates being oxidized by the mitochondria (3, 4).

In general, protein phosphorylation is considered to be a response to extracellular signals, a part of the signal transduction mechanisms, that regulates cellular metabolism (5). For example, phosphorylation (inactivation) of mammalian mtPDC is indirectly regulated by insulin. Diabetes causes a near complete inactivation of the enzyme that is reversed by the *in vivo* feeding of insulin (6).

The effect of extracellular signals on the steady-state mtPDC activity in plants has never been examined. Since light is a primary signal for plants and affects a number of metabolic processes in plants, we undertook a study of the influence of light on the steady-state level of mtPDC activity. Experiments on the potential role of light to alter the mtPDC activity were done *in vivo* due to the interassociating organelles involved in the process of photorespiration (7) and the source–sink interactions involved in photosynthate partitioning, which are altered when detached leaves are used (8). The results reported here indicate that in the light there is a reduction in mtPDC activity *in vivo*. The mechanism by which the steady-state activity of this mitochondrial enzyme is changed in the light is apparently through the process of photosynthetic carbon metabolism.

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MATERIALS AND METHODS

Plant Materials. Green pea (*Pisum sativum* L., cv Little Marvel) seedlings were grown in a growth chamber (10-hr photoperiod, 250 $\mu E \cdot m^{-2} \cdot s^{-1}$, 18°C) for 14–17 days before harvesting. Etiolated pea seedlings were grown in the dark (21°C) for 7–9 days.

Radiochemicals. [1-^{14}C]Pyruvate was purchased (NEN) in the solid crystalline form. Aliquots (50 μCi; 1 Ci = 37 GBq) of [^{14}C]pyruvate were dissolved in 6 ml of 20 mM sodium pyruvate containing 3 mM HCl and stored at −20°C. This acidification was necessary to stabilize pyruvate that otherwise, upon storage, forms compounds inhibitory to the pyruvate dehydrogenase complex (PDC).

Extraction and Assay. To determine the *in vivo* steady-state activity of PDC, a quick extraction technique was developed. Pea leaves [≈2 g (fresh weight)] were removed from seedlings and placed in the well of an ice-cold stainless steel cylinder with a 0.5-mm hole in the bottom covered by cheesecloth. The sap from the leaves was expressed by applying pressure with a stainless-steel piston attached to a drill press. The sap was collected in a microcentrifuge tube and an aliquot was assayed immediately for PDC activity by using a radiometric assay. The pH of the sap was 6.8 ± 0.1. A 50-μl aliquot of the expressed sap was injected into a serum-stoppered 20-ml glass scintillation vial. The vial was equipped with a hanging center well (Kontes) containing a 2.5-cm^2 piece of filter paper impregnated with 70 μl of 5 M ethanolamine to trap CO_2. The vial contained the following assay reagents: 80 mM Tes·NaOH (pH 7.6), 0.1% Triton X-100, 0.5 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 2 mM β-NAD, 0.12 mM lithium coenzyme A, 2 mM cysteine, and 1 mM sodium [1-^{14}C]-pyruvate (750–1000 dpm/nmol) in 1.0 ml [Tes = *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]. The assays were performed in a shaking water bath (30°C). Assays were initiated by the addition of the leaf sap. It took approximately 8 s to prepare the crude extract and initiate the reaction. The basis for the rapid sampling of the tissue is to minimize any changes in the phosphorylation state of mtPDC between the intact tissue state and assay state. Extended extractions usually resulted in fully inactive mtPDC. For each analysis a control assay was performed in the absence of added extract or with boiled extract. After 2 min, the reaction was stopped by the addition of 50 μl of 6 M HCl. After 20 min, the paper used to trap CO_2 was removed and assayed by liquid scintillation counting. Each data point represents the mean of duplicate assays. Each experiment was repeated at least three times.

The trapping of $^{14}CO_2$ from the enzymatic decarboxylation of pyruvate was linear with increasing amount of enzyme (up

Abbreviations: PDC, pyruvate dehydrogenase complex; mtPDC, mitochondrial PDC; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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to 0.1 ml) and with time (up to 2 min). Deviations from linearity with extended time or larger amounts of enzyme were due to product inhibition (2). When using isolated mitochondria, the activity of the mtPDC assayed by this radiometric procedure was 90–95% of the rate determined by the standard spectrophotometric assay (3). Components of the reaction medium inhibit pyruvate dehydrogenase kinase (pyruvate and sodium thiamine pyrophosphate; refs. 1 and 9) and pyruvate dehydrogenase phosphatase (Triton X-100; ref. 3) thereby effectively trapping the *in vivo* steady-state level of phosphorylation of the mtPDC (3).

Organelle Isolation. Intact mitochondria were isolated and purified using two consecutive discontinuous Percoll gradients (10). Intact chloroplasts were isolated using two consecutive low-speed centrifugations (5 min, 4°C, 3000 × *g*) and further purified on a discontinuous Percoll gradient (10 min, 4°C, 4500 × *g*).

Reduced Photorespiration. Reduced photorespiratory conditions were set up in a growth chamber with outside air circulating into the chamber. Bottled gas mixtures of 21% O_2/1% CO_2/78% N_2 or 1% CO_2/99% N_2 were used. A concentration of 1100 ppm CO_2 was maintained by adjusting the flow of bottled gas and amount of outside air circulating into the chamber. The concentration of CO_2 within the chamber was continuously measured using a gas analyzer (Licor, Lincoln, NE) and the concentration of oxygen was calculated. Plants were preequilibrated in the dark for 20–30 min under the specified atmospheric conditions before being illuminated.

RESULTS

Relative Distribution. Plant cells contain two spatially separated and distinct types of PDCs (11). The plant mtPDC, like the complex from animal mitochondria, is regulated by reversible phosphorylation (1, 3, 4, 9) and product inhibition (2). The plastid PDC is regulated by product inhibition but *not* by phosphorylation (11). To address the question as to what effect light has on the steady-state activity of the mtPDC, we determined the relative contribution of each isozyme to the maximum potential total PDC activity in crude leaf extracts. The PDC activities of isolated mitochondria and plastids were measured using conditions optimal for the mtPDC (3). The specific activities for PDC from isolated organelles were 220 and 2.5 nmol per min per mg of organellar protein for the mitochondria and plastid, respectively. Based on chlorophyll and protein measurements (14 mg of plastid protein per mg of chlorophyll and 25 mg of cellular protein per mg of chlorophyll), we calculated that PDC activity measured in our extracts would be 11% from plastid PDC and 89% from mtPDC. The contributions from the plastid PDC would be 1.4 nmol per min per mg of crude protein and this activity is subtracted from total PDCs to calculate mtPDCs. In each experiment, the total PDC activity was determined and the

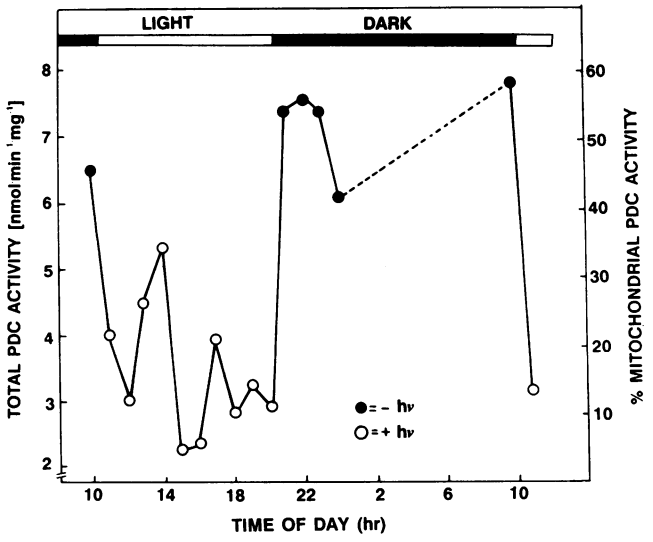

FIG. 1. Diurnal change in PDC activity. Growth conditions were 10 hr of light (250 $\mu E \cdot m^{-2} \cdot s^{-1}$, 18°C). The left axis indicates total PDC activity and the right axis indicates the percent mtPDC activity that was calculated based on a chloroplastic PDC (background) rate of 1.4 nmol per min per mg of protein. ●, Dark; ○, light.

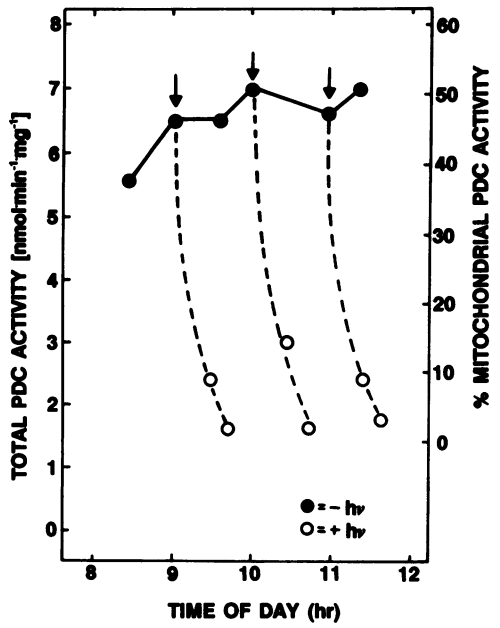

FIG. 2. Light-dependent mtPDC inactivation. Arrows indicate the time at which three pots of green pea seedlings were removed from the dark. One sample (●) was assayed immediately. The others (○) were placed in the light (250 $\mu E \cdot m^{-2} \cdot s^{-1}$, 25°C) and samples were assayed after 30 and 40 min in the light.

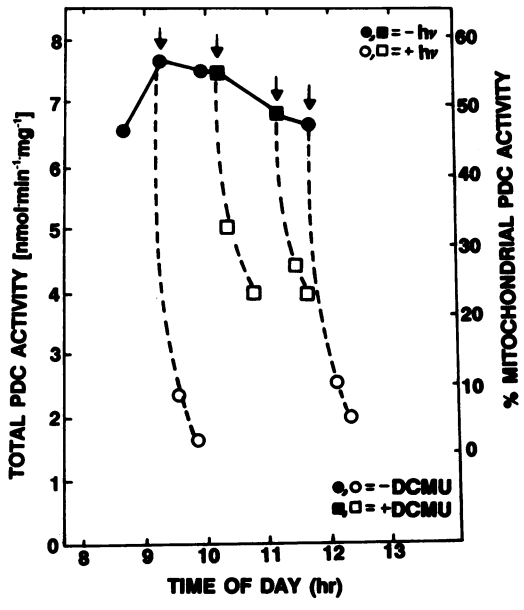

FIG. 3. Effect of DCMU on the light-dependent inactivation of mtPDC activity. Pea seedlings were watered the previous evening with 0.2 mM DCMU (□, ■) or with distilled water (○, ●) before being placed in the dark. Other experimental conditions were as in Fig. 2. Solid symbols, dark; open symbols, light.

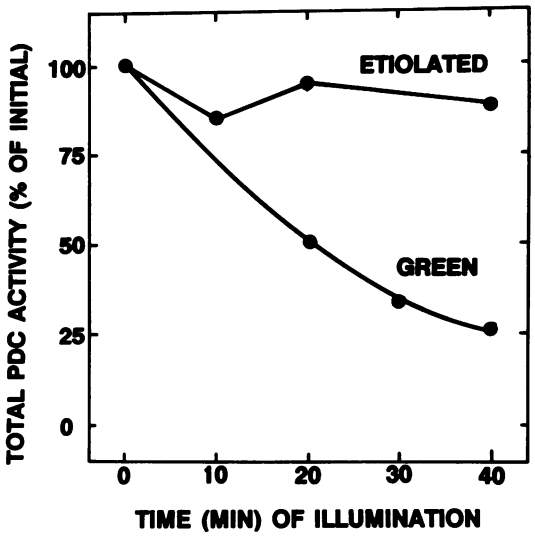

FIG. 4. Light-dependent inactivation of mtPDC activity in green versus etiolated pea seedlings. The seedlings were illuminated at 250 $\mu E \cdot m^{-2} \cdot s^{-1}$, 25°C. Initial PDC activity was 10.4 and 7.5 nmol per min per mg of protein for etiolated and green seedlings, respectively.

percent mtPDC was calculated. The results in Figs. 1–3 present the total PDC activity and the percent mtPDC activity calculated to be in the active form. Since plastid PDC is not inactivated by phosphorylation (11), fully active mtPDC was calculated to be 11 nmol per min per mg of crude protein based on a 20-fold purification of the mitochondria. In isolated pea leaf mitochondria the PDC is fully active (3).

Light-Dependent Inactivation. When the activity of PDCs from pea seedlings was monitored over a 24-hr time period, the steady-state activity of the mtPDC was 40–60% of its maximum when the plants were in the dark (Fig. 1). Upon exposure to the light, the mtPDC activity quickly dropped. During continuous illumination the activity oscillated and these oscillations dampened to a low level. If the plants were kept in the light for more than the standard 10-hr cycle, the mtPDC activity remained low (data not shown). Upon returning the plants to the dark, the mtPDC activity recovered to the level of the previous night. When the plants were illuminated the next morning, the mtPDC activity dropped once again.

Considering the oscillatory behavior of the mtPDC activity, the apparent light-dependent inactivation of the mtPDC was examined further. The decrease in the mtPDC steady-state activity upon the initial exposure to light took place regardless of the time of day at which plants, previously in the dark, were exposed to light (Fig. 2). Therefore, the decrease in mtPDC activity was indeed light-dependent.

Role of Light. To determine whether light was acting directly on the mitochondria or indirectly by way of the chloroplast and the processes of photosynthesis, the effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on the initial light-dependent inactivation of mtPDC activity was examined. DCMU is a specific inhibitor of photosystem II (Q_B-binding site) of photosynthesis (12). In contrast to control samples, plants that were watered the previous night with DCMU exhibited an inhibition of the light-dependent inactivation of the mtPDC (Fig. 3). DCMU did not affect the steady-state mtPDC activity until the plants were illuminated (compare control samples in the dark ± DCMU). In the presence of DCMU, the inactivation of the mtPDC was inhibited about 50%. The reason DCMU did not completely inhibit the light-dependent inactivation may be due to incomplete saturation by DCMU when used *in vivo*. For short-term exposure, the effect of DCMU on photosynthesis is generally much less on intact plant tissue versus leaf discs or isolated chloroplasts (13).

The light-dependent inactivation of activity was also examined with etiolated versus green pea seedlings. Etiolated seedlings lack chlorophyll, the photoreceptor of light for photosynthesis, and also the complete carbon metabolism associated with photosynthesis. Therefore, if the mechanism of the light-dependent inactivation of the mtPDC is by way of photosynthesis, etiolated seedlings should not exhibit the light-dependent inactivation. Fig. 4 shows that upon illumination, total PDC activity from control green seedlings declined by 75% in 40 min whereas there was only a 10% decline in activity from etiolated seedlings. These results, along with those using DCMU, suggest that the photosynthetic apparatus is a part of the light-dependent mtPDC inactivation and that light was acting indirectly on the mtPDC.

Role of Photorespiration. In C_3 type plants, photosynthesis is accompanied by photorespiration that is initiated by the bifunctional enzyme ribulosebisphosphate carboxylase/oxygenase using O_2 rather than CO_2. Conditions that favor photorespiration are high O_2, low CO_2, or both (14). The photorespiratory cycle involves coordinated metabolism between the plastids, peroxisomes, and mitochondria. Within the mitochondria glycine is converted to serine by glycine decarboxylase and serine hydroxymethyltransferase with a concomitant production of NADH. Much of this NADH can be utilized within the mitochondria to produce ATP (15), which in part could be used by pyruvate dehydrogenase kinase to inactivate mtPDC (3, 4). We examined the possibility that the role of light in the light-dependent inactivation of mtPDC activity may be due to mitochondrial metabolism associated with photorespiration. The initial drop in mtPDC activity upon illumination was examined under conditions that should reduce photorespiration (i.e., increased CO_2 concentration, reduced O_2 concentration, or both). In comparison to control samples, increasing the concentration of atmospheric CO_2 or increasing the concentration of CO_2 and lowering the concentration of O_2 inhibited the initial light-dependent drop in mtPDC activity (Table 1). These data support the hypothesis that photorespiration could be at least partially responsible for the light-dependent inactivation of the mtPDC. The inhibition of PDC inactivation was not large (23% with 1100 ppm CO_2 and 44% with decreased O_2 and

Table 1. Effect of reduced photorespiration on the light-dependent inactivation of mtPDC activity *in vivo*

Illumination, min	Control (21% O_2/340 ppm CO_2)		Reduced photorespiration			
			21% O_2/1100 ppm CO_2		≈7% O_2/1100 ppm CO_2	
	Total activity	% initial mtPDC	Total activity	% initial mtPDC	Total activity	% initial mtPDC
0	5.8 ± 0.3	100	5.7 ± 0.5	100	6.7 ± 1.2	100
30	3.4 ± 0.4	45	3.8 ± 0.7	56	4.5 ± 0.7	58
40	2.1 ± 0.2	16	2.9 ± 0.2	35	4.2 ± 0.2	53

Pea seedlings were illuminated (250 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 18°C. Plants were preequilibrated in the dark for 20 min under the specified atmospheric conditions. Values for total activity are the mean ± SD (n = 4) and represent total PDC activity (mtPDC + chloroplast PDC) in nmol per min per mg of protein. Percentage of initial mtPDC was calculated for that portion of the total PDC activity that is due to mtPDC.

increased CO_2) and may reflect the fact that photorespiration is not completely inhibited under these conditions (16) and that regulation of mtPDC is by multiple processes.

DISCUSSION

Despite the spatial separation of the mitochondria from the primary events of photosynthesis taking place within the chloroplast, the mtPDC responds to the stimulus of light and the processes of photosynthesis. The mechanism of this light-dependent inactivation appears to be indirect.

Previous studies have indicated that pyruvate is a competitive inhibitor of mtPDC phosphorylation (1). Phosphorylation results in inactivation of the complex (1, 3, 4, 9). When isolated mitochondria are generating ATP from the oxidation of substrates (other than pyruvate), the mtPDC is inactivated by phosphorylation and reactivates upon the addition of pyruvate (4). Pyruvate dehydrogenase kinase is stimulated by ammonium ions (9), which are also produced in the photorespiratory glycine decarboxylase reaction (7, 17). During photosynthesis, the concentration of pyruvate decreases (18), the mitochondrial ATP/ADP ratio increases (15, 19), and the concentration of mitochondrial ammonium increases due to photorespiration (7, 17). The changes in pyruvate, ATP, and ammonium reported (1, 3, 4, 9) to occur during photosynthesis and their ability to regulate the mtPDC phosphorylation state allow us to suggest that they may be the means by which light causes an inactivation of the mtPDC.

Another potential mediator of the light-dependent inactivation may be the concentration of cytosolic phosphate. Most of the pyruvate used by the mtPDC is thought to be derived from malate (20). Malate is transported into the mitochondria in exchange for P_i with mitochondria taking up cytosolic phosphate in exchange for the hydroxide ion (21). It has been suggested that the slow diffusion of vacuolar phosphate into the cytosol would result in oscillatory behavior with respect to photosynthesis (22). The mtPDC activity also oscillates early in the period of illumination and these oscillations dampen similarly to those of photosynthesis but over a longer time period. We do not know the cause of these oscillations in PDC activity. The loss of these oscillations indicates that there may be more than one signal regulating mtPDC activity and these signals are not synchronized.

There has been a long-standing controversy in plant sciences as to whether or not the Krebs cycle, mitochondrial respiration, or both are inhibited during periods of photosynthesis (23). Krömer *et al.* (24) reported that photosynthesis was inhibited in the presence of inhibitors of mitochondrial oxidative phosphorylation. This would certainly indicate that mitochondrial electron transport for ATP formation is necessary during photosynthesis. However, this study did not determine the source of the electrons (e.g., Krebs cycle activity versus glycine decarboxylase), supporting this mitochondrial ATP formation.

Inhibition of mtPDC during photosynthesis could limit carbon flow into the Krebs cycle and consequently the Krebs cycle as a source of electrons to drive ATP formation. The drop in mtPDC activity we have seen (Fig. 1 and 2) during the illumination of the seedlings should not be construed to mean that mitochondrial respiration (i.e., oxidative phosphorylation) was inhibited during photosynthesis. Most of the ATP produced in the mitochondria during steady-state photosynthesis is probably a result of photorespiration—i.e., the conversion of glycine to serine with concomitant NADH production (15).

Furthermore, there can also be partial Krebs cycle activity in which Krebs cycle intermediates are utilized instead of pyruvate. Some studies on the effect of light on Krebs cycle activity have used [^{14}C]pyruvate (23). Considering the results reported here (light-dependent inactivation) and the role of pyruvate in regulation mtPDC activity (1, 3, 4, 9), the use of exogenous pyruvate (and potentially other metabolites) should be avoided. We feel that our results support the hypothesis that carbon flow into the Krebs cycle from pyruvate could be curtailed during photosynthesis without concomitant loss of oxidative phosphorylation.

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