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Light Activation of Maize Phosphoenolpyruvate Carboxylase Protein-Serine Kinase Activity Is Inhibited by Mesophyll and Bundle Sheath-Directed Photosynthesis Inhibitors

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ABSTRACT

C₄ phosphoenolpyruvate carboxylase (PEPC) is post-translationally regulated by reversible phosphorylation of a specific N-terminal seryl residue in response to light/dark transitions of the parent leaf tissue. The protein-serine kinase (PEPC-PK) that phosphorylates/activates this mesophyll-cytoplasm target enzyme is slowly, but strikingly, activated by high light and inactivated in darkness \textit{in vivo} by a mechanism involving cytoplasmic protein synthesis/degradation as a primary component. In this report, evidence is presented indicating that the inhibition of Calvin cycle activity by a variety of mesophyll (3-(3,4-dichlorophenyl)-1,1-dimethylurea, isocil, methyl viologen) and bundle sheath (α-glyceraldehyde)-directed photosynthesis inhibitors blocks the light activation of maize (\textit{Zea mays L.}) PEPC-PK and the ensuing regulatory phosphorylation of its target enzyme \textit{in vivo}. Based on these and related observations, we propose that the Calvin cycle supplies the C₄ mesophyll cell with \textit{a} signal that interacts with the cytoplasmic protein synthesis event to effect the light activation of PEPC-PK and the concomitant phosphorylation of PEPC, and \textit{b} high levels of known positive effectors (e.g. triose-phosphate, glucose-6-phosphate) that interact directly with the carboxylase. The combined result of this complex regulatory cascade is to effectively desensitize PEPC to feedback inhibition by the millimolar levels of L-malate required for rapid diffusive transport to the bundle sheath during high rates of C₄ photosynthesis.

PEPC\textsuperscript{1} is one of the key target enzymes involved in the light/dark regulation of C₄ photosynthesis and CAM (7, 13, 18). Both \textit{in vitro} (11, 12, 20) and \textit{in vivo} (10, 15, 21) studies have established that the light-induced phosphorylation of C₄ PEPC at a specific N-terminal seryl residue (Ser-15 or Ser-8 in the maize or sorghum enzymes, respectively) results in an increase in catalytic activity, and, more notably, a decrease in malate inhibition of this cytoplasmic MC enzyme. The partially purified maize leaf PEPC-PK that phosphorylates/activates this target enzyme is not affected by a number of putative, light-modulated cytoplasmic effectors, including Ca\textsuperscript{2+}/calmodulin, fructose 2,6-P\textsubscript{2}, PPI, and thioredoxin h (3, 11, 20). However, this kinase activity is reversibly light activated \textit{in vivo} by a mechanism involving cytoplasmic protein synthesis and degradation as primary components (5, 14, 20).

Although it is not known at present how the light signal is transduced during the activation of PEPC-PK and the resulting regulatory phosphorylation of C₄ PEPC, \textit{in vivo} studies with several C₄ species have shown that inhibitors of noncyclic electron flow from H\textsubscript{2}O to NAD\textsubscript{P} (e.g. DCMU, MV) and of the Calvin cycle (DL-glyceraldehyde [24]) are equally effective in blocking the light activation of PEPC in the MC cytoplasm (16, 20, 22). These findings indicate that the light receptor and at least part of the signal transduction pathway are associated with chloroplasts and the photosynthetic apparatus. Because the type 2A protein phosphatase activity that dephosphorylates PEPC (2, 20) does not appear to be significantly affected by light/dark transitions of maize leaf tissue (14, 20), we have continued to focus our attention on the reversibly light-activated PEPC-PK.

In the present study, we pretreated detached maize leaves with a variety of photosynthesis inhibitors and measured both the \textit{in vivo} and \textit{in vitro} PEPC-PK activity in the treated dark- and light-adapted leaf tissue. The results show that PSI\textsubscript{II} (DCMU, isocil) and PSI (MV)-directed electron transport inhibitors and DL-glyceraldehyde block both the light activation of PEPC-PK and the ensuing regulatory phosphorylation of PEPC. Based on these findings and related observations, we propose that Calvin cycle activity and a concerted functioning of the MC and BSC are necessary for the \textit{in vivo} activation of the protein-serine kinase in the maize MC cytoplasm.

MATERIALS AND METHODS

Materials

Maize (\textit{Zea mays L.}, cv Golden Cross Bantam) plants were grown as described (10, 11). [γ-\textsuperscript{32P}]ATP (3 Ci/mmol) was purchased from Amersham, and microcystin-LR from Calbiochem. Recrystallized DCMU was from DuPont and isocil was obtained from the Weed Science Laboratory at the Uni-

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\textsuperscript{3} Abbreviations: PEPC, phosphoenolpyruvate carboxylase; PEPC-PK, phosphoenolpyruvate carboxylase protein-serine kinase; MV, methyl viologen; isocil, 5-bromo-3-isopropyl-6-methyluracil; BSC, bundle-sheath cell; MC, mesophyll cell; IC\textsubscript{50}, inhibitor concentration required for 50% inhibition of PEPC activity.
versity of Nebraska-Lincoln. All other biochemical reagents were obtained from Sigma. Dark-form maize PEPC was purified according to standard procedures (11, 15).

**Feeding of Photosynthetic Inhibitors to Leaf Tissue**

Preilluminated (about 12 h in growth chamber with a light intensity of about 300–500 μmol·m$^{-2}$·s$^{-1}$; see ref. 10) leaves from 3- to 4-week-old maize plants were excised under water at the leaf base and the distal part of the leaf was removed and discarded. Each detached leaf section (about 25 cm long) was then immersed in a 100-mL graduated cylinder that contained 100 mL of either 0.6% (v/v) ethanol (solvent control), 0.2 mM DCMU, 0.2 mM isocil, or 0.2 mM MV (all in solvent). Following 90 min of incubation in the dark at room temperature, each leaf section and its corresponding solution were transferred to a 125-mL flask. In the case of δ1-glyceraldehyde, detached leaves were inserted directly into 125-mL flasks containing 100 mL of water (control) or 0.1 mM δ1-glyceraldehyde. The flasks were then placed in a darkened fume hood overnight. On the following morning, one-half of the treated leaf tissue was kept in the dark for 90 min while the other half was illuminated at a PPFD of 600 to 800 μmol·m$^{-2}$·s$^{-1}$ for 90 min (see ref. 14 for details). Leaf extracts were then prepared as described below.

**Preparation of Leaf Extracts**

Darkened or illuminated leaf tissue (0.2 g fresh weight) was chopped and ground at 4°C in a prechilled mortar with 1 mL of 0.1 m Tris-HCl, pH 8.0, 20% (v/v) glycerol, 10 mM MgCl$_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, 0.2% (w/v) insoluble PVP, and some washed sand. The crude homogenate was centrifuged immediately at 8700g at room temperature for 90 s. A 0.4-mL aliquot of the supernatant fluid was rapidly desalted at about 4°C on a Sephadex G-25 column (1 × 5 cm) equilibrated with 0.1 m Tris-HCl, pH 7.5, 20% (v/v) glycerol, 10 mM MgCl$_2$, and 0.5 mL of the green protein eluent was collected in a graduated 1.5-mL microfuge tube. After this desalting step, both PEPC and PEPC-PK activities were measured immediately.

**Assays**

Assay of PEPC activity was performed at 30°C in a 1-mL reaction mixture that contained 50 mM Hepes-KOH, pH 7.3, 2.5 mM PEP, 5 mM MgCl$_2$, 1 mM NaHCO$_3$, 0.2 mM NADH, 10 units of NADH-malate dehydrogenase, and various concentrations of pH-adjusted l-malate (10, 14). The reaction was initiated by addition of 10 to 20 μL of desalted leaf extract. The malate concentration required for 50% inhibition of PEPC activity under the above assay conditions was determined graphically and defined as the malate IC$_{50}$ value.

PEPC-PK activity was measured by $^{32}$P-incorporation from [γ-$^{32}$P]ATP into purified dark-form maize leaf PEPC in the presence of an ADP-scavenging system (5, 14) and microcystin-LR, a potent and specific inhibitor of type 1 and 2A protein phosphatases (8, 19). If necessary, the soluble protein content was equalized in all desalted samples for each set of experiments. For each assay, 60 μL of desalted leaf extract was incubated with about 20 μg of purified PEPC that had been desalted using a Centricon 30 microconcentrator (Amicon) into 0.1 m Tris-HCl, pH 7.5/20% (v/v) glycerol/10 mM MgCl$_2$, and 0.25 mM PPI(di(adenosine-5')-pentaphosphate, 4 mM phosphocreatine, 10 units of creatine phosphokinase, 10 mM microcystin-LR (in 20% methanol), 28 μM [γ-$^{32}$P]ATP (about 10 μCi), all in a final volume of 120 μL. After 45 min of incubation at 30°C, the phosphorylation reaction was stopped by addition of 40 μL SDS sample buffer (0.25 m Tris-HCl, pH 6.8, 8% [w/v] SDS, 40% [w/v] glycerol, 20% [w/v] 2-mercaptoethanol, 0.04% [w/v] bromphenol blue), followed by immediate boiling for 2 min. Aliquots (40–100 μL) of each denatured sample were analyzed by SDS-PAGE and autoradiography. The relative intensity of the $^{32}$P-signal in the 110-kD PEPC-subunit band was taken as the in vitro PEPC-PK activity (5, 14, 20).

Protein concentrations were determined by a sensitive dye-binding method (1) using crystalline BSA as the standard.

**SDS-PAGE and Autoradiography**

Vertical SDS-PAGE was performed as described (5, 17). After staining with Coomassie brilliant blue R-250 and thorough destaining for 60 to 70 h, autoradiographs were prepared from the dried gels with Kodak X-Omat AR film and two Lightning Plus intensifying screens (DuPont) at −80°C for 12 to 24 h.

**RESULTS**

**Effects of Photosynthetic Inhibitors on the Light-Induced Decrease in Malate Sensitivity of Maize Leaf PEPC**

It is well established that PEPC extracted from illuminated maize leaves has a two- to threefold lower malate sensitivity (i.e., a greater malate IC$_{50}$ value) than that from the corresponding dark-adapted tissue (4, 9, 14, 15, 20, 21). Both in vivo (11, 12, 20) and in vitro (10, 15, 21) studies indicate that this light-induced change in the regulatory properties of C$_4$ PEPC is due to posttranslational modification of the enzyme, i.e., phosphorylation of a specific N-terminal seryl residue (see ref. 13 for a recent review). Based on the related in vivo observations that the light activation of PEPC in several C$_4$ species is sensitive to both photosynthetic electron transport (16, 20, 22) and Calvin cycle inhibitors (22), we investigated the effects of such inhibitors in vivo on the apparent phosphorylation status (i.e. malate sensitivity) of maize leaf PEPC during dark to light transitions. Consistent with previous observations on the light activation of C$_4$ PEPC in Setaria verticillata (22), we found that the light-induced increase in the malate IC$_{50}$ value of the maize enzyme was prevented in the detached leaves that were pretreated with either PSII (DCMU, isocil), PSI (MV), or Calvin cycle (δ1-glyceraldehyde) inhibitors (Table I). As expected, none of these photosynthetic inhibitors affected the malate IC$_{50}$ values of PEPC from the pretreated darkened samples (Table I).

**Effects of Photosynthetic Inhibitors on the Light Activation of Maize PEPC-PK Activity**

The degree of malate sensitivity of C$_4$ PEPC is correlated inversely with the phosphorylation status of the enzyme (10-
of PEPC-PK activity varied to some extent from experiment to experiment, presumably due to inhibitor-uptake phenomena, the illuminated leaf tissue that had been fed photosynthesis inhibitors always displayed a direct correlation between the malate IC₅₀ values of the endogenous PEPC (Table I) and the PEPC-PK activity measured in vitro (Figs. 1, 2). In other words, low IC₅₀ values were correlated with a relatively low PEPC-PK activity, and vice versa.

The simultaneous addition of DCMU and MV (0.1 mM, each) to the in vitro phosphorylation assay mixture containing the illuminated control leaf extract had no effect on PEPC-PK activity (data not shown). This observation precludes the remote possibility that these inhibitors interact directly with PEPC or its protein-serine kinase.

**DISCUSSION**

During C₄ photosynthesis in NADP-malic enzyme-type species such as maize and sorghum, the Calvin cycle in the BSC and the intercellular C₄ cycle in the MC-BSC are highly interdependent. Whereas the latter provides NADPH and substrate CO₂ to the "agranal," PSII-deficient bundle-sheath chloroplasts by the sequential action of stromal NADPH-malate dehydrogenase (MC) and NADP-malic enzyme (BSC), the BSC chloroplasts are the source of pyruvate and 3-P-glycerate for the granal, PS II-competent mesophyll chloroplasts (7, 18, 23) (Fig. 3). This intimate functional interaction between these two metabolic pathways is enabled by rapid diffusive metabolite transport between the two photosynthetic cell types (7, 18, 23). Therefore, it is evident that for C₄ photosynthesis to proceed most effectively (e.g. at high light intensity), the relative rates of the Calvin cycle and intercellular C₄ cycle must be highly coordinated (3, 7, 18).

The photosynthesis-inhibitor results presented in this report indicate that the direct (DL-glyceraldehyde) or indirect (DCMU, isocit, MV) inhibition of Calvin cycle activity in the maize BSC chloroplast blocks the light-induced regulatory phosphorylation of PEPC in the mesophyll cytoplasm (see Table I and refs. 16, 20, 22). More notably, this apparent inhibition of PEPC phosphorylation is the result of a marked inhibition of the light activation of PEPC-PK activity (Figs. 1, 2). We have recently reported that the reversible light activation of this protein-serine kinase (5) involves cytoplasmic protein synthesis and degradation as primary com-

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**Table I. Effects of Feeding PSII, PSI, and Calvin Cycle-Directed Inhibitors to Detached Maize Leaves on the Light-Induced Increase in the Malate IC₅₀ Value of Desalted PEPC**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Malate IC₅₀</th>
<th>Light/Dark</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0.12</td>
<td>0.39</td>
<td>3.3</td>
</tr>
<tr>
<td>DCMU (0.2 mm)</td>
<td>0.12</td>
<td>0.16</td>
<td>1.3</td>
</tr>
<tr>
<td>Isocit (0.2 mm)</td>
<td>0.13</td>
<td>0.12</td>
<td>0.9</td>
</tr>
<tr>
<td>MV (0.2 mm)</td>
<td>0.11</td>
<td>0.12</td>
<td>1.1</td>
</tr>
<tr>
<td>DL-Glyceraldehyde (0.1 m)</td>
<td>0.12</td>
<td>0.13</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*cf. Fig. 1.  *cf. Fig. 2.

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**Figure 1.** Inhibition of PEPC-PK light activation by feeding PSII and PSI-directed inhibitors of noncyclic electron transport to detached maize leaf tissue. D and L indicate the samples from dark- and light-adapted (600–800 μmol·m⁻²·s⁻¹) control leaf tissue, respectively; and Dₜ and Lₜ represent samples from dark- and light-adapted leaf tissue that had been treated with (A) 0.2 mM DCMU and (B) 0.2 mM MV (cf. Table I). The arrow in panel a indicates the position of the 110-kD PEPC subunit. a, SDS-PAGE gel stained with Coomassie blue; b, corresponding autoradiograph.

**Figure 2.** Inhibition of PEPC-PK light activation by feeding DL-glyceraldehyde (0.1 mM), a Calvin cycle inhibitor (24), to detached maize leaf tissue (cf. Table I). See Figure 1 for details.
components (14). Although the nature of the newly synthesized, light-induced protein(s) is not known at present (14), it is clear from the present study that some photosynthesis-related signal emanating from the Calvin cycle likely interacts in some fashion with this protein synthesis event to ultimately effect the light activation of PEPC-PK and its target enzyme in the MC cytoplasm.

Based on the in vivo photosynthesis-inhibitor data presented in this and related (16, 20, 22) reports, and on our repeated, unsuccessful attempts at detecting light activation of PEPC in situ in isolated maize mesophyll protoplasts (3), a working model of the complex signal transduction pathway associated with the light-induced activation of PEPC-PK activity and the phosphorylation of maize PEPC is proposed (Fig. 3). The light signal is perceived by Chl (20) and ultimately leads to the formation of NADPH (and ATP) during noncyclic electron flow from H₂O to NADP. Given that the latter activity is largely, if not entirely, restricted to the granal mesophyll chloroplasts in maize (7), the primary intercellular site of interaction of whole-chain electron transport inhibitors (e.g. DCMU, isocit, MV) would likely be the MC. The NADPH produced in the mesophyll chloroplast in the absence of inhibitor would indirectly serve the reductive needs of the Calvin cycle in the BSC by means of two distinct intercellular metabolite transport cycles, the malate/pyruvate and 3-P-glycerate/triose-P shuttles (7, 18, 23) (Fig. 3). A putative signal derived from Calvin cycle metabolism (e.g. phosphorylated metabolite, amino acid) is then transported to the MC, where it directly or indirectly interacts with the cytoplasmic protein synthesis event (14) that ultimately leads to the activation of PEPC-PK activity and, subsequently, the regulatory phosphorylation of maize PEPC at Ser-15. Thus, any chemical (e.g. the MC- or BSC-directed photosynthesis inhibitors used in the present study) or physical (e.g. darkness, isolation of mesophyll protoplasts [3]) treatment that ultimately inhibits Calvin cycle activity in the BSC and the functional "dialogue" between the two photosynthetic cell types in maize would disrupt this complex intercellular signal transduction pathway and prevent the light activation of PEPC-PK and its target enzyme in the MC cytoplasm.

In the context of this working model, it is important to note that the light-induced regulatory phosphorylation of C₄ PEPC is not viewed as an "off-on" switch per se for this cytoplasmic target enzyme. Indeed, the dephosphorylated dark-form enzyme displays about 50% of the in vitro catalytic activity of fully light-activated PEPC (4, 9, 10, 16, 22). Rather, this sluggish (about 60 min), high light-requiring (>300 μmol·m⁻²·s⁻¹) activation of PEPC-PK and its target enzyme (5, 6, 10, 20–22) are viewed as a primary means by which PEPC can continue to carboxylate PEP at high rates in the presence of millimolar levels of L-malate. Because this C₄ acid is present in the MC cytoplasm of maize at concentrations in excess of 15 to 20 mM when rates of photosynthesis are high in order to enable its rapid diffusive transport to the BSC (7, 18, 23), the regulatory phosphorylation of PEPC and the concomitant decrease in its malate sensitivity are considered critical for the
enzyme’s continued functioning in this increasingly “hostile” environment. In this regard, it is noteworthy that the estimated malate concentration in the maize MC at high light intensity (18, 23) is manifold greater than the malate IC_{50} values reported in this study (Table 1) and elsewhere (9, 10, 14, 20, 21) for the light-activated/phosphorylated enzyme. However, if maize PEPC activity is measured in vitro in the presence of 15 to 20% glycerol, a condition that likely mimics the concentrated nature of the MC cytoplasm in vivo, the malate IC_{50} values increase to about 1.9 mM for purified dark-form PEPC and to about 10 mM for the in vitro phosphorylated enzyme (J-A Jiao, unpublished data). Moreover, physiological concentrations of certain phosphorylated metabolites (e.g. triose-P, glucose 6-P) are known to relieve the in vitro inhibition of light-form maize PEPC by L-malate (4). Thus, it is believed that the high light-induced activation of PEPC-PK and the ensuing regulatory phosphorylation of its target enzyme, together with these light-modulated positive effectors (7, 18, 23), act in concert to effectively desensitize PEPC to inhibition by the millimolar levels of L-malate required for rapid diffuse intercellular transport during C_{4} photosynthesis at high light intensity (Fig. 3). We propose that the Calvin cycle is the common link between these two interactive modes of light regulation of PEPC activity in the C_{4} mesophyll cytoplasm. This metabolic pathway not only supplies the MC with a putative signal that interacts with the protein synthesis event to effect the light activation of PEPC-PK and, thus, its target enzyme (see above), but also with millimolar levels of these phosphorylated positive effectors (7, 18, 23) that interact directly with light-activated PEPC to further increase its malate IC_{50} value (Fig. 3).

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LITERATURE CITED