Reversible light activation of the phospho*enol*pyruvate carboxylase protein-serine kinase in maize leaves

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C_4-leaf phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) undergoes reversible, light-induced increases in its activity–seryl phosphorylation-status in vivo. We now report that the PEPC-protein kinase activity in desalted crude extracts of light-adapted maize leaves is several-fold greater than that from the corresponding dark tissue when in vitro phosphorylation assays are performed with either endogenous or purified dark-form maize PEPC as substrate, both in the absence or presence of okadaic acid, a potent inhibitor of the PEPC type 2A protein phosphatase(s). These and related results indicate that the PEPC protein-serine kinase(s) per se is reversibly light activated in vivo by either covalent modification, protein turnover or, less likely, a tight-binding effector.

Phospho*enol*pyruvate carboxylase; Protein-serine kinase; Regulatory protein phosphorylation; C_4 photosynthesis; Light–dark regulation; Maize (*Zea mays* L.)

1. INTRODUCTION

Control of C_4 photosynthesis is, in part, mediated by the light–dark regulation of phospho*enol*pyruvate (PEP) carboxylase (PEPC; EC 4.1.1.31) activity [1–3]. The nature of this regulation of PEPC in the C_4 mesophyll-cell cytoplasm is posttranslational [2,3]. Specifically, the activity and regulatory properties of this initial carboxylating enzyme vary in accord with the physiological activity of C_4 photosynthesis: PEPC is more active and less sensitive to feedback inhibition by L-malate in the light than in darkness. A wealth of in vivo and in vitro data has recently accumulated in support of the view that the reversible phosphorylation of a specific, N-terminal regulatory serine residue in PEPC (e.g. Ser-15 in the maize enzyme [4] is the unifying molecular mechanism that mediates the posttranslational regulation of the carboxylase by light–dark and night–day transitions in C_4 and Crassulacean acid metabolism plants, respectively (reviewed in [2,3]; [5]).

Light activation of PEPC in C_4 plants is related, either directly or indirectly, to photosynthetic electron transport and/or photophosphorylation, and is modulated by several photosynthesis-related environmental variables, including light intensity, CO_2 concentration and temperature [2,3,6–8]. It is notable that when compared to the activation of photoregulated C_4 mesophyll-chloroplast stromal enzymes (e.g. pyruvate, P_i dikinase (PPDK), NADPH-malate dehydrogenase [1,2]), light activation of this cytoplasmic enzyme is relatively slow, taking as long as 60 min for completion [6,7,9].

In order to account for the reversible, light-induced increases in activity–phosphorylation-status of C_4 PEP carboxylase, regulation of the PEPC protein-serine kinase(s) [10,11] and/or type 2A protein phosphatase(s) [5] must be inferred. While no information is available concerning the regulatory properties of this okadaic acid-sensitive protein phosphatase [5], the partially purified protein kinase (PK) is not activated by a variety of putative, light-modulated cytoplasmic effectors [2,11]. In contrast to these negative findings, we now report that the PEPC-PK activity in desalted crude extracts of light-adapted maize leaves is several-fold greater than that from the corresponding dark tissue when in vitro phosphorylation assays are performed with either endogenous or purified dark-form maize PEPC as substrate, both in the absence and presence of okadaic acid. These and related observations indicate that the activity of the protein-serine kinase(s) per se is light–dark modulated in vivo by some mechanism and imply that the light signal and ensuing activation–seryl-phosphorylation of PEPC in the C_4 mesophyll-cytoplasm involves a bicyclic regulatory cascade.

2. MATERIALS AND METHODS

2.1. *Materials*

PEP (mono(cyclohexylammonium) salt), disodium ATP, L-malate, P^1P^5-di(adenosine-5')-pentaphosphate (AP_5A), phospho-

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creatine, creatine phosphokinase, NADH and pig heart malate dehydrogenase were obtained from Sigma. Rabbit antiserum, raised against the PEPC holoenzyme purified from green *Sorghum* leaves, was obtained as described [12]. Okadaic acid ([5,13]; in DMSO) was the generous gift of Dr Steven C. Huber (North Carolina State University). Maize (*Zea mays* L., cv. Golden Cross Bantam) plants were grown from seed in an illuminated growth room (14-h photoperiod, 27°C day/24°C night) at about 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ (400-700 nm) at the plant top.

2.2. *Preparation of leaf extracts*

For most experiments uppermost leaf tissue (0.3 g fresh wt from 4–6-week-old plants collected, unless noted otherwise, after 4–6 h in the dark or light) was chopped and ground thoroughly at 4°C in a chilled mortar with washed sand, 2% (w/v) insoluble PVP, and 1.5 ml of buffer A (0.1 M Tris-HCl, pH 7.5, 20% (v/v) glycerol, 1 mM EDTA, 10 mM $MgCl_2$ and 14 mM 2-mercaptoethanol). The homogenate was filtered through an 80-μm nylon net, and a 0.2-ml aliquot of the filtrate was rapidly desalted on a Sephadex G-25 column (1 × 5 cm) equilibrated with buffer B (buffer A lacking -SH reagent).

For rapid determination of malate inhibition of PEPC activity, leaf tissue (80 mg fresh wt) was chopped, ground in a chilled mortar with washed sand and 0.5 ml of buffer B, centrifuged for 1.5 min a 12 000 × g, and a 10-μl aliquot of the crude supernatant fluid used immediately for PEPC assays at pH 7.3 and 30°C [9], ± 1 mM L-malate.

2.3. *Phosphorylation assays*

For most experiments an aliquot of the desalted soluble leaf protein sample (35 μl) was incubated at 30°C with about 0.2 U (7 μg) of purified dark-form maize PEPC ([11]; dialyzed against buffer B), 0.25 mM AP_5A (to inhibit endogenous adenylate kinase activity [10]), a phosphocreatine (4 mM)-creatine phosphokinase (10 U) ADP-scavenging system [10], 25 μM ATP and 3 μCi [γ-^{32}P]ATP (Amersham) in a final volume of 60 μl. After 45 min the assays were stopped by addition of 15 μl of buffer C (50 mM Tris-HCl, pH 8.8, 50% glycerol, 5% SDS) plus 10% 2-mercaptoethanol, the samples were boiled for 2 min at 90°C, and subjected to denaturing PAGE [14] and autoradiography [10].

2.4. *Immunoprecipitation of PEPC*

After the 45-min phosphorylation period some samples were incubated for 12 h at 4°C with rabbit anti-PEPC antiserum, and then centrifuged for 10 min at 12 000 × g. The immunoprecipitates were washed twice with 0.6 ml of 0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl, 1% Triton X-100, once with 0.6 ml of 0.1 M Tris-HCl, pH 8.0, and finally resuspended in 50 μl of 0.1 M Tris-HCl, pH 8.0, 15 μl of buffer C plus 10% 2-mercaptoethanol for boiling.

3. RESULTS AND DISCUSSION

In the present study, in vitro phosphorylation assays were performed using rapidly prepared and desalted extracts of light- and dark-adapted green maize leaves as the source of crude PEPC-PK. In most of these experiments, the assays were supplemented with the purified dark-form target enzyme (see Fig. 1a, lanes 1,2 versus 3–8) in order to negate any variation in the endogenous PEPC protein-substrate (e.g. light PEPC is enriched in phosphoserine relative to the dark enzyme-form [6,9]). When such reconstituted phosphorylation assays were performed, the apparent PEPC-PK activity in the light extracts was several-fold greater than that present in the corresponding dark samples when using either endogenous or exogenous PEPC as substrate (Fig. 1, lanes 1–4). This striking observation was not due to an excessive protein phosphatase activity in the dark tissue extracts since (i) okadaic acid, at levels known to completely inhibit types 1 and 2A plant protein phosphatases (up to 1 μM [5,13]), was completely without effect (Fig. 1b, lanes 3–8), and (ii) purified dark-form maize PEPC, specifically ^{32}P-labeled on Ser-15 [4], was not significantly dephosphorylated by either of these dilute leaf extracts (Y. Samaras and C. Echevarría, unpublished). Similarly, the ADP-dependent threonyl-phosphorylation of the 95-kDa PPDK subunit [see 1,2] was not a confounding factor in these experiments since (i) the PPDK and 109-kDa PEPC monomers were sufficiently resolved by SDS-PAGE (Fig. 1a), (ii) an ADP-scavenging system and an adenylate kinase inhibitor [10] were routinely included in the phosphorylation assays, and, most notably, (iii) the striking light-dark difference in PEPC-PK activity was still observed when PEPC-immunoprecipitates

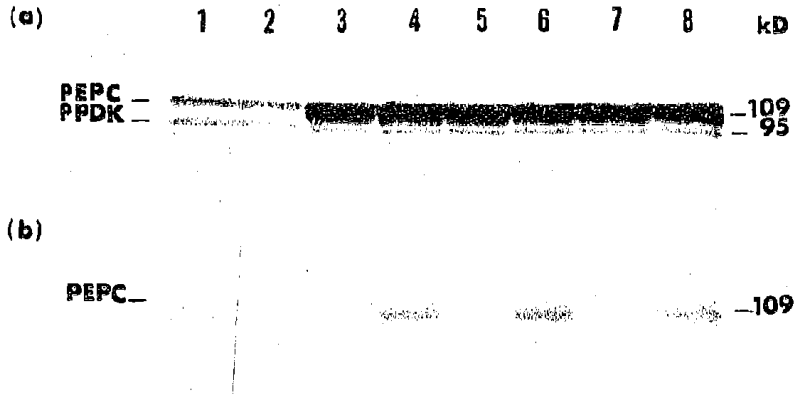

Fig. 1. PEPC-PK activity in desalted crude extracts of illuminated or darkened leaves and the effect of okadaic acid. SDS-PAGE analysis of phosphorylation assays with dark (lanes 1,3,5,7) or light (2,4,6,8) tissue extracts, supplemented with (3–8) or without (1,2) purified dark-form PEPC. The assays were done in the absence (1–4) or presence of either 25 nM (5,6) or 1 μM (7,8) okadaic acid. (a) Coomassie blue-stained gel. (b) Autoradiograph.

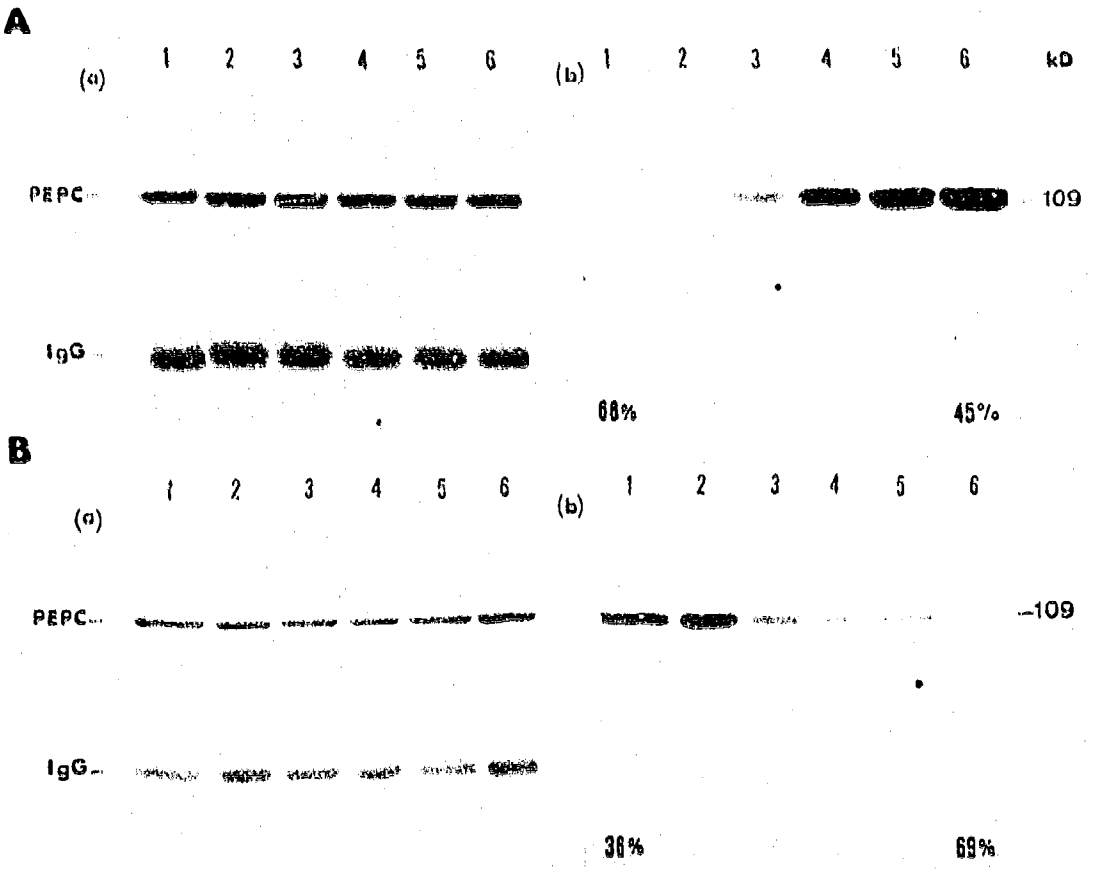

Fig. 2. Changes in PEPC-PK activity in vivo during a dark to light (A) or light to dark (B) transition of the parent leaf tissue. SDS-PAGE analysis of PEPC immunoprecipitates from phosphorylation assays supplemented with purified dark-form PEPC. (A) Leaves in darkness overnight [10–15 h (time '0'), lane 1] and after 15, 30, 45, 60 and 120 min in the light (2–6, respectively). (B) Leaves at the end of the 14-h photoperiod (time '0', lane 1) and after 15, 30, 45, 60 and 120 min in the dark (2–6, respectively). (a) Coomassie blue-stained gels. (b) Autoradiographs. In parallel experiments, the corresponding in vivo phosphorylation-status of PEPC was assessed by the sensitivity of the target enzyme's activity to L-malate using crude extracts rapidly prepared at either time '0' or after 120 min in the light (A) or dark (B). The % inhibition by 1 mM L-malate is indicated at the bottom of the corresponding lane (1 and 6 in Experiments A and B) in (b).

were analyzed (Fig. 2). Thus, these collective findings indicate a striking, reversible light activation of PEPC protein-serine kinase activity in vivo.

Given the relatively slow, reversible light activation of PEPC in the C_4 mesophyll-cytoplasm in vivo [6,7,9], it was of obvious importance to determine the kinetics of the light-dark effects on PEPC-PK activity. The results presented in Fig. 2A (dark to light transition) and 2B (light to dark transition) indicate that the reversible light activation of the PEPC protein-serine kinase in vivo was similarly slow, taking as long as 60 min to near completion. Thus, there is excellent agreement between the known kinetics of the light-dark changes in malate sensitivity-seryl phosphorylation-status of the target enzyme in vivo [6,9] and those anticipated for PEPC-PK activity.

In an attempt to gain further insight into the reversible light activation of the PEPC protein-serine kinase and its possible relationship to the previously reported calcium–calmodulin-dependent PEPC protein kinase activity in crude sorghum leaf extracts [14], we next evaluated the effects of putative, light-modulated cytoplasmic effectors on PK activity in vitro. Inclusion of either dithiothreitol (DTT) or EGTA into the standard phosphorylation assay had no major effect on the relative light–dark PEPC-PK activity (Fig. 3A, lanes 3–8), thus suggesting that changes in cytoplasmic -SH status (e.g. via the extrachloroplastic NADP–thioredoxin *h* system [15]) and Ca^{2+} levels are not responsible for the differential PEPC-PK activity in light and darkness. Similarly, ammonium sulfate precipitation of the crude extracts prior to desalting had no effect on the light–dark activity-ratio of the kinase (Fig. 3B, lanes 9–12), thereby arguing against the involvement of a tight-binding PEPC-PK effector analogous to the nocturnal inhibitor of the chloroplast stromal enzyme, Rubisco [16,17].

In summary, the present results indicate that the PEP carboxylase protein-serine kinase(s) per se is reversibly light activated in vivo by either covalent modification,

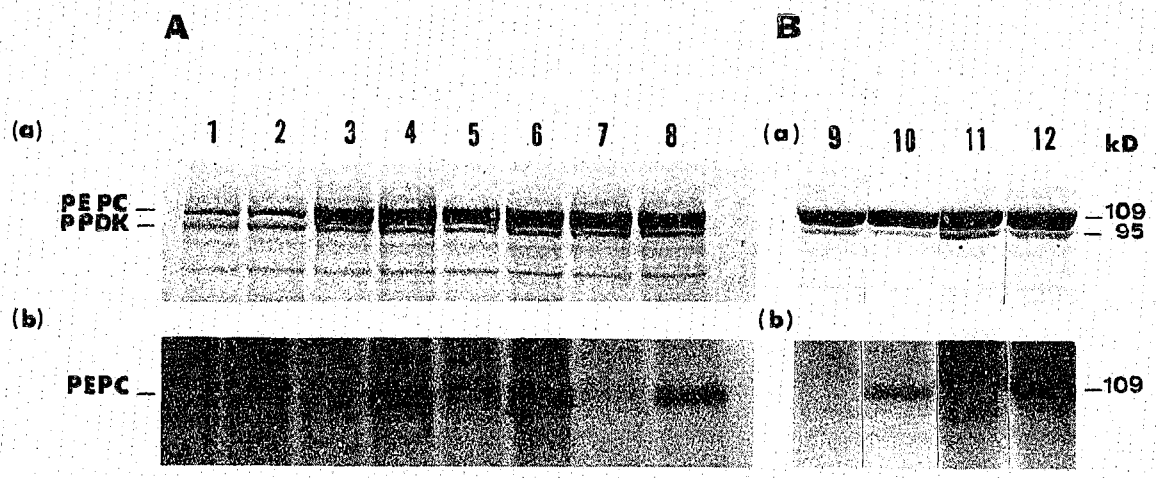

Fig. 3. Effect of DTT, EGTA (A) and ammonium sulfate precipitation of the crude filtrates (B) on PEPC-PK activity. SDS-PAGE analysis of phosphorylation assays with dark (lanes 1,3,5,7,9,11) or light (2,4,6,8,10,12) tissue extracts, supplemented with (3-12) or without (1,2) purified dark-form PEPC. In (A), the phosphorylation assays were performed in the absence (1-4) or presence of 20 mM DTT (5,6) or 4 mM EGTA (7,8). In (B), 0.2 ml of the crude filtrate was either directly desalted (9,10) or first precipitated with 60% saturation $(NH_4)_2SO_4$ (45 min, 4°C), centrifuged, and the pellet resuspended in 0.2 ml of buffer B prior to desalting (11,12). (a) Coomassie blue-stained gels. (b) Autoradiographs.

protein turnover or, less likely, a tight-binding effector, thus implying that the light signal and ensuing activation-seryl-phosphorylation of PEPC in the C_4 mesophyll-cytoplasm involves a bicyclic regulatory cascade. We are now attempting to establish by what specific mechanism(s) the kinase activity in vivo is slowly, but strikingly increased in the light and decreased in darkness.

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