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Title:

In vivo Phosphorylation of Phospho*enol*pyruvate Carboxylase in Guard Cells of *Vicia faba* L. is Enhanced by Fusicoccin and Suppressed by Abscisic Acid.[1]

Authors:

Zhirong Du, Karthik Aghoram and William H. Outlaw Jr.[2]

Department of Biological Science, Florida State University, Tallahassee, FL 32306-3050

Running Title:

PHOSPHORYLATION OF GUARD-CELL PHOSPHO*ENOL*PYRUVATE CARBOXYLASE

Corresponding Author:

William H. Outlaw Jr.

Department of Biological Science, Florida State University, Tallahassee, FL 32306-3050
Phone: (904) 644-4020
Fax: (904) 644-0481
Email: outlaw@bio.fsu.edu

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ABSTRACT

Plants regulate water loss and CO_2 gain by modulating the aperture sizes of stomata that penetrate the epidermis. Aperture size itself is increased by osmolyte accumulation and consequent turgor increase in the pair of guard cells that flank each stoma. Guard-cell phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31), which catalyzes the regulated step leading to malate synthesis, is crucial for charge and pH maintenance during osmolyte accumulation. Regulation of this cytosolic enzyme by effectors is well documented, but additional regulation by posttranslational modification is predicted by the alteration of PEPC kinetics during stomatal opening (FEBS Lett. **352**, 45-48). In this study, we have investigated whether this alteration is associated with the phosphorylation status of this enzyme. Using sonicated epidermal peels ("isolated" guard cells) pre-loaded with $^{32}PO_4$, we induced stomatal opening and guard-cell malate accumulation by incubation with 5 μM fusicoccin (FC). In corroboratory experiments, guard cells were incubated with the FC antagonist, 10 μM abscisic acid (ABA). The phosphorylation status of PEPC was assessed by immunoprecipitation, electrophoresis, immunoblotting, and autoradiography. PEPC was phosphorylated when stomata were stimulated to open, and phosphorylation was lessened by incubation with ABA. Thus, we conclude that regulation of guard-cell PEPC *in vivo* is multifaceted; the effects of regulatory metabolites and the activation status of the enzyme are integrated to control malate synthesis. These results, together with the coincident alteration in the kinetics of the enzyme (FEBS Lett. **352**, 45-48), constitute the first unequivocal demonstration of regulatory posttranslational modification of a guard-cell protein that is specifically implicated in stomatal movements.

Key Words: phospho*enol*pyruvate carboxylase; PEPC; phosphorylation; fusicoccin; FC; abscisic acid; ABA; guard cells; stomata

Stomata in the leaf epidermis provide the major pathway for gas exchange between plants and their environment. As stomata are non-selective, water vapor exits while CO_2 enters through open stomata. To effect a compromise between the conflicting requirements for CO_2 uptake and water conservation, plants have evolved mechanisms to regulate stomatal-aperture size. The regulation is implemented through changes in osmotic pressure of the pair of guard cells that flank each stoma. In brief, during stomatal opening, guard cells accumulate solutes, primarily K^+ salts (1). Uptake of K^+ through channels is driven by membrane hyperpolarization, which results from H^+ extrusion by the plasmalemma H^+-ATPase. As one means to maintain cellular pH during proton extrusion, guard cells synthesize and accumulate organic anions, predominantly malate (2). That is, for each carboxylate accumulated, a proton is released to the cytosol as compensation for proton extrusion. Overall, the decrease in guard-cell solute potential causes osmotic water uptake. Thus, guard cells swell, distending their walls asymmetrically and widening the aperture. Stomatal closure is essentially the reverse as guard cells shrink due to solute and water dissipation.

Phospho*enol*pyruvate (PEP)[3] carboxylase (PEPC, EC 4.1.1.31) is a cytosolic enzyme that catalyzes the branch-point step in the malate-accumulation pathway in guard cells during stomatal opening. This guard-cell enzyme is regulated by cytosolic pH and the allosteric effectors (e.g., 3-5), glc-6-P (an activator) and malate (an inhibitor). PEPC isoforms exist in other plant systems where they have been studied extensively. In the C_4 and CAM auxiliary photosynthetic pathways, atmospheric CO_2 is initially fixed by PEPC. In roots, nodules, and C_3 leaves, PEPC plays a critical role in anaplerosis. Like that in guard cells, these other PEPCs are regulated allosterically (6, 7). In addition, regulatory phosphorylation of PEPC isoforms of the C_4 and the

CAM pathways has been extensively documented (7-10). Phosphorylation of a specific serine residue embedded in a plant-invariant motif near the amino terminus renders the isoforms up to 7- or 10-fold (C_4, 11; CAM, 12) less sensitive to malate under physiological (but suboptimal) assay conditions. More recently, a similar type of regulatory phosphorylation of some non-photosynthetic isoforms of PEPC has also been reported. As examples, an early report (13) showed that purified PEPC from tobacco leaves can be phosphorylated *in vitro*. Phosphorylation of wheat-leaf PEPC *in vivo* results in increased enzyme activity and decreased sensitivity of the enzyme to malate (14). Similar results were obtained with PEPC from barley mesophyll protoplasts (15) and germinating wheat seeds (16). In addition, PEPC from nodules is phosphorylated *in vitro* (17, 18) and *in vivo* (19), and the phosphorylation results in decreased sensitivity of the enzyme to malate (17, 19). However, in other work (20, and references in 21), kinetics modifications indicating regulatory phosphorylation of C_3-leaf PEPC were not demonstrated. A detailed explanation of the possible reasons for this apparent discrepancy can be found in (21).

The possibility that the kinetics properties of guard-cell PEPC are altered by reversible posttranslational modification has been the subject of many studies (cited in 2, and unpublished work cited in 10, 20). However, only in our work (23) did a stomatal-opening stimulus result in a lessening of malate inhibition at suboptimum pH and substrate concentration, the effect that is the kinetics correlate of phosphorylation of all the other isoforms of PEPC (7). Molecular studies on the guard-cell protein indicate that it can be phosphorylated *in vitro* (24) and *in vivo* (24, and unpublished results cited in 10). However, phosphorylation either was not correlated with the

physiological state of the tissue (24) or the time course for phosphorylation was not coordinate with stomatal movements (10).

In the present study, we obtained direct evidence that PEPC was phosphorylated *in vivo* when "isolated" guard cells were treated with fusicoccin (FC), which stimulates stomatal opening (25). The phosphorylation was positively correlated with guard-cell malate content and with stomatal-aperture size. We also observed that abscisic acid (ABA), which causes stomatal closure (26), suppressed FC effects on phosphorylation of guard-cell PEPC and on malate accumulation. To our knowledge, this is the first direct and unequivocal evidence of a regulatory posttranslational modification of a guard-cell protein that is specifically involved in stomatal movements.

MATERIALS AND METHODS

Guard cells were "isolated" from abaxial epidermes of fully expanded leaflets of 21-day-old *Vicia faba* L. cv. Longpod plants, which were cultured essentially as described (5). Epidermal peels were detached at 3 hours into the dark period, when stomata were closed. The peels were brushed in distilled water to remove mesophyll contamination; then, the peels were cut into 0.25-cm^2 squares and stored, 25 each, in scintillation vials that contained incubation buffer (10 mL 10 mM MES-KOH, pH 6.1, 0.1 mM $CaSO_4$). Throughout, the incubation-buffer solutions containing the peels were swirled frequently to avoid anoxia. As a means of destroying cells other than guard cells, the peels were sonicated (3 x 3 s, Cavitator (Mettler Electronics Corp.), cf.. 27). After they were rinsed, the peels, 50 each, were transferred to 4 mL incubation buffer in 5-mL filter units. (Filter units facilitated solution transfer; each was made from the outlet

end of a syringe barrel, which was fitted with a nylon-mesh disk.) During a subsequent 1-h acclimation period, peels were screened for guard-cell viability (using the criteria of chloroplast integrity and of neutral-red uptake) and for contamination. The peels in any filter unit that contained viability < 90 % or contamination (cell basis) > 0.5 % were discarded. Altogether, preparation of isolated guard cells required 4 h and was conducted in darkness or in weak green light as were the treatments described in the following sections.

Two parallel treatments with complementary aims were conducted. In one treatment (for assay of ^{32}P-PEPC), the incubation buffer was replaced by 1.5 mL fresh incubation buffer that included 14 nM $^{32}P_i$ (300 TBq·mmol^{-1}). Pre-incubation to load the cells with $^{32}P_i$ was for 1 h. The other treatment (for assay of malate) was identical except that non-radioactive P_i was substituted for $^{32}P_i$, and some peels were removed for aperture-size measurements and viability assessments at the end of treatments. For both treatments, at the end of the 1-h pre-incubation period, the solutions containing the peels was altered to include 1 mM $MgSO_4$ and, as indicated (Fig. 1, 2), 5 μM FC (which stimulates stomatal opening and consequent malate accumulation, 28) or its antagonist, 10 μM ABA. (Stock solutions of 1 mM FC (a gift of E. Marré) and of 10 mM S(+)ABA (a gift of Abbott Laboratories) were made in 0.5 (v/v) % ethanol and in methanol, respectively.)

For malate assays (Fig. 1), peels (25 cm^2 per sample) were frozen in liquid N_2, powdered in solid CO_2, and extracted in 1 M $HClO_4$ at -4 °C (29). Neutralized extracts were assayed enzymatically (30).

For ^{32}P-PEPC assays (Fig. 2), peels (12.5 cm^2 per sample) were rinsed twice with 2 mL incubation buffer that included 1 mM $MgSO_4$ and 14 nM P_i (non-radioactive), and, as

appropriate, 5 μM FC. Then, the peels were frozen in liquid N_2, freeze-dried, and PEPC was extracted at 4 °C in 250 μL extraction cocktail (100 mM Tris-HCl, pH 8.0, 10 mM EDTA-Na, 10 mM DTT, 50 mM NaF, 100 μM Na_3VO_4, 2 mM PMSF, 1 $\mu g \cdot mL^{-1}$ butylated hydroxytoluene and 20 $\mu g \cdot mL^{-1}$ chymostatin). The extract was centrifuged for 10 min twice at 13,600g. PEPC was immunoprecipitated from 120 μL of supernatant by addition of 7 μL rabbit antiserum (prepared against maize PEPC, a gift of R. Chollet (31)). After 1.5 h with vortexing every 15 min, 6 mg Protein A-Sepharose was added as a 12-μL aqueous mixture. After an additional 1.5 h with vortexing every 5 min, the immunocomplexed beads were pelleted (13,600g, 2 min). Then, the pellet was washed twice with 0.8 mL Tris-buffered saline (10 mM Tris-HCl, pH 8.3, 0.85 % NaCl) that contained 0.5 % (v/v) Triton X-100 and 0.02 % (w/v) SDS and subsequently twice more with only Tris-buffered saline. Following a final wash with 0.8 mL 60 mM Tris-HCl (pH 6.8) the pellet was heated to 100 °C for 5 min in 30 μL of a dissociation buffer (60 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 10 mM DTT and 15 % (v/v) glycerol). Proteins in the supernatant, which contained PEPC and dissociated antibodies, were separated in 10 % SDS-PAGE mini-slab gels (35 x 55 x 0.5 mm) as described by Poehling and Neuhoff (32) except the stacking current was 8 mA (30 min) and the resolving current was 9 mA (75 min). Resolved proteins were blotted onto nitrocellulose membranes (pore size, 0.45 μm; Schleicher and Schuell) at 4 °C (100 V, 40 min). The transfer buffer was 50 mM Tris-glycine, pH 8.3, that contained 20 % (v/v) methanol and 0.1 % (w/v) SDS. (All subsequent procedures were conducted at room temperature, except as noted.) Air-dried blots were incubated for 2 h with blocking buffer (5 % (w/v) nonfat dry milk in Tris-buffered saline). Then, the blots were incubated overnight with a 5,000x dilution of polyclonal rabbit anti-maize-PEPC serum made in

blocking buffer. After they were rinsed with fresh blocking buffer for 20 min, the blots were incubated for 2 h with a 1,000x dilution of Sigma (A-3687) alkaline-phosphatase-labeled goat anti-rabbit IgG made in blocking buffer. PEPC was detected by the NBT-BCIP substrate system. Dried blots were exposed to Kodak X-Omat film with an enhancing screen (Quanta Fast Detail, NEN) at -80 °C for 2 weeks to detect phosphorylated PEPC.

RESULTS AND DISCUSSION

Stomatal opening results from the accumulation of osmotica, prototypically K^+ salts, in guard cells. This accumulation is a result of activation of the universal H^+-ATPase on the guard-cell plasmalemma. In guard cells of *V. faba*, up to 3 pmol H^+ per guard-cell pair (2) are extruded, hyperpolarizing the plasmalemma, during the accumulation of nominally 300 mM K^+ (1). A mechanism to counter the resulting charge perturbation is the synthesis and accumulation of organic anions, predominantly malate, by the guard cells (33). Two H^+ equivalents per malate synthesized (from starch breakdown) are released into the cytosol, balancing the pH. Guard cells of open stomata (about 10 μm) of *V. faba* contain approximately 5-fold as much malate as those of closed stomata (30). FC, a fungal toxin that activates the plasmalemma H^+-ATPase, stimulates dark $^{14}CO_2$ fixation into the C-4 position of malate in *V. faba* guard-cell protoplasts, where malate accumulates at a rate of 9 fmol·guard-cell pair^{-1}·min^{-1} (28). These data are consistent with: a) an activation guard-cell PEPC, the committed enzyme in malate synthesis, and b) coupling of PEPC activity and H^+ extrusion, during stomatal opening. In the present study, we observed a 4-fold increase in *V. faba* guard-cell malate content (Fig. 1) when these cells were stimulated with FC, which caused the

stomata to open from 2±0.1 to 8±0.2 (SE) μm in 60 min. The malate accumulation rate was estimated to be about 3 fmol·guard-cell·pair^{-1}·min^{-1}. Both these observations are consistent with the earlier literature (28, 30).

In contrast to the opening process, stomatal closure requires depolarization of the guard-cell membrane potential (34), which leads to efflux of solutes and water, and, consequently, shrinkage of guard cells. Parallel to these processes, anions, predominantly malate, are dissipated spatially or metabolically (2, 33). ABA, an antagonist of FC, induces stomatal closure and inhibits stomatal opening. Treatment with ABA results in the loss of malate from epidermal strips of *Commelina communis* and most of the malate lost is by release into the surroundings (35). This ABA effect on malate efflux results from Ca^{2+}-mediated activation of non-selective anion channels on the plasmalemma, and from an unknown mechanism across the tonoplast (Ward *et al.*, 1995). Consistent with the above findings (34, 35), we observed that ABA treatment suppressed malate accumulation in FC-stimulated guard cells (Fig. 1). Incubation with ABA also caused a decrease of nominally 60% in malate content of unstimulated guard cells.

PEPC isoforms in C_3, C_4 and CAM mesophyll, and in nodules are regulated by reversible protein phosphorylation (7). In order to determine whether guard-cell PEPC is also subject to posttranslational regulation, we examined the *in vivo* phosphorylation status of PEPC in guard cells treated with FC or ABA (Fig. 2). Conspicuous phosphorylation of PEPC was observed in guard cells treated with FC for 30 min; the phosphorylation increased steadily during the next 30 min of treatment. This increase in phosphorylation correlated well with FC-stimulated malate accumulation (Fig. 1). In contrast, PEPC was not phosphorylated in unstimulated guard cells, which also did not accumulate malate. Both malate content and phosphorylation status of PEPC

were also correlated with stomatal aperture size (data not shown). These correlations indicated that, during FC-induced stomatal opening, guard-cell PEPC was phosphorylated and activated, resulting in malate accumulation. These results corroborate strong indications of posttranslational activation of guard-cell PEPC during stomatal opening induced by light and low CO_2 (23).

ABA, which inhibited malate accumulation (Fig. 1), also lessened phosphorylation of guard-cell PEPC (Fig. 2). Whereas incubation with FC for 60 min caused a remarkable increase in the phosphorylation of PEPC, incubation with ABA for the same time did not have an effect. In addition, ABA treatment suppressed FC-induced PEPC phosphorylation. This suppression may be a result of ABA-induced dephosphorylation, or alternatively, ABA inhibition of phosphorylation.

In summary, our results on the phosphorylation of guard-cell PEPC were consistent with well established effects of FC and ABA on the functioning of stomata. These results demonstrated that: a) PEPC was activated (as manifested by malate accumulation) through phosphorylation during FC-induced stomatal opening, and b) ABA treatment suppressed both phosphorylation and malate accumulation.

Aspects of early reports of posttranslational modification of guard-cell PEPC require explanation. First, light (unpublished results cited in 22, *Pisum sativum*; and unpublished results cited in 10, *C. communis*) or FC (10) treatment of guard-cell protoplasts resulted in a 2-fold decrease in K_m, implying regulation by a posttranslational modification. A statistically significant difference in the K_m (PEP·Mg) at pH 7 of guard-cell PEPC, however, could not be detected in either single-cell microassays (0.7±0.1 mM, opening stomata vs. 0.75±0.3 mM, closed stomata; 4, *V. faba*) or assays of guard-cell protoplast extracts (0.6±0.1 mM, dark vs. 0.5±0.1 mM, light; 5,

V. faba). Second, enhanced phosphorylation of PEPC was observed (10) when labeled guard-cell protoplasts of *C. communis* were treated with light or FC for up to 3 h. However, the concomitant decrease in V_{max}, unaltered malate sensitivity of the enzyme, and lack of a temporal correlation between phosphorylation and stomatal opening precluded a definite conclusion of enzyme regulation by phosphorylation, as discussed by the authors (10). Third, PEPC from *V. faba* epidermal peels is phosphorylated *in vitro* and PEPC from guard-cell protoplasts is phosphorylated *in vivo* (24). The phosphorylation status of the enzyme was, however, not altered by either activating (light) or deactivating (dark) conditions, and the authors (24) hypothesized that they had demonstrated only a basal level of phosphorylation. Moreover, phosphoproteins from guard-cell protoplasts under stimulatory conditions do not include PEPC or a protein of requisite molecular mass (36, 37). Contrary to the above findings (10, 24, 36, 37), we demonstrated a significant decrease in malate sensitivity of PEPC from guard cells of opening stomata (23) as well as an increase in phosphorylation of PEPC when guard cells were treated with FC (Fig. 2). The discrepancies between the results discussed above (10, 24, 36, 37) and ours (23 and Fig. 1, 2) cannot be readily explained, except on the basis of distinct experimental systems. For instance, the signal transduction pathway could be affected during protoplast isolation (24) or the change in phosphorylation status of guard-cell PEPC may not be apparent (36, 37) when a crude extract of guard-cell phosphoproteins is used for gel electrophoresis (Du and Outlaw, unpublished).

Whereas it is clear that guard-cell PEPC is regulated by phosphorylation (23 and Fig. 1, 2), modulation of the enzyme by metabolites and cytosolic pH is also established (3-5). Thus, *in planta* regulation may be an integration of these mechanisms, as reported for C_4-leaf PEPC

(11, 38, 39). Phosphorylation of sorghum-leaf (38) or maize-leaf (39) PEPC causes a 3-fold increase in K_i (malate) of the enzyme. In presence of glc-6-P (4 mM), however, phosphorylation of the sorghum-leaf enzyme causes a 15-fold increase in the K_i (malate) (38). Consistently, phosphorylation of a recombinant sorghum-leaf PEPC not only increases the K_i (malate) by more than 6-fold but also decreases the K_a (glc-6-P) by more than 4-fold (11). A mixture of metabolites (5 mM glycine, 3 mM glc-6-P and 20 mM alanine) added to the reaction mixture yields a K_i (malate) of 6 mM for the dephosphorylated maize-leaf PEPC, but 13 mM for the phosphorylated enzyme (39). These values are comparable to published values of cytosolic malate concentration (2-8 mM, 40 and references therein). Quantitatively assessing the contribution of each mechanism to PEPC regulation is difficult, as a precise value of cytosolic malate concentration in C_4 mesophyll cells is lacking. Similar assessments for regulation of the guard-cell enzyme are more problematical, as data on K_i (malate) and K_a (glc-6-P), and those on the dynamics of cytosolic metabolites during stomatal movements, are insufficient. Further study on regulation of guard-cell PEPC should thus focus on various aspects of the integration of these complementary mechanisms and on the signal transduction pathway that activates PEPC.

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[2]To whom correspondence should be addressed. FAX: (904) 644-0481

[3]Abbreviations used: PEP, phospho*enol*pyruvate; PEPC, PEP carboxylase; FC, fusicoccin; ABA, abscisic acid

Figure Legends

Fig. 1. The antagonistic effects of fusicoccin (5 μM) and abscisic acid (10 μM) on malate accumulation by "isolated" guard cells. Abaxial epidermal peels of *Vicia faba* were sonicated to destroy selectively all cells except guard cells. After 1 h of dark preincubation to mimic the ^{32}P-protein-labeling protocol (see Fig. 2), fusicoccin or abscisic acid was added to the incubation medium as indicated. Enzymatic analysis of the malate contents showed that fusicoccin stimulated, and abscisic acid inhibited, malate accumulation. Three experiments, corresponding to the Arabic numerals by each symbol, were designed to provide at least duplication of each treatment.

Fig. 2. The antagonistic effects of fusicoccin (5 μM) and abscisic acid (10 μM) on the *in vivo* phosphorylation of phospho*enol*pyruvate carboxylase of "isolated" guard cells. Sonicated epidermal peels were pre-loaded with ^{32}P-orthophosphate for 1 h before incubation with effector. SDS-PAGE immunoblot analysis of immunoprecipitated phospho*enol*pyruvate carboxylase confirmed constancy of protein loading (top panel) and ^{32}P-autoradiograms (middle) showed that fusicoccin stimulated, and abscisic acid suppressed, phosphorylation. Two immunoreactive bands (110 kDa and 106 kDa) were detected (top panel), but only the lower (major) band was phosphorylated (middle panel). Symbols on the time and treatment scales (bottom panel) identify lanes and correspond to malate contents (Fig. 1).