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THE INTERACTION OF SHIKIMIC ACID AND PROTEIN PHOSPHORYLATION WITH PEP CARBOXYLASE FROM THE C_4 DICOT *AMARANTHUS VIRIDIS*

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Key Word Index—*Amaranthus viridis*; Amaranthaceae; C_4; PEPC; protein kinase A; protein phosphorylation; shikimic acid.

Abstract—Shikimic acid has been described as a potent competitive inhibitor of the activity of C_4 phospho*enol*pyruvate carboxylase (PEPC) from *Amaranthus viridis*. In the present study, the effects of shikimic acid were examined further with the dephospho (dark-form) and *in vitro* phosphorylated forms of homogeneous PEPC from *A. viridis*. Kinetic analysis showed that the inhibitory effect of shikimic acid was dependent on the phosphorylation state of the enzyme. Thus, the I_{50} value of shikimic acid for dark-form PEPC was six times lower than that for the phosphorylated enzyme (12 vs 71 μM, respectively). When Glc6P, an activator of C_4 PEPC, was present in the assay medium, the I_{50} value increased 2- and 3-times with the phospho and dephospho PEPC-forms, respectively. Shikimic acid also markedly decreased ^{32}P incorporation from Mg[γ-^{32}P]ATP into the dark-form of C_4 PEPC, but not casein, catalyzed by protein kinase A. In this way, shikimic acid mimics the behaviour of L-malate, a well-known inhibitor of PEPC, in that it decreases both the enzyme's activity and phosphorylatability. Based on these data, a possible role for shikimic acid in the regulation of PEPC activity in plants is suggested.

INTRODUCTION

Phospho*enol*pyruvate (PEPC; EC 4.1.1.31) catalyses the irreversible β-carboxylation of PEP by HCO_3^- in the presence of a divalent cation to yield oxaloacetate (OAA) and Pi [1]. This cytosolic reaction has an essential role in C_4 metabolism as it catalyses the primary incorporation of atmospheric CO_2 in the photosynthetic process. It is well known that changes in the intracellular concentrations of L-malate and Glc6P (metabolite inhibitor and activator of PEPC, respectively) participate in the modulation of PEPC activity *in vivo* [2, 3], but these effects are influenced profoundly by the phosphorylation state of the enzyme [3–5]. For example, the dephospho C_4 enzyme from dark-adapted *Amaranthus viridis* leaves is more susceptible to inhibition by L-malate, less sensitive to activation by Glc6P, and less active than the phosphorylated enzyme from illuminated leaves [6].

PEP is one of the initial substrates for the shikimic acid pathway [7], and is, thus, a common metabolite of the C_4 and secondary metabolism pathways in plants. Flavonoids (end products of one of the shikimic-acid pathway branches) and shikimic acid have been reported to be competitive inhibitors of C_4 PEPC activity from *A. viridis* [8, 9]. The 50% inhibition constants (I_{50} values) of these aromatic compounds are in the micromolar range, which makes them potent inhibitors of PEPC activity. The purpose of the present investigation was to reveal possible relationships between shikimic acid and the regulation of C_4 PEPC activity by seryl-phosphorylation. A possible regulatory system for linking both C_4 and secondary plant metabolism is suggested.

RESULTS AND DISCUSSION

The phosphorylation site on plant PEPC is established as an invariant Ser near the N-terminus of the protein [5]. Depending on the plant species this residue is located at position 8 (C_4 sorghum [3]), 11 (C_4 *Amaranthus* [10]; *Mesembryanthemum* [11]) or 15 (C_4 maize [12]), within a highly conserved motif of E/DR/KxxSIDAQL/MR in various plants [13]. One of the most problematic aspects in the study of plant PEPC is the loss of this N-terminal phosphorylation domain by limited proteolysis during time-consuming puri-

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fication steps [3, 5, 11]. The FPLC protocol presented here, which is a modification of that reported by Wang *et al.* [14, 15], decreases the time required for purification to 12 h, thus providing an enzyme preparation which is suitable for phosphorylation experiments. The use of a proteinase inhibitor cocktail containing chymostatin is also essential to minimize loss of the N-terminus [5].

Dark-form PEPC (dephospho enzyme) from the C_4 dicot *Amaranthus viridis* was phosphorylated *in vitro* using mammalian protein kinase A (PKA). This heterologous protein-Ser/Thr kinase has been shown previously to specifically phosphorylate the target Ser residue in C_4 PEPC [3, 12, 16, 17]. The time course for apparent phosphorylation of PEPC was monitored by measuring increases in enzyme activity after different incubation times [3, 17]. A plateau was reached after ca 65 min (Fig. 1), indicating that the phosphorylation of PEPC was complete after this time. Similarly, the changes in the percentage inhibition of PEPC activity by shikimic acid, previously reported as a potent inhibitor of the C_4 enzyme [1], showed a parallel profile over this same time frame (Fig. 1). Thus, the seryl phosphorylation of C_4 PEPC not only changes its sensitivity to L-malate and Glc6P [3, 5, 13, 15, 17] but also to inhibition by this key aromatic precursor of chorismate.

After complete phosphorylation, the V_{max} value for the enzyme increased from 81.7 to 145 nkat/mg (Table 1, Fig. 1), measured at the suboptimal pH of 7.1 [6]. These data are consistent with the results reported previously by Podestá *et al.* [6] with PEPC purified from dark- and light-adapted *Amaranthus* leaves. Similarly, the I_{50} value of shikimic acid varied from 12 (dephospho form) to 71 μM (phospho form) before and after these *in vitro* phosphorylation experiments (Table 1). As a control, shikimic acid was substituted with L-malate, a PEPC inhibitor for which a ca 10-fold increase in I_{50} following *in vitro* phosphorylation of C_4 PEPC by PKA has been documented [3]. In the presence of this physiological negative effector, a profile similar to that presented for shikimic acid in

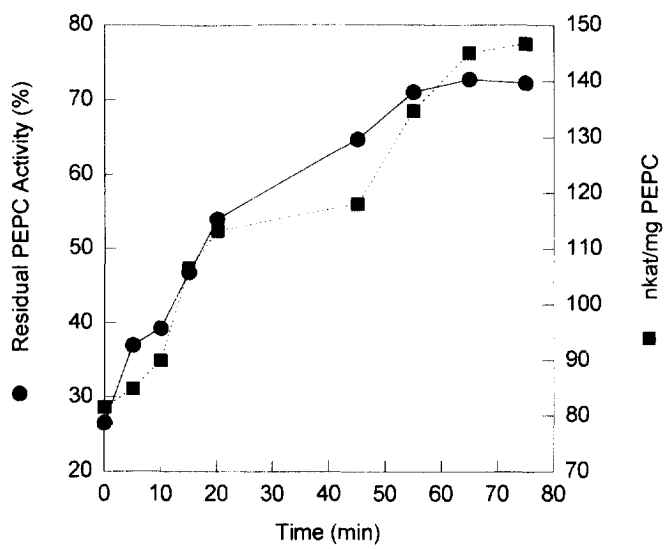

Fig. 1. Time-course of apparent phosphorylation of purified PEPC by PKA and MgATP. The circles represent the changes in residual activity (%) when 50 μM shikimic acid was present in the PEPC assay medium. The squares represent the specific activity values of PEPC (in the absence of inhibitor) during the *in vitro* phosphorylation reaction. For explanation, see Experimental.

Table 1. I_{50} values for inhibition of C_4 PEPC activity by shikimic acid and L-malate (control) before and after *in vitro* phosphorylation by PKA and MgATP. Where indicated, Glc6P (4 mM) was included in the PEPC assay medium

		I_{50} (μM)			
Enzyme form	Activity* (nkat/mg)	Shikimate	Shikimate +Glc6P	L-malate	L-malate +Glc6P
Phospho form	145	71	134	270	555
Dephospho form†	81.7	12	37	31	120

* Determined in the absence of effector at pH 7.1 and 4 mM PEP.

† Purified from dark-adapted (8 h) leaves.

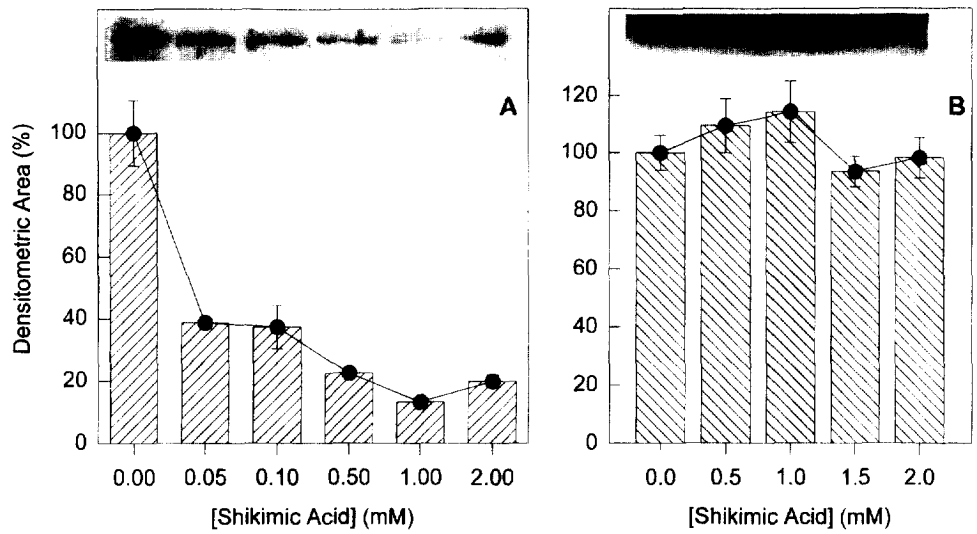

Fig. 2. Autoradiography and the corresponding densitometric analysis (% of control) of incorporation of ^{32}P into PEPC (A) or casein (B) in the presence of 0–2 mM shikimic acid, PKA, and Mg[γ-^{32}P]ATP. For explanation, see Experimental. No radiolabel was incorporated into either target protein in the absence of PKA.

Fig. 1 was observed (data not shown). Controls lacking PKA in the phosphorylation medium revealed that PEPC was completely stable during the 80 min required to perform the experiment. The presence of Glc6P in the PEPC assay medium caused an increase in the I_{50} value for shikimic acid with both the dephospho and phospho enzyme forms (Table 1). However, the effect on the dephospho form was ca 65% higher than with the phospho form. These results were similar to those obtained with L-malate, $\pm$Glc6P, during the PEPC assays (Table 1).

When purified PEPC was assayed for incorporation of ^{32}P from Mg[γ-^{32}P]ATP and PKA, shikimic acid behaved as an inhibitor of this process. As shown in Fig. 2, 50 μM shikimic acid caused a marked decrease in ^{32}P incorporation when PEPC was used as substrate in the phosphorylation reaction (panel A), but not when casein replaced PEPC as the target protein for PKA (panel B). Therefore, it is likely that the *in vitro* interaction of C_4 PEPC with either shikimic acid (Fig. 2) or L-malate [14, 17] perturbs the accessibility of the N-terminal phosphorylation domain to protein-Ser/Thr kinases.

Although considerably more research on this subject is necessary, we suggest that the inhibition of C_4 PEPC activity and its *in vitro* phosphorylatability by shikimate is very similar to that by L-malate [3, 14, 15, 17]. Clearly, as with L-malate, its inhibitory effect on PEPC activity depends on the phosphorylation state of the enzyme and the presence or absence of the activator Glc6P (Fig. 1, Table 1).

The total intracellular shikimic acid concentration has been determined in cell suspension cultures of buckwheat (*Fagopyrum esculentum*) to be ca 0.04 μmol/g fr. wt, and 56% of this metabolite was located in the vacuole [18]. Considering that water can comprise up to 80% of the tissue fresh weight, an estimation of the shikimic acid concentration is possible. Thus, ca 0.018 μmol/g fr. wt is distributed to the plastid and cytosol. Based on this estimation and the data presented in this paper and elsewhere [9], a putative regulatory role for shikimic acid on C_4 PEPC activity, regulation, and/or PEP partitioning between photosynthesis and aromatic secondary products can be postulated (Fig. 3).

EXPERIMENTAL

Plant material

Amaranthus viridis L. plants were collected from the field and grown in a local greenhouse (16 h light/8 h dark; 30° day/15° night).

Enzyme purification

PEPC was extracted from mature leaves of *A. viridis* plants that were dark-adapted for 8 h. Leaf tissue (10–20 g fr. wt) was chopped and homogenized in liquid N_2 and the enzyme was extracted with 3 volumes of buffer A (100 mM Tris-HCl, pH 7.5, 20% glycerol, 5 mM L-malate, 15 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 10 μg/ml chymostatin, 10 μg/ml leupeptin) in a Waring blender. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000 *g* for 10 min. The crude supernatant fluid was fractionated with PEG 8000. The 5–20% PEG ppt. was redissolved in 5 ml of buffer B (20 mM Tris-HCl, pH 8.0, 5% glycerol, 2 mM L-malate, 1 mM DTT, 0.1 mM EDTA, 5 μg/ml chymostatin, 5 μg/ml leupeptin). The clarified solution was applied to a column (1.5 × 12 cm) of Q-Sepharose Fast Flow con-

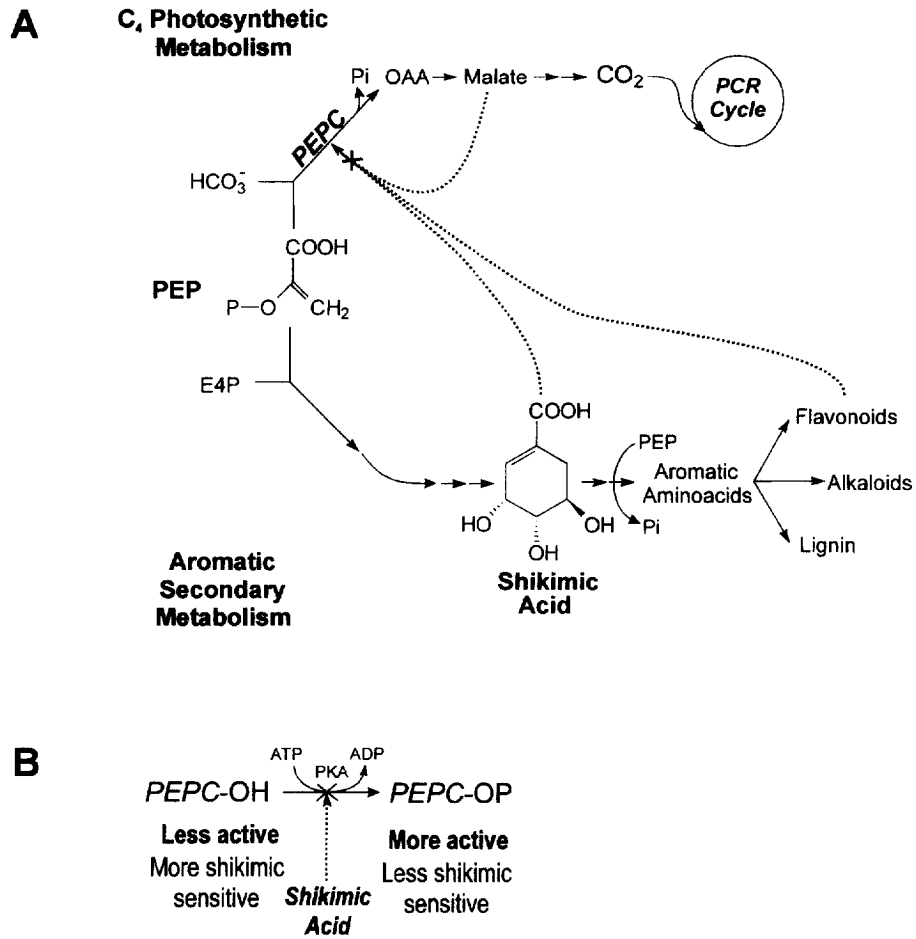

Fig. 3. A. Scheme showing the possible partitioning of PEP between photosynthetic and aromatic secondary metabolism in C_4 plants. Arrows do not indicate single enzymatic reactions. E4P, erythrose 4-P; PCR, photosynthetic carbon reduction. B. Effects of shikimic acid on protein phosphorylation of C_4 PEPC.

nected to an FLPC system, and eluted with a linear gradient of 0–0.4 M NaCl in buffer B. The fractions with PEPC activity were pooled and brought to 60% satn with $(NH_4)_2SO_4$. The ppt. was redissolved in buffer B plus 0.5 M $(NH_4)_2SO_4$ and applied to a phenyl Sepharose column (1.5 × 4 cm) [15]. A descending linear gradient of 0.5–0 M $(NH_4)_2SO_4$ in buffer B was used to elute the enzyme. The PEPC activity peaked at around 0 M $(NH_4)_2SO_4$. The PEPC-containing fractions from hydrophobic-interaction FPLC were pooled and applied directly to the Q-Sepharose column described above. The fractions with activity were diluted 1:5 with buffer B and reapplied to the anion-exchange column. The fractions with PEPC activity were concentrated using a Centricon 30 (Amicon). The resulting preparation was more than 95% pure when analyzed by SDS-PAGE.

PEPC activity assay

The routine, 1 ml assay medium contained 50 mM Tris-HCl, pH 7.1, 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1–2 μg purified PEPC, and 4 mM PEP. The reaction was started by the addition of enzyme. All measurements were performed at 30° and followed the formation of OAA at 272 nm ($\varepsilon = 910\ M^{-1}\ cm^{-1}$) [19] in a Hitachi 150-20 dual-beam spectrophotometer. The reaction proceeded linearly during the first 120 s. The I_{50} values were determined using 0.3 mM PEP and shikimic acid or L-malate concentrations around the I_{50} value. Kinetic analysis of the results was performed using the computer program developed by Brooks [20].

Time course of in vitro *phosphorylation*

Approximately 100 μg of purified dephospho (dark form) PEPC were incubated for 80 min at 30° in 200 μl of 50 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 0.1 mM ATP, and 4 U of the catalytic subunit of cAMP-dependent protein kinase (PKA) from bovine heart (prepared/activated for 2 h at 25° in 50 mM phosphate buffer, pH 7.0, containing 1 mM DDT and 20% glycerol). Aliquots (5 μl) were withdrawn at the specified times and assayed immediately for PEPC activity and its inhibition by shikimic acid. Determination of the

I_{50} value was performed at 0 and 80 min. Parallel control experiments were performed with a well characterized inhibitor of PEPC, L-malate [3, 14, 15, 17], and also with the same phosphorylation medium minus PKA; the latter caused no changes in PEPC activity or sensitivity to inhibitors. One unit (U) of PKA will transfer 1.0 picomole of phosphate from [γ-^{32}P]ATP to hydrolyzed, partially dephosphorylated casein per min at 30°.

In vitro *phosphorylation with* [γ-^{32}P]ATP

In vitro radiolabeling of purified PEPC (dark-form, 6 μg) was performed at 30° for 30 min in 45 μl of a medium containing 50 mM Tris-HCl, pH 7.0, 2 μCi [γ-^{32}P]ATP, 0.1 mM ATP, 5 mM $MgCl_2$, 5 mM DTT, 4 U of PKA (prepared/activated as above) per μg of PEPC, and 0–2 mM shikimic acid. The reaction was stopped by the addition of 5 μl of SDS sample-buffer [21]. The mixture was heated for 3 min at 100° and subjected to SDS-PAGE. As a control in these ^{32}P-labeling experiments with PKA, PEPC was replaced by casein (10 μg).

SDS-PAGE analysis

Electrophoresis in 8% (or 15%) polyacrylamide gels under denaturing conditions was performed as described by Laemmli [21]. Gels were stained with Coomassie blue, dried, and autoradiographed at −80° using Kodak X-Omat film and an intensifying screen.

Densitometric analysis

The autoradiographs were analysed densitometrically using a scanner (Hewlett-Packard ScanJet 3C). The images were processed and analysed by QuantiScan software (Microbial System Ltd).

Protein determination

Protein concentrations were measured according to Lowry *et al.* [22]. BSA was used as standard.

Chemicals

Tris, PEP, casein, chymostatin, leupeptin, the catalytic subunit of PKA from bovine heart, phenyl Sepharose CL-4B, Kodak X-Omat film, PEG 8000, ATP, shikimic acid, and L-malate were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). [γ-^{32}P]ATP was purchased from NEN/Dupont. Q-Sepharose Fast Flow was purchased from Pharmacia. All other reagents were of analytical grade.

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