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In vitro phosphorylation of purified tobacco-leaf phospho*enol*pyruvate carboxylase

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C_3-leaf phospho*enol*pyruvate (PEP) carboxylase (PEPC) was purified about 1,000-fold from tobacco and displayed a final specific activity of 35 μmol/min/mg protein, an apparent K_m (total PEP) of 95 mM (both at pH 8.0, 30°C), and an I_{50}(L-malate) value of 0.14 mM at pH 7.3, 0.2 mM PEP. The rapid, 5-step protocol involved polyethylene glycol fractionation and sequential FPLC on hydroxylapatite, phenyl-Sepharose, Mono Q and Superose 12. The electrophoretically pure protein and purified C_4-leaf PEPC were phosphorylated in vitro in a reconstituted system with PEPC-kinase isolated from illuminated tobacco and maize leaves. These reciprocal phosphorylation experiments (i) indicate that Ser^{11} of tobacco PEPC is the likely target residue, situated in the plant-invariant Glu/Asp-Lys/Arg-X-X-Ser phosphorylation motif near the N-terminus, and (ii) lend support to the recent hypothesis that C_3-leaf PEPC is subject to regulatory phosphorylation in vivo.

Phospho*enol*pyruvate carboxylase; Protein kinase; Protein phosphorylation; C_3 plant; Tobacco (*Nicotiana tabacum* L.)

1. INTRODUCTION

PEPC (EC 4.1.1.31) is a ubiquitous, cytosolic enzyme in bacteria, algae, and higher plants [1,2]. While this carboxylase is best known for its cardinal role in catalyzing the fixation of atmospheric CO_2 (as HCO_3^-) during C_4 photosynthesis and CAM [1–3], it also functions in C_3-plant carbon and nitrogen metabolism in general, and in the more specialized cases of legume root-nodules and leaf guard-cells [4–6].

In leaf tissue of C_4 and CAM species, PEPC activity is regulated posttranslationally by at least two interactive mechanisms. The enzyme is subject to allosteric control by positive (glucose-6-P, triose-P) and negative (L-malate) metabolite effectors [1–3], and an increasingly complex regulatory phosphorylation cycle that principally modulates the enzyme's sensitivity to L-malate [7,8]. In marked contrast, relatively little is known with respect to the possible posttranslational regulation of the non-photosynthetic C_3 enzyme. While it appears likely that this PEPC enzyme-form is also subject to allosteric control by metabolite effectors [5,6,9,10], only a single report has appeared pertaining to the phosphorylation of the C_3-leaf enzyme (in wheat) [11]. Given this paucity of information on the possible covalent modification of non-C_4/CAM PEPC, we have investigated the in vitro phosphorylation of the purified tobacco-leaf enzyme by both an endogenous protein kinase and C_4-leaf PEPC-kinase. Notably, tobacco PEPC, like all other C_3, C_4 and CAM isoforms, has a putative phosphorylation domain encompassing a target serine residue (i.e. Ser^{11}) in the plant-invariant Glu/Asp-Lys/Arg-X-X-Ser motif near its N-terminus ([12,13] and references therein).

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Abbreviations: PEPC, phospho*enol*pyruvate carboxylase; CAM, Crassulacean acid metabolism; PK, protein kinase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PEG, polyethylene glycol; DTT, dithiothreitol; FPLC, fast-protein liquid chromatography; PEP, phospho*enol*pyruvate.

2. EXPERIMENTAL PROCEDURES

2.1. *Purification of tobacco-leaf PEPC*

Tobacco (*Nicotiana tabacum* L.) leaf tissue (120 g), harvested during the photoperiod from greenhouse-grown plants, was homogenized in 300 ml of extraction buffer (100 mM MOPS-KOH, pH 7.3, 10 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM L-malate) containing 12 g polyvinylpolypyrrolidone and 1 mM fresh PMSF (from an ethanolic 0.1 M solution). The homogenate was filtered through 4 layers of cheesecloth and then centrifuged at 30,000 × *g* for 15 min. The supernatant fraction was brought to 8.5% (w/v) PEG by addition of powdered PEG-8000 (J.T. Baker). The suspension was stirred gently for 15 min at 0–4°C, the precipitate collected by centrifugation at 30,000 × *g* for 10 min, and finally resuspended in Buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 5 mM L-malate) plus 1 mM fresh PMSF. After clarification, the sample was loaded at a flow rate of 0.8 ml/min onto an hydroxylapatite (Bio-Gel HTP; Bio-Rad) column (2.5 × 10 cm) that was pre-equilibrated with Buffer A. The column was washed with 50 ml Buffer A and then a 150-ml linear gradient of 0–0.4 M KP_i was developed by mixing an increasing percentage of Buffer B (0.4 M KH_2PO_4–K_2HPO_4, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 5 mM L-malate) against Buffer A. The PEPC activity-peak fractions were pooled, PMSF was added to 1 mM, and finally brought to 0.5

M $(NH_4)_2SO_4$ by addition of solid, ultrapure $(NH_4)_2SO_4$ with gentle stirring. The sample's pH was adjusted to 8.0 with 2 M Tris-base solution. After clarification, the supernatant fluid was loaded at a flow rate of 1 ml/min directly onto a phenyl-Sepharose CL-4B (Sigma) column (1.6 × 5 cm) that was pre-equilibrated with Buffer C (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 5% (v/v) glycerol, 1 mM DTT, 2 mM L-malate) plus 0.5 M $(NH_4)_2SO_4$. After washing with 10 ml of the same solution, the column was eluted sequentially with a 30-ml gradient of 0.5–0.25 M $(NH_4)_2SO_4$, 20 ml 0.25 M, and a 30-ml gradient of 0.25–0 M, all in Buffer C. PEPC activity was eluted with Buffer C. The activity-peak fractions were pooled, filtered, and loaded directly onto a prepacked Mono Q HR 5/5 (Pharmacia) column pre-equilibrated with Buffer C at a flow rate of 0.5 ml/min. A 20-ml linear gradient of 0–0.35 M NaCl in Buffer C was developed at a flow rate of 1 ml/min. The PEPC activity-containing fractions were pooled and either diluted 1:1 with Buffer C and rechromatographed on Mono Q or concentrated with a Centricon 30 microconcentrator (Amicon) and chromatographed on a prepacked Superose 12 HR 10/30 (Pharmacia) column pre-equilibrated with Buffer C plus 50 mM NaCl at a flow rate of 0.25 ml/min. The PEPC activity-peak fractions were pooled, concentrated, and desalted into Buffer D (20 mM Tris-HCl, pH 8.0, 1 mM DTT, 20% (v/v) glycerol) with a Centricon 30. All of the above purification steps were carried out at 4°C, and all the chromatographic columns were linked to an automated FPLC system (Pharmacia).

2.2. *PEPC activity and in vitro phosphorylation assays, partial purification of maize PEPC-kinase, and purification of dark-form sorghum and maize PEPCs*

See Wang and Chollet [14] and Wang et al. [15] for details.

2.3. *Partial purification of tobacco-leaf PEPC-kinase*

Mature, greenhouse-grown tobacco leaves were harvested after 5 h illumination at ~600 $\mu E \cdot m^{-2} \cdot s^{-1}$. About 200 g leaf tissue was used as starting material. The partial-purification protocol was similar to that used for C_4 PEPC-kinase [14] except for the following modification: after high-salt elution from the blue dextran-agarose (Sigma) column, tobacco-leaf PEPC-PK was loaded onto a phenyl-Sepharose CL-4B column (1.0 × 3 cm) that was pre-equilibrated with Buffer E (see [14]) plus 0.5 M NaCl. After loading, the column was washed sequentially with 10 ml, each, of 0.5, 0.25 and 0 M NaCl in Buffer E. Finally, ice-cold distilled water was used to elute PEPC-PK activity, and the eluant was immediately buffered by addition of one-fourth volume of Buffer E [14]. The PK-sample was concentrated and desalted into Buffer D (see Section 2.1.) with a Centricon 10.

3. RESULTS AND DISCUSSION

3.1. *Purification and characterization of tobacco-leaf PEPC*

This C_3-leaf PEPC was purified about 1,000-fold, with a 13% overall recovery, by PEG fractionation and a rapid 4-step, FPLC-based protocol (see Section 2.1., Table I, Fig. 1). The purification strategy for this relatively low-abundance PEPC isoform was designed in such a way that the sample from each step was used directly in the next, thus eliminating time-consuming concentration and dialysis procedures that could lead to proteolysis of PEPC and, thus, loss of its N-terminal phosphorylation domain (see [15–17]). The purified tobacco-leaf enzyme had a subunit molecular weight of ~110,000, which was identical to the maize monomer (Fig. 1, lanes 7,8). This ~110-kDa polypeptide was the predominant PEPC subunit-form in crude tobacco-leaf extracts analyzed by SDS-PAGE/immunoblotting with antibody against maize C_4-PEPC (data not shown). The tobacco enzyme had a stronger binding affinity for Mono Q than did sorghum or maize PEPC, eluting at approximately 0.24 M (C_3) versus 0.18 M (C_4) NaCl in Buffer C. Steady-state kinetic analysis indicated that the purified tobacco-leaf PEPC had a K_m (total PEP) of 95 μM and a specific activity of 35 U/mg at pH 8.0, 30°C. These kinetic properties are essentially identical to those reported previously for the enzyme purified from suspension-cultured tobacco cells [9]. In contrast, the corresponding values for dark-form sorghum PEPC were 1.25 mM and 47 U/mg, respectively [15]. Under suboptimal assay conditions, tobacco-leaf PEPC had an I_{50}(L-malate) value of 0.14 mM (at 0.2 mM PEP (2-times K_m), pH 7.3), while that for the dark-form sorghum enzyme was 0.15 mM (at 2.5 mM PEP (2-times K_m), pH 7.3) [15]. This high sensitivity to inhibition by L-malate suggests that the purified tobacco-leaf enzyme was isolated with its N-terminal regulatory-phosphorylation domain intact (see [7,15–17]).

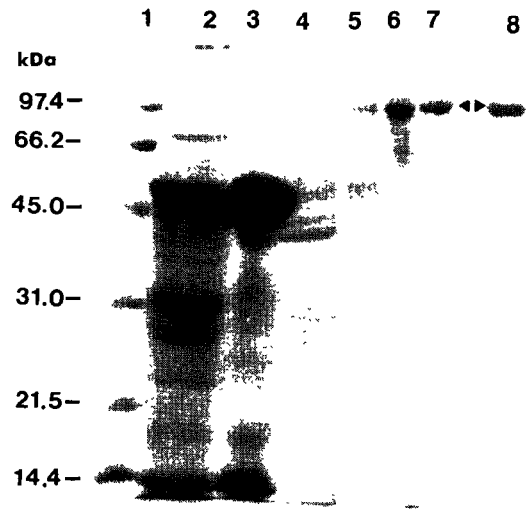

Fig. 1. Purification of tobacco-leaf PEPC. Samples from the various purification steps (see Section 2.1. and Table I) were analyzed by 12% SDS-PAGE [14] and stained with Coomassie brilliant blue. Lane 1, mol.wt. markers (values in kDa); Lane 2, crude extract (200 μg protein); Lane 3, PEG fractionation (200 μg); Lane 4, hydroxylapatite (20 μg); Lane 5, phenyl-Sepharose (20 μg); Lane 6, Mono Q (10 μg); Lane 7, Superose 12 (8 μg); Lane 8, purified dark-form maize PEPC (15 μg). Filled arrowheads indicate the ~110-kDa PEPC monomer.

3.2. *In vitro phosphorylation of tobacco-leaf PEPC*

The purified tobacco enzyme was readily phosphorylated by the Ca^{2+}-independent [14] maize-leaf PEPC-kinase in a reconstituted, in vitro phosphorylation system (Fig. 2B, lane 2). The maize PK *exclusively* phosphorylates Ser^8 and Ser^{15} of sorghum and maize C_4-PEPC, respectively [15,18]. Thus, by analogy, the in vitro phosphorylation of tobacco-leaf PEPC by this C_4 PEPC-kinase likely occurs at Ser^{11}, the enzyme's corre-

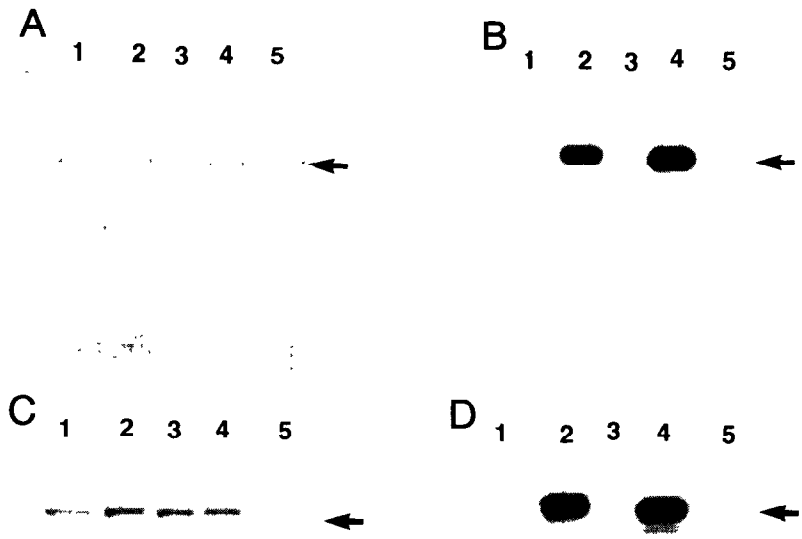

Fig. 2. Phosphorylation of tobacco and maize-leaf PEPCs by maize (A,B) or tobacco-leaf (C,D) PEPC-kinase. Purified PEPC (2 μg) was incubated in the 40-μl reconstituted, in vitro phosphorylation system (pH 8.0, 0.1 mM [γ-^{32}P]ATP, 5 mM $MgCl_2$, 0.1 mM EGTA, 10 nM microcystin-LR, 30°C (see details in [14]) with (lanes 2,4) or without (lanes 1,3) PEPC-PK from maize (A,B) or tobacco (C,D). Lanes 1,2: tobacco-leaf PEPC; Lanes 3,4: dark-form maize PEPC; Lanes 5: PEPC-PK without PEPC. (A,C) Coomassie blue-stained gels; (B,D) Corresponding autoradiographs. The PEPC subunit is indicated by arrows.

sponding structural homolog located within its Glu-Lys-Leu-Ala-Ser phosphorylation motif ([12] see also [13,18]).

In addition to this heterologous, reconstituted C_3/C_4 phosphorylation system, a tobacco-leaf PK was partially purified by exploiting a protocol similar to that recently developed for maize-leaf PEPC-kinase (see Section 2.3. and [14]). Chromatographically, the tobacco PK was very similar to that from illuminated maize leaves in that both eluted well within the fractionation range of an Ultrogel AcA 54 size-exclusion column (90-kDa exclusion limit), both were eluted from an hydroxylapatite column at 0.08–0.1 M phosphate, and both bound relatively weakly to blue dextran-agarose and strongly to phenyl-Sepharose (data not shown) (see [14]). Notably, the partially purified tobacco-leaf PK phosphorylated purified tobacco PEPC, as well as the dark-form maize enzyme, in a Ca^{2+}-independent manner (Fig. 2D, lanes 2 and 4, respectively).

3.3. *Concluding remarks*

This study represents the first report of the in vitro phosphorylation of purified C_3-leaf PEPC. The findings from the reciprocal phosphorylation reactions with the tobacco and maize protein-substrates and PEPC-kinases (Fig. 2) indicate that Ser[11] of tobacco PEPC is the likely target residue, situated in the plant-invariant Glu/Asp-Lys/Arg-X-X-Ser phosphorylation motif near the N-terminus [12,13,18]. Since the more primitive PEPC enzyme-forms in bacteria and cyanobacteria lack this N-terminal phosphorylation domain ([7,13] and references therein), we propose that the addition of this motif was an early step in the molecular evolution of C_3, C_4 and CAM PEPC. In addition, the isolation of a protein kinase from illuminated tobacco leaves with properties very similar to C_4-leaf PEPC-kinase (see Section 3.2.

Table I

Purification of PEP carboxylase from tobacco leaves (120 g)

Purification step[a]	Protein (mg)	Activity[b] (U)	Specific activity[b] (U/mg)	Recovery (%)	Purification (fold)
Crude extract	1,680	49.2	0.029	100	1.0
0–8.5% PEG	1,200	46.3	0.039	94	1.3
Hydroxylapatite	45.5	27.9	0.61	57	21.0
Phenyl-Sepharose	5.9	15.2	2.58	31	89.0
Mono Q	0.52	11.1	21.3	23	734
Superose 12	0.22	6.2	28.2[c]	13	972

[a]See Fig. 1 and Section 2.1. [b]Assayed at pH 7.3, 2.5 mM PEP, 30°C. One unit (U) = μmol/min. [c]At pH 8.0, this value was 35 U/mg.

and [14]) lends support to the recent hypothesis that C_3-leaf PEPC, like the C_4 and CAM isoforms [7,8], is also subject to regulatory phosphorylation in vivo [11,19].

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