Phosphorylation of Phosphoenolpyruvate Carboxylase from *Crassula argentea*

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Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) was purified to electrophoretic homogeneity from *Crassula argentea* leaves according to established methods incorporating DEAE, hydroxylapatite, and Mono Q chromatography. The purified enzyme had a specific activity of 20–25 units/mg of protein. Purified PEPC was further processed by blue agarose affinity chromatography and gel filtration to help ensure the removal of potential contaminating kinases. Autoradiography revealed that phosphorylation of PEPC occurred when the purified enzyme was incubated with $[\gamma\text{-}^{32}P]ATP\text{-}Mg^{2+}$. Radiolabel was not incorporated when $[\alpha\text{-}^{32}P]ATP\text{-}Mg^{2+}$ was utilized as substrate. Phosphorylation of the PEPC culminated in its activation: the K_i for L-malate increased 2.5-fold while the maximum inhibition dropped from 73 to 39% and the K_m for magnesium phosphoenolpyruvate dropped from 69 to 53 μM. These data are consistent with phosphorylation sensitive PEPC from *C. argentea* possessing an auto-kinase function or, alternatively, the copurification of a kinase that possesses a high specific activity and tightly associates with PEPC.

Keywords: *Phosphoenolpyruvate carboxylase; phosphorylation*

INTRODUCTION

Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) facilitates the initial fixation of atmospheric CO_2 during C_4 and Crassulacean acid metabolism (CAM) photosynthesis (Andreo et al., 1987; Hatch, 1992; O'Leary, 1982; Ting, 1985) by catalyzing the irreversible carboxylation of phosphoenolpyruvate (PEP) to yield oxaloacetate and P_i. L-Malate, a principal intermediate in these adaptive photosynthetic pathways, negatively controls PEPC activity.

The diurnal fluctuation of PEPC properties in C_4 and CAM leaves, however, is regulated primarily through the reversible phosphorylation of an N-terminal serine residue (Nimmo et al., 1984; Jiao and Chollet, 1991; Jiao et al., 1991a; Lepiniec et al., 1994; Brulfert et al., 1986; Kluge et al., 1988). Upon phosphorylation, PEPC expresses a lower K_m for PEP and is less sensitive to feedback inhibition by L-malate, resulting in increased catalytic activity under physiological assay conditions (suboptimal pH and at rate-limiting concentrations of PEP) (Jiao and Chollet, 1991; Jiao et al., 1991a; Nimmo et al., 1986).

The phosphorylation state of PEPC in C_4 and CAM plants appears to be governed largely by the activity of a Ca^{2+}-independent PEPC–(serine)kinase. A protein–serine kinase able to phosphorylate PEPC has been partially purified from *Mesembryanthemum crystallinum* (Li and Chollet, 1994), *Bryophyllum fedtschenkoi* (Carter et al., 1991), an inducible CAM plant, and maize (Wang and Chollet, 1993; McNaughton et al., 1991), a C_4 plant. The kinase has an approximate molecular weight of 30–35 kDa.

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Our studies with electrophoretically pure CAM PEPC, isolated from *Crassula argentea*, showed that phosphorylation of the enzyme occurred without addition of an exogenous kinase. This observation led us to examine whether the observed phosphorylation generated the requisite change in PEPC activity necessary to be considered "regulatory" and to determine if PEPC phosphorylation may have resulted from a contaminating copurified kinase or an undisclosed bifunctional activity for this PEPC.

MATERIALS AND METHODS

Buffers. Extraction buffer: 50 mM Hepes–NaOH (pH 8.0), 1 mM EDTA, 2 mM DTT, 200 mM KCl, 10 mM $MgCl_2$, 5 mM L-malate, 0.1 mg/mL MBTZ, 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL leupeptin, 5 μg/mL aprotinin, 1% (w/v) soluble PVP, and 1% (w/v) PEG-8000. Buffer A: 50 mM Hepes–NaOH (pH 7.0), 1 mM EDTA, 1 mM DTT, 5 mM L-malate, 1 mM NaF, and 10% (w/v) glycerol. Buffer B: 50 mM potassium phosphate (pH 7.4), 5 mM l-malate, 1 mM EDTA, 1 mM DTT, 1 mM NaF, and 10% (w/v) glycerol. Buffer C: 20 mM Hepes–NaOH (pH 8.0), 2 mM L-malate, 1 mM EDTA, 1 mM DTT, and 5% (w/v) glycerol.

Purification of PEPC. PEPC was purified from leaves of the CAM plant *C. argentea* by modifying the procedure of Meyer et al. (1988). Leaves harvested in the early afternoon were placed immediately on ice, rinsed twice with cold distilled water, and blotted dry. Leaf material (250 g) was frozen with liquid nitrogen and ground to a fine powder in a Waring commercial blender (model 33BL12) set on medium speed. The pulverized leaf material was added to 500 mL of extraction buffer and homogenized with a Polytron homogenizer. The homogenate was filtered through four layers of cheesecloth and then centrifuged at 16000*g* for 25 min to remove insoluble material. The supernatant was fractionated with PEG-8000 in increments of 5 and 13% (w/v), respectively. Following centrifugation, the 5–13% PEG precipitate was resuspended in buffer A. The suspension was clarified by centrifugation at 10000*g* for 10 min and applied to a DEAE-Fractogel 650M

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column (1.5 × 15 cm) equilibrated with buffer A. Contaminating proteins were eluted from the column in a stepwise fashion by increasing the NaCl concentration in buffer A to 50, 100, and 200 mM. PEPC was eluted either with a linear 200−300 mM NaCl gradient or with a 250 mM NaCl batch wash. The flow rate was 1 mL/min. PEPC was concentrated by bringing the active fractions to 60% saturation with ammonium sulfate. Following centrifugation, precipitated protein was suspended in buffer B and the buffer change completed by passage through a Bio-Rad, Econo-Pac 10DG desalting column, also equilibrated in buffer B. This sample was loaded onto an HAP column (0.5 × 18 cm) equilibrated with buffer B. The column was washed with buffer B at a flow rate of 0.5 mL/min, and then the phosphate concentration in buffer B was increased to 100 mM. PEPC was eluted with a 100−300 mM potassium phosphate linear gradient. Active fractions were pooled, and the protein was concentrated by precipitation with 60% ammonium sulfate. The pellet was suspended in a minimal volume of buffer C and the buffer change completed by using a Bio-Rad desalting column. This sample was applied to a Pharmacia Mono Q HR 5/5 anion-exchange column equilibrated with buffer C plus 100, 200, and 300 mM NaCl. PEPC was eluted by applying a 300−500 mM NaCl linear gradient. Fractions with PEPC activity were pooled, concentrated, and then equilibrated in a storage buffer of 50 mM Hepes−NaOH (pH 7.2), 0.5 mM EDTA, and 1 mM DTT with a Centricon 100 (Amicon) membrane filtration apparatus. To reduce the possibility of contamination by a protein−serine kinase, the purified PEPC was applied to an Affi-Gel blue affinity column followed by gel filtration; these techniques were central to the purification of the PEPC protein−serine kinase from maize leaves (Wang and Chollet, 1993; McNaughton et al., 1991; Jiao and Chollet, 1989), *M. crystallinum* (Li and Chollet, 1994), and *B. fedtschenkoi* (Carter et al., 1991). PEPC, equilibrated with 50 mM Tris-HCl (pH 7.5), 1 mM DTT, and 5% (v/v) glycerol, was applied to a Bio-Rad Affi-Gel blue (4%) agarose column (0.5 × 10 cm) at a flow rate of 0.5 mL/min. The column was washed with equilibration buffer until the absorbance baseline at 254 nm was re-established, at which point 500 mM NaCl was passed through the column to ensure protein elution. PEPC (present in the wash fractions) was concentrated with a Centriconn 100 (Amicon) membrane filtration unit and applied to a Superdex 200 HR 10/30 (Pharmacia) FPLC gel filtration column connected to an HPLC system (Waters 600 E system controller and 486 tunable absorbance detector, adjusted to 280 nm, and Hewlett-Packard 3396A integrator). The column, equilibrated with 10 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 1 mM 2-mercaptoethanol, and 150 mM NaCl, was calibrated with Bio-Rad protein standards and operated at a flow rate of 0.5 mL/min. Fractions containing PEPC activity were concentrated with a Centricon 100 (Amicon) membrane filtration apparatus. Protein chromatograms were derived from the absorbance obtained either at 254 nm (when using a Pharmacia single path UV-1 monitor) or at 280 nm (when using the Waters tunable absorbance detector).

Chromatofocusing. PEPC (purified by DEAE, HAP, and Mono Q chromatography) was applied to a Mono P HR 5/5 Pharmacia chromatofocusing column connected to the HPLC system described above. The chromatofocusing column was equilibrated with 25 mM histidine−HCl (pH 6.5). The pH gradient was established by passing 25 mL of a 1/8 PBE 74 (pH 4.0) solution over the column at a flow rate of 1 mL/min. PEPC was phosphorylated in a reaction medium of 50 mM Hepes (pH 7.5), 1 mM DTT, 5% (v/v) glycerol, 10 mM MgCl₂, and 0.5 mM ATP (control samples had no ATP) for 10 or 30 min prior to chromatofocusing. The ATP concentration used was 10-fold higher than the concentration of PEPC and should not be regarded as limiting as the reported $K_{m,ATP}$ for PEPC−kinase is 25 μM (Baur et al., 1992).

Assays. PEPC was assayed according to the method of Meyer et al. (1991) in a Perkin-Elmer Lambda 4B UV−vis spectrophotometer or a Hewlett-Packard 8452A diode array spectrophotometer by monitoring the decrease in absorbance at 340 nm from the oxidation of NADH in an assay system coupled to MDH or MDH and LDH. The temperature was maintained at 25 °C by a circulating water bath. The routine assay (1 mL) included 50 mM Aces (pH 7.0), 5 mM free Mg²⁺ (8.34 mM total Mg²⁺), 5 mM HCO₃⁻, 0.2 mM NADH, 1 unit of MDH or 1 unit each of MDH and LDH, and 2.61 mM Mg−PEP (7.61 mM total PEP). Reactions were initiated by the addition of PEPC. Rates were typically calculated between 0 and 2 min. One unit of PEPC activity is defined as the amount of enzyme that corresponds to the oxidation of 1 μmol of NADH/min by the coupling enzyme(s) at 25 °C.

Radiometric Kinase Assay. Phosphorylation of PEPC was performed in conditions based on those of Jiao and Chollet (1989). PEPC (5−10 μg) was incubated in a standard assay medium of 70 mM potassium phosphate (pH 7.5), 2 mM DTT, 10 mM MgCl₂, 10% (v/v) glycerol, and 60 μM ATP (10 μCi of [γ-³²P]ATP, specific radioactivity = 4500 Ci/mmol, ICN) in a total volume of 22 μL. The total concentration of ATP used was at least 10-fold higher than the concentration of PEPC. Incubation was carried out at 25 °C for 30 min. Reactions were terminated by adding an equal volume of 2× SDS sample buffer [60 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 20% (v/v) glycerol, 1 mM 2-mercaptoethanol, and 0.009% (w/v) bromophenol blue] and boiling for 5 min. The samples were electrophoresed by SDS−PAGE and stained for protein with Coomassie Blue. ³²P incorporation was detected by autoradiography on X-ray film (Kodak X-OMAT AR) at room temperature for 8−12 h with an intensifying screen. Factors influencing the rate of ³²P incorporation were assessed by substituting [α-³²P]ATP or [α-³²P]dATP or supplementing the standard assay with 10 mM L-malate, 10 mM EDTA, or 5 mM arsenate. The level of incorporation of radiolabel was measured by scanning the autoradiographic film on an FB910 densitometer (FisherBiotech), which permitted the relative intensities to be quantified, or by Cerenkov counting of the PEPC subunit band excised from the destained gel.

Protein Concentration. Soluble protein was determined with the Bio-Rad protein assay procedure, based on the dye-binding method of Bradford (1976) and using bovine serum albumin as the protein standard.

Electrophoresis. SDS−PAGE was performed in a polyacrylamide slab gel (158 × 140 × 1.0 mm) according to the method of Laemmli (1970). The gel contained a 10% acrylamide resolving gel and a 4.5% acrylamide stacking gel. Protein samples were diluted 1:1 with 2× SDS sample buffer, heated at 100 °C for 5 min, and electrophoresed at a constant 100 V. Gels were stained with Coomassie Blue, dried, and autoradiographed. Native-PAGE was conducted in the same apparatus with a 7% acrylamide resolving gel and a 3% acrylamide stacking gel (Willeford and Wedding, 1987). The gel was soaked in radiometric kinase assay medium for 20 min, proteins were fixed, and the gel was washed with an acetic acid/methanol solution [10%/50% (v/v), respectively], then stained for protein, washed exhaustively with 10% (v/v) acetic acid, then washed as before with acetic acid/methanol, and autoradiographed.

Effect of Phosphorylation on the Kinetics of PEPC. PEPC was phosphorylated by incubating the enzyme in a solution containing 5.0 mM MgCl₂, 1.0 mM DTT, and 0.5 mM ATP. A control set of enzyme samples was prepared by incubating PEPC in a solution containing 5.0 mM MgCl₂ and 1.0 mM DTT. Mg−PEP saturation profiles ranging from 0.01 to 3 mM Mg−PEP were generated with and without L-malate (0.5 and 1.5 mM). These data were fit to the Michaelis−Menten equation; activation by phosphorylation was determined by the difference in V_{max}. The K_i for L-malate was determined by generating a malate saturation profile (0−10 mM L-malate) in an assay containing 1.3 mM Mg−PEP; data were fit to a modified form of the Michaelis−Menten equation (Meyer et al., 1991)

$$\%I = \frac{\%I_{max}[I]^{nH}}{K_i^{nH} + [I]^{nH}}$$

where $\%I = (V_0 - V_i)/V_0$; V_0 = rate in the absence of inhibitor velocity, V_i = rate in the presence of inhibitor, I_{max} = maximum

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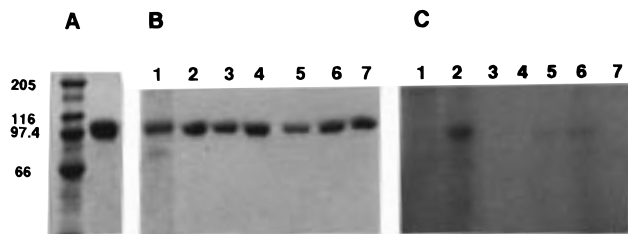
Figure 1. SDS−PAGE electrophoretogram and autoradiogram of *Crassula* PEPC. Panels A and B show an SDS−PAGE electrophoretogram stained with Coomassie Blue. PEPC (25 μg) was electrophoresed alongside molecular standards (myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; and bovine serum albumin, 66 kDa) in panel A. Panel C shows the corresponding autoradiogram for panel B. Ten microgram samples of PEPC from maize [C_4 (lane 1)] and *Crassula* [CAM (lanes 2−7)] were incubated in a phosphorylation reaction medium containing [γ-^{32}P]ATP (lanes 1, 2), [α-^{32}P]ATP (lane 3), [γ-^{32}P]ATP + 2 mM EDTA (lane 4), [γ-^{32}P]-ATP + 5 mM arsenate (lane 5), [γ-^{32}P]ATP + 10 mM L-malate (lane 6), and [γ-^{32}P]ATP + 2X SDS buffer at time 0 min (lane 7); see Materials and Methods for details.

percent inhibition, I = concentration of inhibitor, K_i = inhibition constant, and nH = the Hill number indicating cooperativity of the reaction.

RESULTS AND DISCUSSION

Purification of Phosphoenolpyruvate Carboxylase. PEPC, isolated from the light-adapted leaves of *C. argentea*, was purified to apparent electrophoretic homogeneity and possessed a maximal specific activity of 25.2 units/mg. PEPC appeared as a single protein band with a molecular weight of ∼100 kDa on SDS−PAGE (Figure 1A). CAM plants are ecologically adapted to arid environments where water conservation is critical for survival. Carbon dioxide is fixed only during the cool nights when gaseous exchanges are permitted through stomatal opening. PEPC, therefore, is most active during the night, when it is poised in the phosphorylated tetrameric state. The relatively inactive day-form of PEPC is dimeric and unphosphorylated. The purified unphosphorylated day-PEPC was used as substrate for monitoring PEPC−serine kinase activity. Surprisingly, exogenous addition of kinase was not required for PEPC phosphorylation. To separate PEPC from possible contamination by the low-abundance PEPC−kinase, the purified PEPC was subjected to blue agarose affinity chromatography and gel filtration; similar procedures were found to be effective previously (Li and Chollet, 1994; Wang and Chollet, 1993; McNaughton et al., 1991; Jiao and Chollet, 1989). PEPC, as expected, did not bind to the blue agarose column (an "affinity" resin for the serine−kinase) and resolved as a single 400 kDa protein peak from the gel filtration column (Figure 2). Protein eluted from the gel filtration column as the PEPC homotetramer, expressing a specific activity of 22 units/mg. Evidence of a copurifying protein was not found. It remains possible, however, that under these relatively mild, native conditions a small amount of contaminating kinase could still be present. It would, however, have to be tightly associated with the PEPC to avoid (1) binding to the large excess of "affinity" matrix (blue agarose), (2) separation from PEPC in the presence of 150 mM NaCl during size exclusion chromatography (a kinase with a MW of 30−35 kDa should easily be resolved from 400 kDa PEPC), and (3) separation from PEPC during concentration/
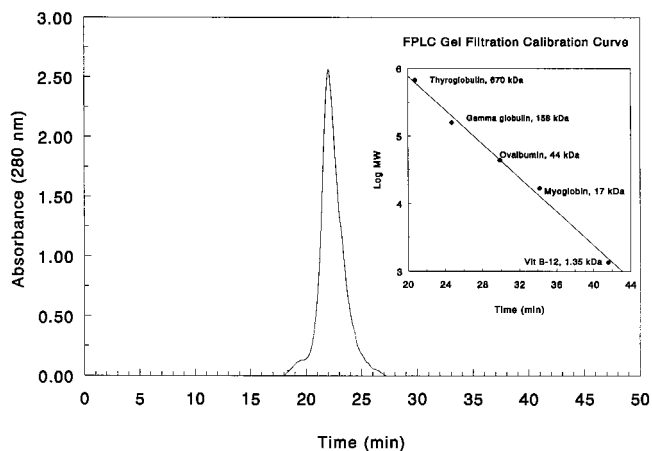
Figure 2. FPLC gel filtration chromatogram of purified PEPC. PEPC which did not bind to the Affi-gel blue agarose "affinity" matrix was concentrated and then analyzed by gel filtration chromatography with a Superdex 200 HR. A 4.8 mg sample was loaded and eluted at 0.5 mL/min.
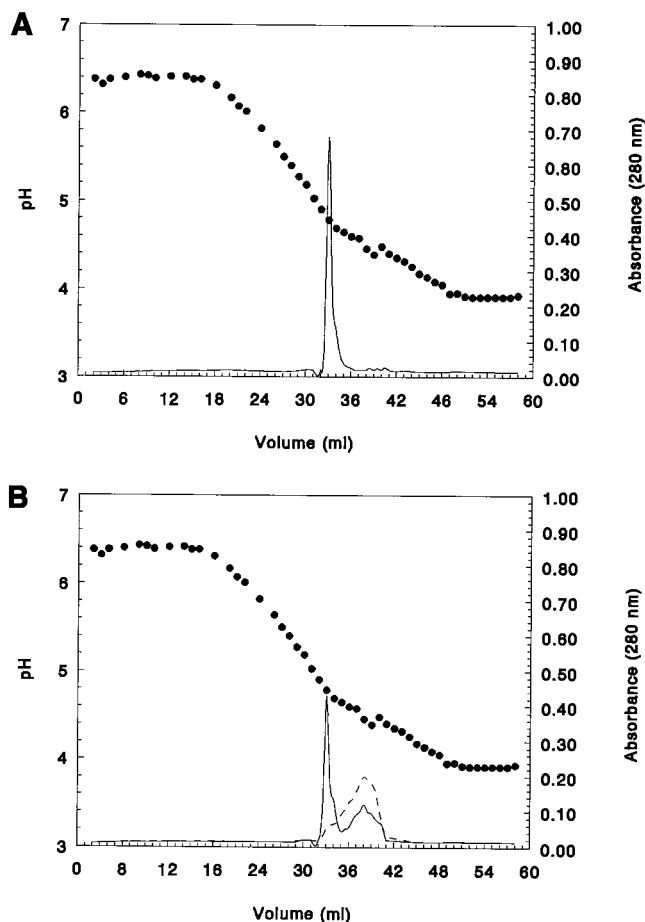
dialysis through a filter that has an approximate molecular weight cutoff of 100 kDa (Centricon 100) when initial salt concentrations were appreciably >1 M.

Kinase activity could not be eliminated from preparations of PEPC from the facultative CAM plant *M. crystallinum* L. (Baur et al., 1992) or from banana fruit (Law and Plaxton, 1997) even though blue dextran/agarose affinity chromatography and gel filtration were employed. Kinases appear to bind target proteins through a specific binding groove and hydrogen bonding (Kemp et al., 1995). If the PEPC−PEPC kinase interaction is stable throughout the purification protocol, it may also serve an in vivo role in regulating PEPC activity.

Chromatofocusing. PEPC, incubated with and without Mg-ATP, was resolved by chromatofocusing (pH 6.5−4.0) into two distinct protein peaks. PEPC samples not exposed to Mg-ATP eluted as a single sharp peak with a pI of 4.8 (Figure 3A). When Mg-ATP was introduced for 10 min, a portion of the PEPC resolved with a pI of 4.3 (Figure 3B). After 30 min, 90% of the protein profile shifted in the acidic direction, consistent with a phosphorylation event. Unfortunately, instability of enzyme activity due to low pH made this procedure undesirable as a means for isolating bulk quantities of phosphorylated PEPC for purposes of kinetic analysis. The results, however, support the observation that the PEPC purified was a single protein entity, which upon incubation with Mg-ATP became more negatively charged (e.g., phosphorylated). The breadth of the phosphorylated PEPC peak (Figure 3B) may be attributed to either the phosphorylated state of tetrameric enzyme, which allows for the addition of one to four phosphates, or a reflection of the pH slope, which has an inflection point in this region.
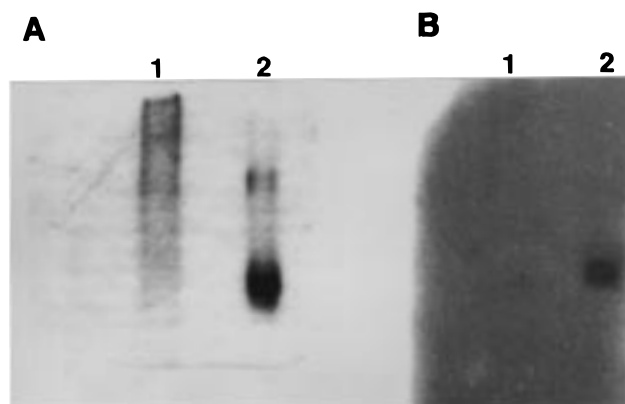
Radiometric Kinase Assay. PEPC was phosphorylated by incubating purified PEPC with [γ-^{32}P]ATP as described under Materials and Methods. Phosphorylation was detected by autoradiography after resolution by SDS−PAGE. PEPC became intensely labeled with ^{32}P, a result that remained evident even after blue agarose and gel filtration chromatography (Figure 1C, lane 2). Cerenkov counting revealed that the maximal molar ^{32}P incorporation was 0.87 per 100 kDa subunit. PEPC remained unlabeled when [γ-^{32}P]ATP was substituted in the incubation medium with [α-^{32}P]ATP

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Figure 3. PEPC was incubated in a Mg-ATP phosphorylation reaction medium for 10 or 30 min and resolved by analytical chromatofocusing (pH 6.5−4.0, ●). Panel A shows how PEPC resolves without phosphorylation. Panel B shows how incubation with Mg-ATP induces an acidic modification: 10 min (—); 30 min (- - -). Protein elution was monitored at 280 nm.

Figure 4. Panel A shows a native-PAGE electrophoretogram stained for protein with Coomassie Blue for 20 μg samples of maize PEPC purchased from Sigma Chemical Co. (lane 1) and *Crassula* PEPC purified as described under Materials and Methods (lane 2). Panel B shows the corresponding autoradiogram.

(Figure 1C, lane 3) or [α-^{32}P]dATP (data not shown), confirming a terminal phosphate transfer. When 10 mM EDTA or 5 mM arsenate was included in the phosphorylation assay medium, incorporation of label into PEPC was inhibited by 100 and 88%, respectively (Figure 1C, lanes 4 and 5, respectively). When 10 mM L-malate was present in the phosphorylation assay medium, incorporation of label into PEPC was inhibited by 91% (Figure 1C, lane 6). Phosphorylation of PEPC was not observed when hot SDS sample buffer was added into the phosphorylation assay medium at time zero (Figure 1C, lane 7). Phosphorylated PEPC, obtained by chromatofocusing (Figure 3B), did not incorporate additional phosphate under the radiometric conditions described because the target N-terminal serine residue was already phosphorylated. Alternatively, diminished kinase activity due to pH denaturation or resolution of kinase activity (which may have a p$I \neq$ 4.3) from phosphorylated PEPC (pI 4.3) could be invoked. When maize PEPC, as purified by us in a similar manner, was incubated with [γ-^{32}P]ATP under the same experimental conditions, no incorporation of radiolabel was observed (Figure 1C, lane 1). A crude maize serine−kinase was prepared by ammonium sulfate fractionation (0−60%) and blue agarose affinity chromatography (Jiao and Chollet, 1989). This crude kinase preparation catalyzed phosphorylation of the purified maize PEPC (data not shown). It was impos-

sible to discern whether the crude maize kinase (or a kinase derived from *Crassula*) specifically phosphorylated the *Crassula* PEPC because its addition was not required for phosphorylation, and the degree of phosphorylation observed for the *Crassula* PEPC already approached the maximal regulatory level of one per subunit. The phosphorylation of the *Crassula* PEPC appeared to be enzymatic in nature. The reaction required a divalent metal (e.g., Mg^{2+}), was inhibited by arsenate (a competitive inhibitor of phosphorylation reactions), and could be halted if the enzyme was denatured by heat. Phosphorylation was not an artifact brought about by a tight association with ATP because only terminally labeled ATP generated phosphorylated enzyme. Procedures previously shown to separate kinase activity from PEPC were used in these experiments. As expected, we verified the importance for kinase-induced phosphorylation in maize, but not in *Crassula*.

Native gel electrophoresis was performed on purified CAM (*Crassula*) and C$_4$ (maize) PEPC in an effort to resolve PEPC from any kinase activity. Autoradiography was conducted after the gel had been incubated in a radiometric Mg-ATP solution for 20 min. Label was incorporated into the *Crassula* PEPC sample (Figure 4). Maize PEPC did not resolve as distinctly as the *Crassula* PEPC, but there was no evidence of incorporated label. Clearly, kinase activity remains associated with our purified *Crassula* PEPC after a variety of native separation protocols. Contaminating proteins (e.g., kinase) were also not evident after SDS−PAGE, even when 20 μg of sample was applied and silver stain was employed to detect protein (data not shown).

Effect of Phosphorylation on the Kinetics of PEPC. Critical to the nature of PEPC phosphorylation is the regulatory control that is brought about. If the observed phosphorylation does not result in the required kinetic control, then the event is meaningless. It has been shown that the phosphorylated and dephosphorylated forms of PEPC express differences in kinetic properties, especially in sensitivity to L-malate inhibition. PEPC extracted from CAM leaves collected at night (which are primarily phosphorylated) expresses lower K_m (PEP) and higher K_i (L-malate) values than the enzyme from leaves harvested during the day (Brulfert et al., 1986; Kluge et al., 1988; Nimmo et al., 1986, 1987; Baur et al., 1992; Kruger and Kluge, 1987;

Table 1. Effects of Mg-ATP-Initiated Phosphorylation on PEPC

	L-malate (mM)					
	control			+ Mg ATP (phosphorylated PEPC)		
	0	0.5	1.5	0	0.5	1.5
$K_{m(Mg-PEP)}$, μM	69	114	224	53	87	107
V_{max}, nmol/min	280	221	217	289	282	268
% inhibition		21	23		2.4	7.3

[a] Mg-PEP isotherms were generated with and without L-malate for PEPC which had been incubated with and without Mg-ATP (see Materials and Methods for details). Assays were initiated by the addition of 14 μg of PEPC.
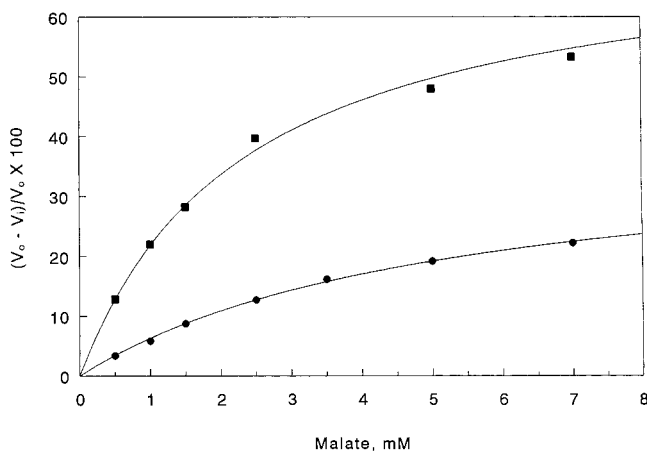
Figure 5. L-Malate inhibition in control (■) and phosphorylated (●) samples of *Crassula* PEPC. L-Malate saturation profiles were generated by assaying PEPC at 1.3 mM Mg-PEP and varying the L-malate concentration from 0 to 10 mM. The data were fitted to a modified Michaelis–Menten equation: $\%I = (V_0 - V_i)/V_0 = (\%I_{max} \times [I]^{nH})/(K_i^{nH} + [I]^{nH})$.

Winter, 1982). Table 1 shows the effect of phosphorylation on the kinetic characteristics of purified *Crassula* PEPC. L-Malate inhibition is not as pronounced when the enzyme is phosphorylated; in the presence of L-malate, the K_m for Mg–PEP is lower and its activity is higher. Phosphorylated enzyme assayed in the presence of 0.5 mM L-malate reveals almost 30% activation over unphosphorylated enzyme and has the same V_{max} as PEPC assayed without L-malate. The apparent K_i for L-malate, determined at a constant level of Mg–PEP (1.3 mM), increased from 2.06 to 5.12 mM upon phosphorylation, a 2.5-fold difference (Figure 5). As much as a 5.3-fold difference was observed. This may reflect some loss of the regulatory N-terminal serine sequence (<20 residues) during purification. Terminal sequences that are not packed into the globular core of the protein are susceptible to proteolysis. In the absence of the protease inhibitor chymostatin, a 128 amino acid N-terminal sequence was removed from the PEPC isolated from *M. crystallinum* L. (Baur et al., 1992). In our preparation, the maximum inhibition induced by L-malate dropped from 73.1 to 38.8% upon phosphorylation, demonstrative of PEPC's reduced sensitivity to L-malate.

CONCLUDING REMARKS

Phosphorylation of a plant-invariant regulatory N-terminal serine residue introduces a negative charge to this domain and activates PEPC. Dephosphorylation apparently results from nonspecific phosphatase activ-

ity, whereas a serine kinase with relative specificity for PEPC is responsible for phosphorylation. Kinase activity remained associated with the purified PEPC isolated from *C. argentea* despite purification attempts that had proven successful for other sources. The enzyme was electrophoretically homogeneous, became phosphorylated when incubated with Mg-ATP, and, when phosphorylated, displayed reduced sensitivity to inhibition by L-malate. The origin and nature of the kinase activity responsible for the phosphorylation of PEPC remain in question. A small amount of copurifying kinase could be present [the PEPC serine–kinase from maize is considered to be a low-abundant protein (Wang and Chollet, 1993)], or this *Crassula* PEPC may have bifunctional activity–carboxylase and kinase. It would be difficult for a small amount of contaminating kinase to phosphorylate all of the PEPC present as the statistical distribution of phosphorylated PEPC to PEPC becomes small. A phosphorylation molar ratio of 0.87 was determined; a value of 1 seems improbable as some of the PEPC may already be phosphorylated or its N terminus truncated. The induction (Jiao et al., 1991b; Carter et al., 1991) or activation of such an efficient PEPC serine–kinase would, however, provide for a very efficient PEPC regulatory cascade.

The data also support the possibility that this PEPC may be bifunctional. Hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate is a bifunctional enzyme that catalyzes both the synthesis and degradation of Fru-2,6-P_2. A point mutation (serine-404 to histidine) converted the monofunctional yeast 6-phosphofructo-2-kinase into the bifunctional enzyme (Kretschmer et al., 1993). Enzymes have also been found to exist in monofunctional or bifunctional forms depending on their tissue source or cellular location (Iwasaki et al., 1993). The insertion of a protein–serine/threonine kinase catalytic domain into this *Crassula* PEPC is highly unlikely because the subunit molecular weight (100 kDa) is consistent with all other CAM, C_4, and C_3 PEPCs. A sequence homology comparison between known kinase domains and *Crassula* PEPC may, however, prove interesting. Regulatory control of such a potential auto-kinase function would also be an effective means by which PEPC activity could be modulated. In any case, the specificity and regulation of the kinase reaction in *Crassula* merit further investigation.

ABBREVIATIONS USED

PEPC, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Aces, *N*-(2-acetamido)-2-aminoethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; MBTZ, 2-mercaptobenzothiazole; PVP, polyvinylpolypyrrolidone; PEG, polyethyelene glycol; PAGE, polyacrylamide gel electrophoresis; CAM, Crassulacean acid metabolism; FPLC, fast protein liquid chromatography; HAP, hydroxylapatite; MDH, L-malate dehydrogenase; LDH, lactate dehydrogenase; PBE, poly buffer exchange.

LITERATURE CITED

Andreo, C. S.; Gonzales, D. H.; Iglesias, A. A. Higher plant phosphoenolpyruvate carboxylase: Structure and regulation. *FEBS Lett.* **1987**, *213*, 1–8.

Baur, B.; Dietz, K. J.; Winter, K. Regulatory protein phosphorylation of phosphoenolpyruvatecarboxylase in the faculta-

tive Crassulacean-acid-metabolism plant *Mesembryanthemum crystallinum* L. *Eur. J. Biochem.* **1992**, *209*, 95−101.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities ofprotein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Brulfert, J.; Vidal, J.; Marechal, P. L.; Queiroz, O.; Kluge, M.; Kruger, I. Phosphorylation/dephosphorylation process as a probable mechanism for the diurnalregulatory changes of phosphoenolpyruvate carboxylase in CAM plants. *Biochem. Biophys. Res. Commun.* **1986**, *136*, 151−159.

Carter, Paul; Nimmo, H. G.; Fewson, Charles; Wilkins, Malcolm. Circadian rhythms in the activityof a plant protein kinase. *EMBO J.* **1991**, *10* (8), 2063−2068.

Hatch, M. D. C_4 Photosynthesis: An unlikely process full of surprises. *Plant Cell Physiol.* **1992**, *33*, 333−342.

Iwasaki, Y.; Shimoi, H.; Saiki, H.; Nishikawa, Y. Tissue-specific molecular diversity of amidatingenzymes (peptidylglycine α-hydroxylating monooxygenase and peptidyl-hydroxyglycine N−C lyase) in *Xenopus laevis. Eur. J. Biochem.* **1993**, *214*, 811−818.

Jiao, J.-A.; Chollet, R. Regulatory seryl-phosphorylation of C_4 phosphoenolpyruvate carboxylase by a soluble protein kinase from maize leaves. *Arch. Biochem. Biophys.* **1989**, *269*, 526−533.

Jiao, J.-A.; Chollet, R. Posttranslational regulation of phosphoenolpyruvate carboxylase in C_4 and Crassulacean acid metabolism plants. *Plant Physiol.* **1991**, *95*, 981−985.

Jiao, J.-A.; Echevarria, C.; Vidal, J.; Chollet, R. Protein turnover as a component in the light/darkregulation of phosphoenolpyruvate carboxylase protein-serine kinase activity in C_4 plants. *Proc. Natl. Acad. Sci. U.S.A.* **1991a**, *88*, 2712−2715.

Jiao, J.-A.; Vidal, J.; Echevarria, C.; Chollet, R. *In vivo* regulatory phosphorylation site in C_4-leaf phosphoenolpyruvate carboxylase from maize and sorghum. *Plant Physiol.* **1991b**, *96*, 297−301.

Kemp, B. E.; Parker, M. W.; Hu, S.; Tiganis, T.; House, C. Substrate and pseudosubstrate interactions with protein kinases: determinants of specificity. *Trends Biochem. Sci.* **1995**, *10*, 440−444.

Kluge, M.; Maier, P.; Brulfert, J.; Faist, K.; Wollny, E. Regulation of phosphoenolpyruvatecarboxylase in Crassulacean acid metabolism: *In vitro* phosphorylation of the enzyme. *J. Plant Physiol.* **1988**, *133*, 252−256.

Kretschmer, M.; Langer, C.; Prinz, M. Mutation of monofunctional 6-Phosphofructo-2-kinase in yeast to bifunctional 6-Phosphofructo-2-kinase/Fructose 2,6-Bisphosphatase. *Biochemistry* **1993**, *32*, 11143−11148.

Kruger, I.; Kluge, M. Diurnal changes in the regulatory properties of phosphoenolpyruvatecarboxylase in plants: Are alternations in the quaternary structure involved? *Bot. Acta* **1987**, *101*, 24−27.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophageT4. *Nature (London)* **1970**, *227*, 680−685.

Law, R. D.; Plaxton, W. C. Regulatory phosphorylation of banana fruit phosphoenolpyruvate carboxylase by a copu-

rifying phosphoenolpyruvate carboxylase-kinase. *Eur. J. Biochem.* **1997**, *247* (2), 642−651.

Lepiniec, L.; Vidal, J.; Chollet, R.; Gadal, P.; Cretin, C. Phosphoenolpyruvate carboxylase: Structure, regulation, and evolution. *Plant Sci.* **1994**, *99*, 111−124.

Li, B.; Chollet, R. Salt induction and the partial purification/characterization of phosphoenolpyruvate carboxylase protein-serine kinase from an inducible Crassulacean-acid-metabolism (CAM) plant, *Mesembryanthemum crystallinum* L. *Arch. Biochem. Biophys.* **1994**, *314*, 247−254.

McNaughton, G. A. L.; MacKintosh, C.; Fewson, C. A.; Wilkins, M. B.; Nimmo, H. G. Illumination increases the phosphorylation state of maize leaf phosphoenolpyruvatecarboxylase by causing an increase in the activity of a protein kinase. *Biochim. Biophys. Acta* **1991**, *1093*, 189−195.

Meyer, C. R.; Rustin, P.; Wedding, R. A simple and accurate spectrophotometric assay for phosphoenolpyruvate carboxylase activity. *Plant Physiol.* **1988**, *86*, 325−328.

Meyer, C. R.; Willeford, K. O.; Wedding, R. Regulation of phosphoenolpyruvate carboxylase from *Crassula argentea*: Effect of incubation with ligands and dilution on oligomeric state, activity, and allosteric properties. *Arch. Biochem. Biophys.* **1991**, *288*, 343−349.

Nimmo, G. A.; Nimmo, H. G.; Fewson, C. A.; Wilkins, M. B. Diurnal changes in the properties of phosphoenolpyruvate carboxylase in *Bryophyllum* leaves: A possible covalent modification. *FEBS Lett.* **1984**, *178* (2), 199−203.

Nimmo, G. A.; Nimmo, H. G.; Hamilton, I. D.; Fewson, C. A.; Wilkinas, M. B. Purification of the phosphorylated night form and dephosphorylated day form of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi. Biochem. J.* **1986**, *239*, 213−220.

Nimmo, G. A.; Wilkins, M. B.; Fewson, C. A.; Nimmo, H. G. Persistent circadian rhythms in the phosphorylation state of phosphoenolpyruvate carboxylase from *Bryophyllum fedtschenkoi* leaves and in its sensitivity to inhibition by malate. *Planta* **1987**, *170*, 408−415.

O'Leary, M. H. Phosphoenolpyruvate carboxylase: An enzymologist's view. *Annu. Rev. Plant Physiol.* **1982**, *33*, 297−315.

Ting, I. P. Crassulacean acid metabolism. *Annu. Rev. Plant Physiol.* **1985**, *36*, 595−622.

Wang, Y.-H.; Chollet, R. Partial purification and characterization of phosphoenolpyruvatecarboxylase protein serine-kinase from illuminated maize leaves. *Arch. Biochem. Biophys.* **1993**, *304*, 496−502.

Willeford, K. O.; Wedding, R. T. Evidence for a multiple subunit composition of the plant NAD malic enzyme. *J. Biol. Chem.* **1987**, *263*, 8423−8429.

Winter, K. Properties of phosphoenolpyruvate carboxylase in rapidly prepared, desalted leafextracts of the Crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. *Planta* **1982**, *154*, 298−308.