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Phosphorylation of phosphoenolpyruvate carboxykinase in plants

Studies in plants with C₄ photosynthesis and Crassulacean acid metabolism and in germinating seeds

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We have previously shown that phosphoenolpyruvate carboxykinase (PEPCK) is phosphorylated *in vivo* in the cotyledons of darkened cucumber seedlings and that phosphorylation is reversed by light [Walker and Leegood (1995) FEBS Lett. 362, 70–74]. In this study the molecular mass of PEPCK was estimated in a range of gluconeogenic seedlings and in leaves of C₄ plants and plants with Crassulacean acid metabolism (CAM). Phosphorylation of PEPCK was studied in these plants by feeding tissues with [³²P]P_i and assessing phosphorylation by SDS/PAGE and autoradiography of either total proteins or of immunoprecipitated protein. In gluconeogenic seedlings and

most CAM plants PEPCK had a molecular mass of 74 kDa, whereas in C₄ grasses the molecular mass of PEPCK was always smaller and varied from 67–71 kDa. In all gluconeogenic seedlings and leaves of CAM plants PEPCK was phosphorylated, but it was not phosphorylated in all species of C₄ grasses studied. In CAM plants, phosphorylation of PEPCK occurred at night and dephosphorylation occurred during the day. In C₄ grasses phosphorylation occurred when leaves were darkened and the enzyme was dephosphorylated following illumination, but it was only phosphorylated in those plants with larger (71 kDa) molecular mass forms of PEPCK.

INTRODUCTION

Phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.49) catalyses the ATP-dependent decarboxylation of oxaloacetate to phosphoenolpyruvate (PEP), which is a key step in photosynthetic CO₂ assimilation in some C₄ and Crassulacean acid metabolism (CAM) plants [1–3]. Although PEPCK has been purified to homogeneity from the leaves of three C₄ grasses [4], later work showed that these purifications yielded a truncated form of the enzyme which had lost the N-terminal region following extraction [5,6]. Arnelles and O'Leary [7] purified PEPCK from leaves of a C₄ plant, *Chloris gayana*, but since their procedure was based on that of Burnell [4], it is likely that PEPCK was also isolated in a truncated form. PEPCK has also been purified from the leaves of the CAM bromeliad, pineapple, although its molecular mass was not reported [8,9]. Recently, we have shown that PEPCK from another CAM bromeliad is rapidly cleaved upon extraction [5]. This phenomenon of rapid proteolysis means that the native form of this enzyme has not been purified from, or studied in, crude extracts of any C₄ or CAM plant.

Perhaps for this reason, we know little about the regulation of PEPCK activity in plants. Its known regulatory properties are insufficient to explain its role in the regulation and control of gluconeogenesis in fat-storing seedlings [5,10–12], or the necessity for light–dark regulation of the enzyme in C₄ plants [3] or in CAM plants (although diurnal variations in the activity of PEPCK have been observed in leaves of *Aloe vera* [13,14]). For example, without regulation, a futile cycle between PEP and oxaloacetate would readily occur in PEPCK-type CAM plants since PEP carboxylase and PEPCK are both cytosolic [15]. In CAM plants PEP carboxylase activity is regulated so that it is active only at night. This regulation is partly achieved by changes in its phosphorylation state [16]. In contrast, PEPCK, which is a decarboxylase, should be active only during the day.

One possibility for the apparent lack of appropriate regulatory properties in PEPCK is that the portion of the enzyme that is proteolytically cleaved during extraction confers regulatory properties on the enzyme which the cleaved form lacks. There are a number of examples in plants (e.g. ADP glucose pyrophosphorylase [17,18], NADP malate dehydrogenase [19] and PEP carboxylase [20]) where proteolytic cleavage of an enzyme in crude extracts does not affect V_{\max} but affects regulatory properties such as sensitivity to effectors.

We have recently shown that regulation of PEPCK might be achieved by light-dependent changes in phosphorylation. Incubation of PEPCK purified from cucumber cotyledons with [γ -³²P]ATP and either PEP carboxylase kinase or mammalian cAMP-dependent protein kinase led to labelling of the enzyme in the part of the molecule (presumably the N-terminal extension) which is cleaved during proteolysis. When darkened cucumber seedlings were fed [³²P]P_i, PEPCK was also phosphorylated; this was reversed by illumination [12].

In this study we have investigated whether PEPCK is phosphorylated in a range of plant tissues and show that PEPCK is phosphorylated in a range of gluconeogenic seedlings, in the leaves of CAM plants and in the leaves of some C₄ plants.

EXPERIMENTAL

Plant material

Seeds of *Melinis minutiflora*, *Chloris gayana*, *Spartina anglica*, *Urochloa panicoides* and *Panicum maximum* were obtained from the Kew Seed Bank (Royal Botanical Gardens, Kew, U.K.). CAM plants were obtained from a local nursery except for *Tillandsia* spp. which were obtained from The Tropical Rain Forest (Leeds, U.K.). Plants were grown in a greenhouse under ambient light. Seeds of cucumber, marrow, onion, oil seed rape, tomato and sunflower were sown on 1% (w/v) agar and grown at 25 °C in the dark.

Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase; CAM, Crassulacean acid metabolism; DTT, dithiothreitol; PEP, phosphoenolpyruvate.

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SDS/PAGE

SDS/PAGE [21] was performed using a 4.7% T/2.7% C stacking gel and a 10.5% T/2.7% C resolving gel. After electrophoresis, polypeptides were fixed in gels by immersion in 50% (v/v) methanol and 12% (v/v) acetic acid. Polypeptides were visualized by colloidal Coomassie Blue G-250 (Sigma, U.K.). Autoradiography of dried gels was performed at -80°C using Kodak X-OMAT LS X-ray film and intensifying screens. Exposure times were 12–72 h.

Immunoblotting

Transfer of polypeptides from an SDS/PAGE gel to Immobilon P membrane (Sigma, U.K.) was done in a Pharmacia Multiphor apparatus. Immunoreactive polypeptides were visualized using an antiserum raised to purified cucumber PEPCK [5] in conjunction with an enhanced chemiluminescence kit (Amersham, U.K.).

In vivo phosphorylation assay

Terminal 4–10 cm portions of leaves of C_4 grasses were excised, placed in a 1 ml cuvette containing 200 μl of water and 50 μCi (5 μl) of [^{32}P]P_i (specific radioactivity 200 mCi \cdot mmol⁻¹; Amersham, U.K.) and incubated under lights (500 μmol quanta \cdot m⁻² \cdot s⁻¹) at 25–30 $^{\circ}\text{C}$ until 90% of the solution had been taken up, a further 200 μl of water was then added and the above procedure repeated. For bromeliads, leaves were excised and fed similarly except that 50 μCi of [^{32}P]P_i was fed in a volume of 50 μl . For *Aloe aristata* and *Hoya carnos*a, slices of leaf were placed on moist filter paper and 50 μCi (5 μl) of [^{32}P]P_i was applied to the cut surface. For gluconeogenic seedlings, seedlings were placed on moist filter paper in a Petri dish and 5 μl of [^{32}P]P_i applied directly to either the endosperm or the cotyledon. For roots and hypocotyls of cucumber seedlings, roots (0.5 cm long) were inserted in capillary tubing containing 5 μl of [^{32}P]P_i until all the solution was absorbed; seedlings were then transferred to a Petri dish containing moist filter paper. In all cases tissue was incubated at 25–30 $^{\circ}\text{C}$ in the dark overnight. At 09.00 a.m. leaves were either left in darkness or illuminated (500 μmol quanta \cdot m⁻² \cdot s⁻¹).

Tissue was homogenized in a mortar with 10 vol. of ice-cold 200 mM 2-amino-2-methyl-1-propanol-HCl (pH 10.5)/1% (w/v) SDS/5 mM dithiothreitol (DTT), then clarified by centrifugation at 20000 g for 5 min at 4 $^{\circ}\text{C}$. For leaves fed [^{32}P]P_i by the transpiration stream, only the basal portion (1–2 cm) was used. Supernatants were added to an equal volume of SDS/PAGE solubilization buffer [62.5 mM Tris/HCl (pH 6.8), 10% (v/v) glycerol, 5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.002% (w/v) Bromophenol Blue], placed at 100 $^{\circ}\text{C}$ for 3 min, centrifuged at 20000 g for 3 min and supernatants analysed by SDS/PAGE. For preparation of the truncated 62 kDa form of PEPCK the above procedure was used except that 100 mM Tris/HCl (pH 7.5)/5 mM DTT was used as extraction buffer. For immunoprecipitation, tissue was homogenized in a mortar with 10 vol. of ice-cold 200 mM Bicine/KOH (pH 9.0)/5 mM DTT and then clarified by centrifuging three times at 20000 g for 5 min at 4 $^{\circ}\text{C}$. To 200 μl of the supernatant was added 30 μl of a rabbit polyclonal antiserum raised against purified cucumber PEPCK [5]. Samples were incubated on a shaker at 25 $^{\circ}\text{C}$ for 1 h, 10 μl of Protein A–Sepharose (Sigma, U.K.) was then added and samples shaken for 15 min. Immune complexes were collected by centrifugation at 20000 g for 5 min at 25 $^{\circ}\text{C}$. Pellets were washed by resuspension in 200 μl of 20 mM Tris/HCl (pH 7.4)/150 mM NaCl and then analysed by SDS/PAGE.

RESULTS

Differences in molecular mass of PEPCK in higher plants

Extracts of cotyledons or endosperm from a range of gluconeogenic seedlings and leaves from a range of C_4 and CAM species were subjected to SDS/PAGE and immunoblotting in order to determine the subunit molecular mass of PEPCK. In a range of gluconeogenic seedlings, PEPCK had an apparently constant molecular mass of about 74 kDa (Figure 1). In a range of CAM plants PEPCK had a molecular mass of 74 kDa or 78 kDa (Figure 1). The molecular mass was 74 kDa in *Aechmea fasciata*, *Billbergia nutans*, *Neoregelia carolinae* and *Nidularium fulgens* (Bromeliaceae); *Aloe aristata*, *Aloe mitriformis*, *Gasteria maculata*, *Haworthia fasciata* and *Haworthia margaritifera* (Lili-

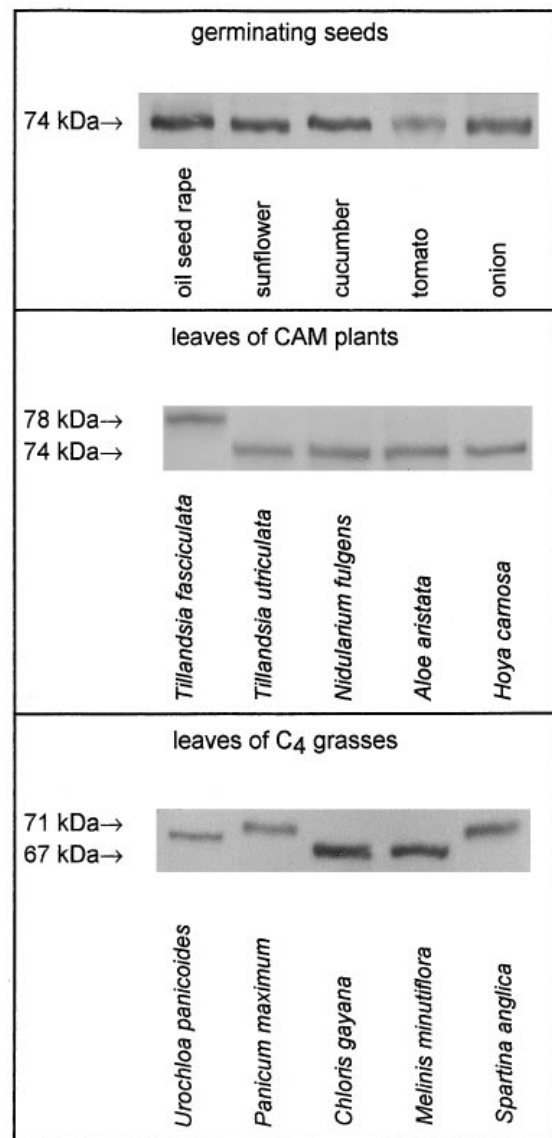


Figure 1 Measurement of the molecular mass of PEPCK in a range of plant tissues

Extracts of cotyledons or endosperm of germinating seeds or of leaves of C_4 grasses and CAM plants were subjected to SDS/PAGE. After transfer of fractionated polypeptides to Immobilon P membrane, PEPCK was visualized using an antiserum raised to purified cucumber PEPCK.

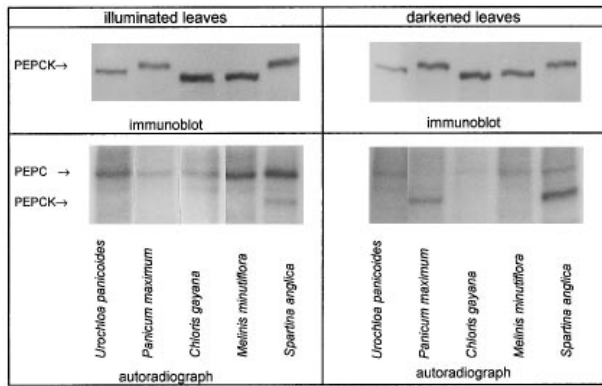


Figure 2 Analysis of *in vivo* phosphorylation of PEPCK in leaves of C_4 grasses

Leaves were fed $[^{32}P]P_i$ and then left in darkness for 15 h. Leaves were then either left in darkness or illuminated for 5 h. Phosphorylation of proteins was assessed by autoradiography of SDS/PAGE-fractionated polypeptides. Immunoblot analysis showed that similar amounts of PEPCK were present in each extract. A polypeptide of 71 kDa, the same size as PEPCK, was heavily phosphorylated in darkened leaves of both *P. maximum* and *S. anglica*.

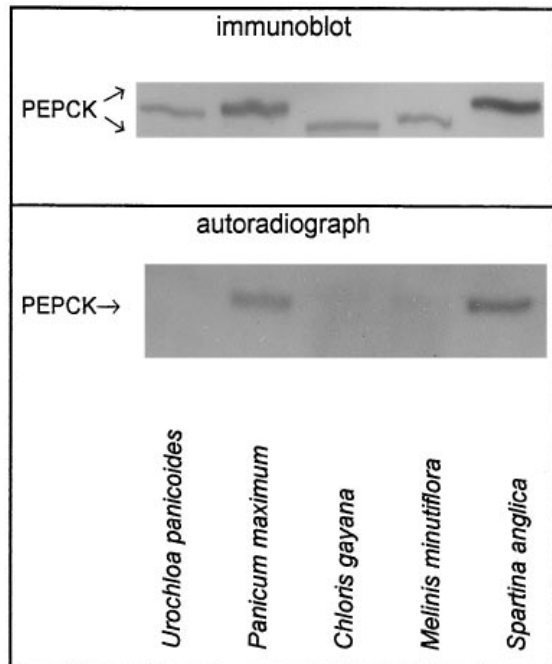


Figure 3 Immunoprecipitation of PEPCK from $[^{32}P]P_i$ -fed leaves of C_4 grasses

Leaves were fed $[^{32}P]P_i$ and then left in darkness for 20 h. PEPCK in extracts was immunoprecipitated by an antiserum raised against cucumber PEPCK. Phosphorylation of immunoprecipitated PEPCK was assessed by autoradiography of SDS/PAGE gels. Immunoblot analysis showed that similar amounts of PEPCK were immunoprecipitated from extracts of each species and that in each case the molecular mass of the enzyme was the same as that of uncleaved PEPCK.

aceae); *Euphorbia grandidens* (Euphorbiaceae); *Hoya carnos*a (Asclepiadaceae) (results not shown). However, in bromeliads of the genus *Tillandsia* there were two forms of PEPCK. One form, with a molecular mass of 74 kDa, was present in *T. aernanthos*, *T. albida*, *T. atroviridipetala*, *T. bulbosa*, *T. incarnata*, *T. ionantha*

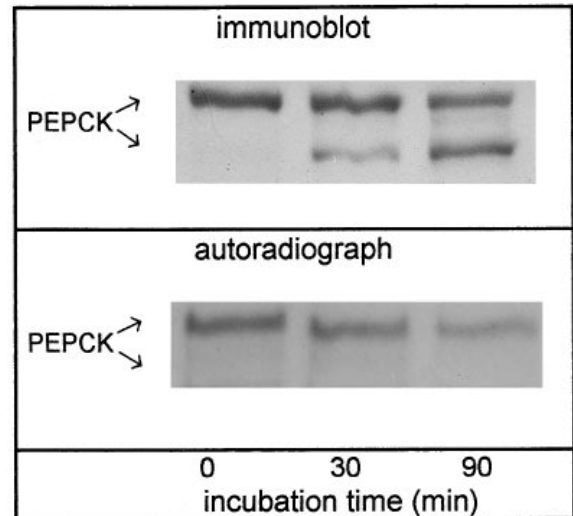


Figure 4 PEPCK is phosphorylated *in vivo* in *P. maximum* leaves at a site contained within the cleaved fragment

P. maximum leaves were fed $[^{32}P]P_i$ and incubated in darkness for 20 h. Leaves were homogenized in buffer at pH 7.5 to facilitate proteolytic cleavage of PEPCK. Portions of the extract were prepared for electrophoresis by boiling in SDS/PAGE sample buffer either immediately after extraction or after incubation at 25 °C for either 30 or 90 min. Polypeptides were then fractionated by SDS/PAGE and the amount and molecular mass of PEPCK assessed by immunoblotting. Phosphorylation of polypeptides was assessed by autoradiography.

ionantha, *T. paleacea*, *T. tenuifolia*, *T. usneoides* and *T. utriculata* (Figure 1), while a form of PEPCK with a molecular mass of 78 kDa was present in *T. brachycaulos abdita*, *T. fasciculata* (Figure 1), *T. juncea* and *T. melanocrater*. Incubation of extracts containing either the 78 or 74 kDa form of PEPCK from species of *Tillandsia* gave rise to a 62 kDa fragment (results not shown). There was much more variation in the molecular mass of PEPCK between different C_4 grasses in which the molecular mass of PEPCK ranged from 67 kDa to 71 kDa (Figure 1).

In vivo phosphorylation of PEPCK in C_4 plants

Illuminated or darkened leaves of C_4 grasses that had been fed $[^{32}P]P_i$ were homogenized in a buffer at high pH containing 1% (w/v) SDS, which minimized proteolysis of PEPCK [5]. Phosphorylation of polypeptides was assessed by SDS/PAGE and autoradiography (Figure 2). In leaves of both *Panicum maximum* and *Spartina anglica* a polypeptide of 71 kDa, which is the same size as PEPCK from these species, was heavily labelled in darkened leaves but labelling was much less in illuminated leaves. No polypeptide of the same size as PEPCK was labelled under any conditions in leaves of *Chloris gayana*, *Melinis minutiflora* or *Urochloa panicoides*. In separate extractions, immunoprecipitation confirmed that, in both leaves of *P. maximum* and *S. anglica*, the 71 kDa polypeptide which was labelled was PEPCK (Figure 3). As a control, a polypeptide of 100 kDa corresponding to PEP carboxylase underwent the expected phosphorylation in illuminated leaves and dephosphorylation in the darkened leaves in all five species (Figure 2), showing that lack of labelling of PEPCK in some species was not due to restricted entry, or metabolism, of $[^{32}P]P_i$. The similar intensity of labelling of PEP carboxylase and PEPCK, which have a comparable abundance, suggests that they are phosphorylated with a similar stoichiometry. Incubation of extracts at pH 7.5, which leads to cleavage of PEPCK [5], showed that loss of label

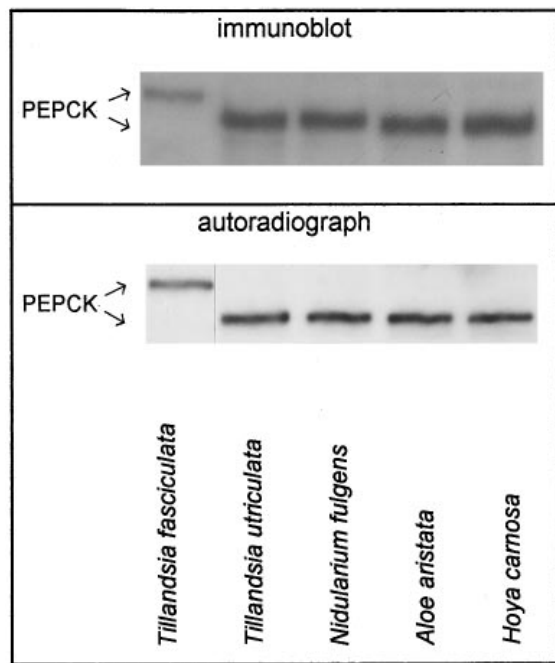


Figure 5 Analysis of *in vivo* phosphorylation of PEPCK in CAM plants

Leaves were fed [^{32}P] P_i and then left in darkness for 20 h. PEPCK in extracts was immunoprecipitated by an antiserum raised against cucumber PEPCK and phosphorylation assessed by autoradiography of SDS/PAGE gels. Immunoblot analysis showed that similar amounts of PEPCK were immunoprecipitated from extracts of each species and that in each case the molecular mass of the enzyme was the same as that of uncleaved PEPCK.

from the 71 kDa polypeptide occurred in parallel with cleavage to the 62 kDa form. The stoichiometry of labelling in the 71 kDa form was similar before and after incubation (Figure 4). No label appeared in the cleaved 62 kDa form. These data indicate that the site phosphorylated *in vivo* is located in the proteolytically cleaved portion.

In vivo phosphorylation of PEPCK in CAM plants

Leaves from a range of CAM plants were fed [^{32}P] P_i and phosphorylation of polypeptides assessed by SDS/PAGE and autoradiography. A polypeptide of the same molecular mass as PEPCK was phosphorylated in leaves from a range of CAM plants [*A. aristata* (Liliaceae), *H. carmosa* (Asclepiadaceae), *N. fulgens*, *T. utriculata* and *T. fasciculata* (Bromeliaceae)]; this polypeptide was confirmed to be PEPCK by immunoprecipitation (Figure 5). As with C_4 grasses, incubation of extracts at pH 7.5, which results in cleavage of PEPCK, indicated that the site phosphorylated *in vivo* is located on the proteolytically cleaved portion (results not shown). We also investigated changes in the phosphorylation state of PEPCK in leaves of *T. fasciculata* during a diurnal cycle. A polypeptide of 78 kDa, which was confirmed to be PEPCK by immunoprecipitation, was heavily labelled at most times during the diurnal cycle; however, the enzyme was dephosphorylated during the late afternoon and phosphorylated again during the night (Figure 6).

In vivo phosphorylation of PEPCK in gluconeogenic seedlings

Seedlings were fed [^{32}P] P_i and phosphorylation of PEPCK assessed by SDS/PAGE and autoradiography of immunopre-

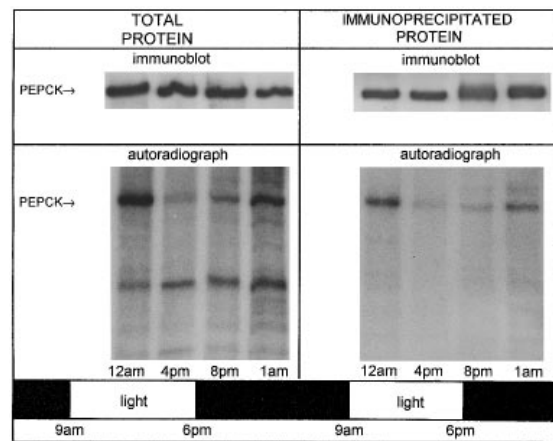


Figure 6 Analysis of *in vivo* phosphorylation of PEPCK in leaves of *Tillandsia fasciculata*

Leaves were fed [^{32}P] P_i and then left in darkness from 06.00 p.m. until 09.00 a.m. Leaves were then illuminated ($500 \mu\text{mol quanta} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) until 06.00 p.m. and then darkened. Phosphorylation of proteins was assessed by autoradiography of SDS/PAGE-fractionated polypeptides. The amount and molecular mass of PEPCK in these leaves was also assessed by immunoblot analysis.

cipitated proteins. PEPCK was phosphorylated in the cotyledons or endosperm of a range of gluconeogenic seedlings [oil seed rape (Cruciferae), onion (Liliaceae), tomato (Solanaceae), sunflower (Compositae) and marrow (Cucurbitaceae)] (Figure 7). During

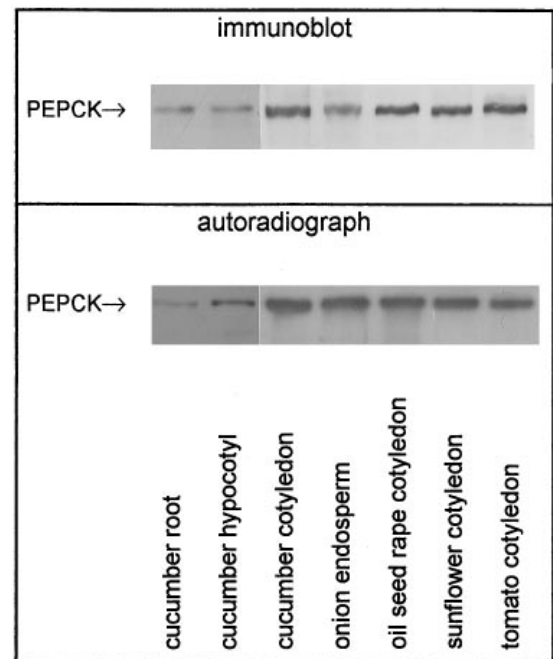


Figure 7 Analysis of *in vivo* phosphorylation of PEPCK in gluconeogenic seedlings

Seedlings were fed [^{32}P] P_i and then left in darkness for 20 h. PEPCK in extracts was immunoprecipitated by an antiserum raised against cucumber PEPCK. Phosphorylation of immunoprecipitated PEPCK was assessed by autoradiography of SDS/PAGE gels. The amount of PEPCK that was immunoprecipitated was estimated by immunoblot analysis.

the germination of some seeds such as oil seed rape [22] storage lipid is also mobilized from tissues such as the root and hypocotyl. PEPCK and isocitrate lyase occur in the hypocotyl and root of cucumber seedlings ([23]; results not shown), where they presumably function in the conversion of lipid into sugar. PEPCK from both the roots and hypocotyl of cucumber seedlings was phosphorylated (Figure 7, lanes 1 and 2).

DISCUSSION

The results presented in this paper demonstrate that the phosphorylation of PEPCK occurs widely in higher plants. PEPCK had a molecular mass of 74 kDa and was phosphorylated in all the gluconeogenic C_3 tissues which were studied from a range of plant families. Similarly, in all the plants with CAM which were studied PEPCK had a molecular mass of 74 kDa (or 78 kDa in some species of *Tillandsia*) and was phosphorylated. The present results do not distinguish whether PEPCK in CAM plants is regulated by light itself or in response to a circadian rhythm, as occurs in the case of PEP carboxylase [24]. The studies in C_4 and CAM plants further demonstrate that regulation of the activity of PEPCK does not occur through changes in the amount of PEPCK protein through light-dark or circadian cycles. However, C_4 plants present a different picture from CAM plants because the molecular mass differed appreciably between different species (67–71 kDa) and only the larger (71 kDa) forms of PEPCK from the C_4 grasses *P. maximum* and *S. anglica* were phosphorylated, whereas the smaller forms of PEPCK from the leaves of other C_4 plants were not. In those C_4 plants in which phosphorylation occurred, the enzyme was phosphorylated in darkness and dephosphorylated following illumination.

ATP-dependent PEPCKs from plants, bacteria and fungi are similar except that the plant enzyme possesses an N-terminal extension [6,23]. The gluconeogenic form of PEPCK is widely distributed among C_3 plants where it plays an important role in the conversion of lipid into sugar during the germination of fat-storing seeds. In every species examined this gluconeogenic form of PEPCK had a subunit molecular mass of 74 kDa. It is likely that the photosynthetic form of PEPCK that is present in leaves of C_4 grasses and CAM plants evolved from this enzyme. The relative constancy in the molecular mass of PEPCK between different C_3 and CAM plants suggests that the structure of the gluconeogenic and CAM enzyme have been maintained, but the smaller molecular mass forms observed in C_4 plants suggest that it has been modified to suit its role in C_4 photosynthesis. Thus the N-terminal extension of PEPCK from the C_4 grass *U. panicoides* has been modified considerably and is much shorter than that of cucumber [6]. Due to the high degree of similarity in the rest of the molecule it is likely that any differences in molecular mass of PEPCK in plants reflect differences in the length of the N-terminal extension. Two pieces of evidence suggest that it is the N-terminal region that is lost from PEPCK upon extraction. First, amino acid sequencing has shown that it is the N-terminal region of PEPCK that is cleaved in extracts of *U. panicoides* leaves [6]. Secondly, cleavage of PEPCK from a range of plants always yields a 62 kDa polypeptide (R. P. Walker and R. C. Leegood, unpublished work). For both cucumber and *U. panicoides* PEPCK the difference in size between the intact and cleaved polypeptides was similar to the size of the N-terminal extension deduced from the cDNA sequence.

Phosphorylation of PEPCK was investigated in five C_4 grasses. Only in the two species that possess PEPCK with the largest molecular mass was the enzyme phosphorylated. A comparison of the sequences of the N-terminal regions of cucumber and *U. panicoides* PEPCK (which was not phosphorylated) show that

the putative phosphorylation motif (Gln-Lys-Lys-Arg-Ser-Thr; [12]) is absent from the latter. We know that C_4 plants of the same biochemical subtype evolved separately many times from C_3 plants and did so quite recently (perhaps no longer than 7–8 million years ago) [25]. These findings suggest that during the evolution of PEPCK-type C_4 grasses there has been a change in the structure and phosphorylation of PEPCK which might be related to its role in C_4 photosynthesis. For example, in C_4 plants, PEP carboxylase and PEPCK are located in different photosynthetic cell types, the mesophyll and bundle-sheath, respectively, whereas in C_3 and CAM plants both enzymes are located in the cytosol of the same cells. It may, therefore, be unnecessary to exert such tight control over the activity of PEPCK in C_4 plants since there is no potential for the operation of a futile cycle with PEP carboxylase. Hence the capacity for phosphorylation may have been lost during the evolution of the C_4 pathway in some plants. It is not unusual for an enzyme to be regulated by phosphorylation in some species but not in others. For example sucrose phosphate synthase is phosphorylated and strongly regulated by metabolites in some plants but not others [16]. Similarly fructose-1,6-bisphosphate phosphatase is regulated by phosphorylation in rat liver but not in mouse or rabbit liver [26,27].

It seems likely that the N-terminal extension confers regulatory properties on PEPCK. It should be noted that a number of other enzymes have regulatory properties conferred on them by phosphorylation of a residue contained within a sequence of amino acids that is absent from enzymes with otherwise very similar sequences. For example, mammals possess cell-specific isoforms of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase. This enzyme contains a highly conserved central core in which, in the various isoforms, C- and N-terminal extensions have been added. Some of these extensions contain phosphorylation sites which regulate the activity of the enzyme [28]. PEP carboxylase from plants has regulatory properties conferred on it by phosphorylation/dephosphorylation of a serine residue contained in a sequence of amino acids at the N-terminus, which are lacking in the bacterial enzyme [29]. PEP carboxylase rapidly loses the N-terminal region, and much of its sensitivity to inhibition by malate, following proteolysis in crude extracts [30].

How phosphorylation influences the regulatory properties of PEPCK is not yet apparent, but the answer may lie in assaying the activity of the enzyme at physiological concentrations of Mn^{2+} . Previous studies have assayed PEPCK at unphysiological concentrations of Mn^{2+} (> 0.5 mM), whereas the concentration of Mn^{2+} in the cytosol of maize roots is submicromolar [31].

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