

Purification and characterization of phosphoenolpyruvate carboxylase from *Brassica napus* (rapeseed) suspension cell cultures

Implications for phosphoenolpyruvate carboxylase regulation during phosphate starvation, and the integration of glycolysis with nitrogen assimilation

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Phosphoenolpyruvate carboxylase (PEPC) specific activity increased by 250% following 8 to 10 days of P_i starvation of *Brassica napus* suspension cells. Densitometric scanning of PEPC immunoblots revealed a close correlation between PEPC activity and the amount of the antigenic 104-kDa PEPC subunit. To further assess the influence of P_i deprivation on PEPC, the enzyme was purified from P_i-sufficient (+P_i) and P_i-starved (–P_i) cells to electrophoretic homogeneity and final specific activities of 37–40 μmol phosphoenolpyruvate utilized per min per mg protein. Gel filtration, SDS/PAGE, and CNBr peptide mapping indicated that the +P_i and –P_i PEPCs are both homotetramers composed of an identical 104-kDa subunit. Respective pH–activity profiles, phosphoenolpyruvate saturation kinetics, and sensitivity to L-malate inhibition were also indistinguishable. Kinetic studies and phosphatase treatments revealed that PEPC of the +P_i and –P_i cells exists mainly in its dephosphorylated (L-malate sensitive) form. Thus, up-regulation of PEPC activity in –P_i cells appears to be solely due to the accumulation of the same PEPC isoform being expressed in +P_i cells. PEPC activity was modulated by several metabolites involved in carbon and nitrogen metabolism. At pH 7.3, marked activation by glucose 6-phosphate and inhibition by L-malate, L-aspartate, L-glutamate, DL-isocitrate, rutin and quercetin was observed. The following paper provides a model for the coordinate regulation of *B. napus* PEPC and cytosolic pyruvate kinase by allosteric effectors. L-Aspartate and L-glutamate appear to play a crucial role in the control of the phosphoenolpyruvate branchpoint in *B. napus*, particularly with respect to the integration of carbohydrate partitioning with the generation of carbon skeletons required during nitrogen assimilation.

Keywords: phosphoenolpyruvate carboxylase; plant glycolysis; phosphate starvation response; carbon–nitrogen interactions; *Brassica napus* (rapeseed).

Phosphoenolpyruvate carboxylase (PEPC) is a ubiquitous cytosolic enzyme in vascular plants that is also widely distributed in green algae, cyanobacteria, and bacteria [1]. It catalyzes the irreversible β-carboxylation of phosphoenolpyruvate (PEP) in the presence of HCO₃[–] and Mg²⁺ to yield oxaloacetate and P_i. The enzyme is particularly abundant in the mesophyll cells of C₄ and crassulacean acid metabolism (CAM) leaves where it participates in photosynthesis by catalyzing the initial fixation of atmospheric CO₂. Both allosteric mechanisms and covalent modification are involved in the regulation of PEPC activity

during C₄ and CAM photosynthesis [1–3]. Early work determined that C₄ and CAM photosynthetic PEPCs were regulated via a diurnal cycle that served to modulate their sensitivity to L-malate inhibition, without affecting V_{max} or the amount of PEPC protein. This has been shown to be due to reversible phosphorylation by an endogenous Ca²⁺-independent PEPC protein kinase and dephosphorylation by a protein phosphatase type 2 A (PP2A) at a key serine residue located near the N-terminus of the 100- to 110-kDa PEPC subunit [1–3].

In contrast to C₄ and CAM leaf PEPCs, the properties, regulation and functions for the enzyme from C₃ plants and nonphotosynthetic tissues of C₄ and CAM plants are less well understood. Proposed roles for the C₃ enzyme are diverse and include: (a) regulation of cellular pH and cation balance, (b) production of dicarboxylic acids used as respiratory substrates by nitrogen-fixing bacteroids of legume root nodules, and/or (c) the anaplerotic replenishment of citric acid cycle intermediates consumed in biosynthesis [1]. The enzyme has been partially or fully purified from several nonphotosynthetic plant tissues including germinating castor seeds [4], soybean root nodules [5], and ripening banana fruit [6]. As with the C₄ and CAM leaf enzyme, reversible phosphorylation and allosteric effectors have been suggested to be important in the regulation of PEPC from C₃ plants [1,4–10]. Radiolabeling studies with ³²P_i have demonstrated the *in vivo* phosphorylation of PEPC in soybean root nodules [5], banana fruit [7], wheat leaves [8], *Vicia faba* guard cells [9], and germinating wheat seeds [10]. In banana

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Abbreviations: AAT, aspartate aminotransferase; APase, acid phosphatase; CAM, crassulacean acid metabolism; GS/GOGAT, glutamine synthetase/glutamine 2-oxoglutarate aminotransferase; +P_i and –P_i, cultured in the presence and absence of 2.5 mM KP_i, respectively; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PK_c, cytosolic isozyme of pyruvate kinase; PP2A, protein phosphatase type 2A1. *Enzymes:* acid phosphatase (EC 3.1.3.2); aspartate aminotransferase (EC 2.6.1.1), glutamine synthetase (EC 6.3.1.2), glutamine 2-oxoglutarate aminotransferase (EC 1.4.1.13); phosphoenolpyruvate carboxylase (EC 4.1.1.31); pyruvate kinase (EC 2.7.1.40); protein phosphatase type 2A1 (EC 3.1.3.16).

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fruit, an endogenous PEPC kinase forms a tight complex with its target enzyme [7]. However, the regulatory properties of plant PEPC that mediate glycolytic flux and PEP partitioning during nutritional P_i deprivation have not been elucidated.

Phosphorous is an essential element for normal growth and metabolism, as it plays a central role in virtually all metabolic processes. Plants preferentially absorb phosphorous from the soil in its fully oxidized anionic form, P_i ($H_2PO_4^-$, orthophosphate). Although widely distributed in the earth's crust, most P_i exists in insoluble mineral forms and, as such, is unavailable to plants [11]. Thus, in many natural environments P_i deficiency is the rule rather than the exception. Plants have evolved to acclimatize to P_i stress to varying degrees through a number of mechanisms, including acid phosphatase (APase) induction [11]. APases can function as intracellular or extracellular P_i salvaging systems that can scavenge P_i from phosphate esters. The plant ' P_i starvation response' also involves the induction of PEPC activity, which has been reported for several C_3 plants including: *Brassica nigra* (black mustard) and *Catharanthus roseus* (periwinkle) suspension cells [12,13], *Lupinus albus* (lupin), *Lycopersicon esculentum* (tomato) and *Cicer arietinum* (chickpea) roots [14,15], and *B. napus* shoots [16]. PEPC induction has been correlated with increases in *in vivo* dark $^{14}CO_2$ fixation and/or levels of PEPC-derived organic acids [13–19]. During P_i deprivation, PEPC (with malate dehydrogenase and NAD-malic enzyme) may provide a metabolic 'bypass' to the ADP-limited cytosolic pyruvate kinase (PK_c) to facilitate continued pyruvate supply to the citric acid cycle, while concurrently recycling the PEPC by-product P_i for its assimilation into the metabolism of the P_i -starved cells [11,13]. In addition, PEPC induction can promote the synthesis and consequent excretion of large amounts of malic and citric acids from roots during P_i stress. This acidifies the rhizosphere, which therefore increases P_i availability to the plant by solubilizing otherwise inaccessible sources of mineral P_i [11,14,16,19]. Increased levels of PEPC protein and mRNA were correlated with enhanced PEPC activity of proteoid roots of P_i -deficient lupin plants [18]. The possible expression of a separate ' $-P_i$ inducible' PEPC isozyme was indicated by the isolation of a PEPC cDNA from the P_i -stressed proteoid lupin roots [19]. In addition, kinetic studies of partially purified PEPC from proteoid lupin roots suggested that during P_i stress the enzyme may be phosphorylated by an endogenous PEPC kinase, resulting in reduced sensitivity of PEPC to inhibition by L-malate [19].

The initial goal of the current study was to investigate the impact of P_i starvation on the PEPC of *B. napus* suspension cell cultures. Such cultures represent an ideal model system for examining the influence of P_i nutrition on plant metabolism, because they contain a homogeneous population of cells, with each cell in direct contact with the culture medium. Moreover, relatively large quantities of cells at a precise nutritional and developmental state can be amassed for use in enzyme purification. Our second goal was to compare the kinetic and regulatory features of the purified PEPC from P_i -sufficient *B. napus* with those being concurrently characterized for the homogeneous PK_c from the same cells [20]. Coordinate regulation of these two PEP utilizing enzymes plays a critical role in the regulation of plant cytosolic glycolytic flux, particularly with respect to the integration of carbon partitioning with the generation of 2-oxoglutarate needed for nitrogen assimilation by Gln synthetase/Gln 2-oxoglutarate aminotransferase (GS/GOGAT) [21,22]. PEPC has an additional metabolic function during nitrogen assimilation to produce oxaloacetate for L-Asp synthesis by Asp aminotransferase (AAT). PK_c and

PEPC appear to be activated (or deinhibited) *in vivo* following nitrogen resupply to nitrogen limited plant tissues and green algae [21]. This not only generates necessary carbon skeletons for AAT and/or GS/GOGAT, but also serves to reduce PEP levels, thereby relieving PEP inhibition of the ATP-dependent phosphofructokinase [22], thus stimulating overall glycolytic flux. Despite the tremendous importance of PEPC and PK_c in controlling interactions between plant carbon and nitrogen metabolism, few workers have attempted to characterize both enzymes from the same plant tissue or cell type [4]. Indeed, the simultaneous complete purification and thorough comparative analysis of PEPC and PK_c from the same vascular plant source has not been described. This is important because vascular plants express tissue- and developmental-specific isozymes of PK_c and PEPC that may display very different physical, kinetic, and regulatory properties [1,21–23]. In particular, the combined results of the present and following [20] papers suggest a pivotal role for L-Asp and L-Glu in the coordinate regulation of *B. napus* PEPC and PK_c .

MATERIALS AND METHODS

Chemicals and plant material

PEP, Coomassie Blue R-250, bistris-propane, Mes, and dithiothreitol were from Research Organics, Inc. Tris base and SDS were from Schwartz/Mann Biotech. Ribi adjuvant (product code R-730) was obtained from Ribi Immunochemical Research. Poly(vinylidene difluoride) membranes (Immobilon, 0.45 μ m pore size) were obtained from Millipore and all solutions were prepared using Milli-Q-processed water. Other biochemicals, coupling enzymes, SDS molecular mass standards, cell culture reagents and alkaline phosphatase-tagged goat anti-(rabbit IgG) IgG were obtained from Sigma Chemical Co. All other reagents were of analytical grade obtained from BDH Chemicals. Purified bovine heart PP2A (catalytic subunit) was a gift of G. Moorhead, University of Calgary (1 U of PP2A dephosphorylates 1 μ mol of bovine glycogen phosphorylase per min at 30 °C).

An embryogenic pollen-derived heterotrophic cell suspension of winter oilseed rape (canola) (*B. napus* L. cv. Jet Neuf) [24] was provided by R. Weselake, University of Lethbridge, Canada. Cells were maintained on a rotational shaker (125 r.p.m.) at 22 °C in NLN media (pH 7.5) containing 6.5% (w/v) sucrose, 3 mM NO_3^- , 5.5 mM L-Gln, 0.5 mg·L⁻¹ α -naphthalene acetic acid, 0.5 mg·L⁻¹ 2,4-dichlorophenoxyacetic acid and 2.5 mM K_2HPO_4 (added from a 200-mM sterile stock at the time of subculturing) as described previously [25]. Subculturing was performed by transferring 10 mL of a 7-day-old cell suspension into 40 mL (125 mL flask) of fresh NLN media containing 2.5 mM K_2HPO_4 . Cells used in time-course studies and PEPC purification were obtained by scaling up the culture volume. Briefly, two 7-day-old 50-mL cultures were combined, concentrated to about 60 mL by removing excess media, and used to inoculate 440 mL of fresh NLN media containing either 2.5 mM K_2HPO_4 (+ P_i cells) or 0 mM K_2HPO_4 ($-P_i$ cells) in 2-L flasks. Cells were harvested on a Buchner funnel fitted with Miracloth, washed with 10 mM $CaCl_2$, frozen in liquid N_2 , and stored at -80 °C.

Enzyme and protein assays

The PEPC and APase reactions were coupled to the malate dehydrogenase and lactate dehydrogenase reactions, respectively, and assayed at 24 °C by monitoring NADH oxidation at

340 nm using a Gilford 260 recording spectrophotometer, in a final volume of 1 mL. Coupling enzymes were desalted before use. Standard assay conditions for PEPC were: 50 mM bistris-propane/HCl (pH 8.4), 10% (v/v) glycerol, 2 mM PEP, 2.5 mM KHCO_3 , 12 mM MgCl_2 , 0.15 mM NADH and 5 U of porcine heart malate dehydrogenase. Assay conditions for APase were: 25 mM bistris-propane/25 mM Mes (pH 5.7), 2 mM PEP, 0.15 mM NADH and 5 U of rabbit muscle lactate dehydrogenase. All assays were: (a) initiated by addition of enzyme preparation, (b) corrected for NADH oxidase activity, and (c) linear with respect to time and concentration of enzyme assayed. To examine the influence of rutin and quercetin on PEPC activity, a 10-mM stock of each flavonoid was prepared in dimethylsulfoxide and stored at -20°C . Control assays containing only dimethylsulfoxide were included. One unit of enzyme activity is defined as the amount of enzyme resulting in the production of 1 μmol of product per min at 24°C .

Protein concentrations were routinely determined using a Dynatech MR-5000 microplate reader and the Coomassie Blue G-250 dye-binding method described by Bollag and Edelstein [26]. Protein concentration of the purified PEPCs was also determined using the bicinchoninic acid method of Hill and Straka [27]. Bovine γ -globulin was used as the protein standard.

Kinetic studies

Kinetic studies were conducted using a Dynatech MR-5000 microplate reader and a final volume of 0.2 mL for the PEPC reaction mixture. Apparent K_m values were calculated from the Michaelis–Menten equation fitted to a nonlinear least-squares regression computer kinetics program [28]. K_a and I_{50} values (concentration of activator and inhibitor producing 50% activation and inhibition of PEPC activity, respectively) were determined using the same computer kinetics program. All kinetic parameters are the means of at least three separate determinations and are reproducible to within $\pm 10\%$ SE.

Preparation of clarified homogenates used in time-course studies

+ P_i or $-\text{P}_i$ *B. napus* cells were ground to a powder in liquid N_2 and homogenized (1 : 2, w/v) using a mortar and pestle and a small scoop of sand in ice-cold 100 mM imidazole/HCl (pH 7.6) containing 1 mM EDTA, 5 mM MgCl_2 , 100 mM KCl, 20 mM NaF, 20% (v/v) glycerol, 10 mM thiourea, 1% (w/v) each of insoluble and soluble polyvinylpyrrolidone, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 5 $\mu\text{g}\cdot\text{mL}^{-1}$ chymostatin, and 50 nM microcystin-LR. Homogenates were centrifuged at 4°C and 14 000 g for 15 min, and the resulting clarified extracts prepared for SDS/PAGE and PEPC immunoblotting, and/or assayed for total protein, PEPC and APase activities.

Buffers used during PEPC purification

Buffers were degassed and adjusted to their respective pH values at 24°C . Buffer A: aforementioned extraction buffer containing 0.1% (v/v) Triton X-100 and 4% (w/v) poly(ethylene glycol) 8000. Buffer B: 50 mM imidazole/HCl (pH 7.1) containing 5 mM MgCl_2 , 1 mM EDTA, 1 mM dithiothreitol, 20 mM NaF and 25% (saturation) $(\text{NH}_4)_2\text{SO}_4$. Buffer C: 50 mM imidazole/HCl (pH 7.1) containing 5 mM MgCl_2 , 1 mM EDTA, 1 mM dithiothreitol, 20 mM NaF and 10% (v/v) ethylene glycol. Buffer D: 100 mM Tris/HCl (pH 8) containing 1 mM EDTA, 5 mM MgCl_2 , 20% (v/v) glycerol, 20 mM NaF and 1 mM

dithiothreitol. Buffer E: 50 mM imidazole/HCl (pH 7.5) containing 15% (v/v) glycerol, 1 mM EDTA, 3 mM MgCl_2 , 50 mM KCl, 0.02% (w/v) NaN_3 , 20 mM NaF and 1 mM dithiothreitol.

Purification of PEPC

All procedures were carried out at 4°C . Identical protocols were employed for the purification of PEPC from 8-day-old + P_i and $-\text{P}_i$ *B. napus* suspension cells, henceforth referred to as the '+ P_i PEPC' and ' $-\text{P}_i$ PEPC' preparations, respectively.

Preparation of clarified extract and poly(ethylene glycol) fractionation

Quick-frozen *B. napus* suspension cells (220 g of + P_i cells or 147 g of $-\text{P}_i$ cells) were ground to a powder in liquid N_2 , homogenized (1 : 2.5, w/v) in buffer A using a Polytron, and centrifuged at 14 000 g for 20 min. Finely ground poly(ethylene glycol) 8000 was added to the supernatant fluid to a final concentration of 24% (w/v). The extract was stirred for 45 min and centrifuged for 20 min 35 000 g. Poly(ethylene glycol) pellets (4–24%; w/v) were stored overnight at -20°C .

Butyl-Sepharose hydrophobic interaction FPLC

Poly(ethylene glycol) pellets were resuspended in buffer B lacking $(\text{NH}_4)_2\text{SO}_4$, but containing 5 $\mu\text{g}\cdot\text{mL}^{-1}$ chymostatin, to yield a protein concentration of about 5 $\text{mg}\cdot\text{mL}^{-1}$. Following centrifugation for 20 min at 35 000 g, the extract was adjusted to 25% (saturation) $(\text{NH}_4)_2\text{SO}_4$ by the addition of solid $(\text{NH}_4)_2\text{SO}_4$. The solution was stirred for 20 min, centrifuged as above, and adsorbed at 4 $\text{mL}\cdot\text{min}^{-1}$ onto a column (3 \times 6.8 cm) of butyl-Sepharose Fast Flow (Pharmacia) preequilibrated with buffer B. The column was connected to a FPLC system, washed with 100 mL of buffer B, and PEPC activity eluted in a stepwise fashion with 60% buffer C (40% buffer B) (flow rate = 4 $\text{mL}\cdot\text{min}^{-1}$; fraction size = 5 mL). Pooled peak PEPC activity fractions were diluted with an equal volume of 50% (w/v) poly(ethylene glycol) 8000, stirred for 30 min, and centrifuged as above. The resulting pellets were stored overnight at -20°C .

Fractogel EMD DEAE-650 (S) anion-exchange FPLC

The poly(ethylene glycol) pellets were solubilized in buffer D to which 5 $\mu\text{g}\cdot\text{mL}^{-1}$ chymostatin was added to yield a protein concentration of about 10 $\text{mg}\cdot\text{mL}^{-1}$, centrifuged as above, and loaded at 1.5 $\text{mL}\cdot\text{min}^{-1}$ onto a column (1.1 \times 6.7 cm) of Fractogel EMD DEAE-650 (S) (Merck) that had been connected to a FPLC system and preequilibrated with buffer D. The column was washed with buffer D until the A_{280} decreased to baseline, and was PEPC eluted with 80 mL of a linear 0–300 mM KCl gradient in buffer D (fraction size = 5 mL). Pooled peak activity fractions were adjusted to contain 5 $\mu\text{g}\cdot\text{mL}^{-1}$ chymostatin and concentrated to about 1 mL using an Amicon XM-50 ultrafilter.

Superdex 200 gel filtration FPLC

The concentrated anion-exchange fractions were passed through a 0.45- μm syringe filter and applied at 0.3 $\text{mL}\cdot\text{min}^{-1}$ onto a column (1.6 \times 51 cm) of Superdex 200 Prep Grade (Pharmacia) that had been attached to a FPLC system and preequilibrated with buffer E (fraction size = 1.2 mL).

Mono-Q anion-exchange FPLC

Pooled peak activity fractions from the Superdex 200 column were immediately loaded at $0.5 \text{ mL}\cdot\text{min}^{-1}$ onto a Mono-Q HR 5/5 column (Pharmacia) preequilibrated with buffer D. PEPC was eluted using 25 mL of a linear 0–300 mM KCl gradient in buffer D (fraction size = 1 mL). Peak activity fractions were pooled, adjusted to $5 \mu\text{g}\cdot\text{mL}^{-1}$ chymostatin, concentrated as above to 0.65 mL, divided into 50- μL aliquots, frozen in liquid N_2 and stored at -80°C . The purified PEPC was stable for at least 6 months when stored frozen.

Determination of native molecular mass via Superdex 200 gel filtration

Native molecular mass estimation for the $+P_i$ and $-P_i$ PEPCs was performed during FPLC on the Superdex 200 Prep Grade column as described above. Native molecular masses were estimated from a plot of K_{av} (partition coefficient) vs. log molecular mass for the following protein standards: ferritin (440 kDa), catalase (232 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). Blue Dextran was used to determine the column's void volume.

Antibody production

Purified $+P_i$ PEPC (500 μg) was dialyzed overnight against NaCl/P_i (20 mM NaP_i , pH 7.4, 150 mM NaCl), filtered through a 0.2- μm membrane, and emulsified in Ribi adjuvant (1 mL total volume). After collection of preimmune serum, the PEPC was injected (0.6 mL subcutaneously, 0.4 mL intramuscularly) into a 2-kg New Zealand rabbit. A booster injection (250 μg) was administered subcutaneously after 6 weeks. Ten days after the final injection, blood was collected by cardiac puncture. After incubation overnight at 4°C , the clotted blood cells were removed by centrifugation at 1500 g for 10 min. The crude antiserum was frozen in liquid N_2 and stored at -80°C in 0.04% (w/v) NaN_3 . For immunoblotting, the anti-(*B. napus* $+P_i$ PEPC) IgG was affinity-purified against 25 μg of purified *B. napus* $+P_i$ PEPC as previously described [29].

Immunotitration of PEPC activity

Immunoremoval of enzyme activity was tested by mixing 0.05 units of homogeneous $+P_i$ PEPC with 25 mM Hepes/NaOH (pH 7.5), containing 0.1 $\text{mg}\cdot\text{mL}^{-1}$ BSA, 10% (v/v) glycerol, 1 mM dithiothreitol, and various amounts of rabbit preimmune or anti-(*B. napus* PEPC) immune serum diluted into NaCl/P_i (total volume = 0.1 mL). The mixtures were incubated at 30°C for 60 min and then for 90 min at 4°C prior to centrifuging for 5 min at 17 000 g. Residual PEPC activity in the supernatant fraction was determined as described above.

Electrophoresis and immunoblotting

SDS/PAGE was performed according to Laemmli [30] using a Bio-Rad mini-gel apparatus. The acrylamide monomer concentration in the 0.75-mm-thick slab gels was 4% and 9% (w/v) for the stacking and separating gels, respectively. Prior to SDS/PAGE samples were incubated for 3 min at 100°C in 50 mM Tris/HCl (pH 6.8) containing 1% (w/v) SDS, 10% (v/v)

glycerol and 100 mM dithiothreitol. Gels were run at a constant voltage of 200 V for 45 min. To determine subunit molecular masses by SDS/PAGE, a plot of relative mobility vs. the log m was constructed using the following protein standards: α_2 macroglobulin (180 kDa), β -galactosidase (116 kDa), phosphofructokinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactate dehydrogenase (36.5 kDa) and triose phosphate isomerase (26.6 kDa).

Nondenaturing PAGE was conducted at 4°C using the highly porous SDS/PAGE system of Doucet and Trifaró [31] except that SDS was omitted from all buffers, and 20% (v/v) glycerol and 10% (v/v) ethylene glycol were included in the stacking and separating gels which contained acrylamide concentrations of 4% and 6% (w/v), respectively. Gels were run at 200 V for 2 h, and either incubated in a PEPC activity stain, or immunoblotted using affinity-purified anti-(*B. napus* $+P_i$ PEPC) IgG. To detect in-gel PEPC activity, a gel was incubated for 30 min at 23°C in 50 mM Tris/HCl (pH 8.4) containing 10% (v/v) glycerol, 12 mM MgCl_2 , 2.5 mM KHCO_3 , 0.15 mM NADH, and 5 U·mL of porcine heart malate dehydrogenase. PEP (2 mM) was added to initiate the reaction and PEPC activity was visualized as dark bands in a fluorescent background using a UV transilluminator.

Immunoblotting was performed by transferring protein from SDS or native gels to poly(vinylidene difluoride) membranes by electroblotting for 75 min or 180 min, respectively, at 100 V. Antigenic polypeptides were visualized using an alkaline phosphatase-tagged secondary antibody [29]. The relative amount of PEPC protein in clarified extracts from 8-day-old $+P_i$ vs. $-P_i$ *B. napus* cells was determined by quantification of the antigenic 104-kDa PEPC subunit on immunoblots (in terms of A_{633}) using an LKB Ultrosan XL laser densitometer and GEL SCAN software (version 2.1) (Pharmacia LKB Biotech). Derived A_{633} values were linear with respect to the amount of the immunoblotted extract. Immunological specificities were confirmed by performing immunoblots in which rabbit preimmune serum was substituted for the affinity-purified anti-(*B. napus* $+P_i$ PEPC) IgG.

Peptide mapping by CNBr cleavage

Polypeptides corresponding to the 104-kDa subunit of purified $+P_i$ and $-P_i$ PEPCs were excised from SDS/PAGE mini-gels and cleaved *in situ* with CNBr. The degradation products were analyzed on a SDS/PAGE 14% mini-gel [32], and stained with silver [33].

Phosphatase treatment

Clarified homogenates of $-P_i$ and $+P_i$ cells were prepared in the presence and absence of 20 mM NaF and 50 nM microcystin-LR. Aliquots (0.5 mL) were desalted by centrifugation at 100 g through 5 mL of Sephadex G-50 [34] that had been preequilibrated in 50 mM bistris-propane/HCl (pH 7.3) containing 10% (v/v) glycerol, 5 mM MgCl_2 , and 1 mM dithiothreitol. The desalted extracts as well as the purified $+P_i$ and $-P_i$ PEPCs (dialyzed free of NaF) were incubated for 1 h at 23°C in the presence and absence of 0.5 U·mL $^{-1}$ of bovine heart PP2A or 2 U·mL $^{-1}$ of bovine intestinal alkaline phosphatase. PEPC activity was determined relative to controls prepared and desalted in the presence of 20 mM NaF and 50 nM microcystin-LR. PEPC assays were conducted at pH 7.3 with subsaturating (0.4 mM) PEP in the presence and absence of 0.1 mM L-malate.

RESULTS

Influence of P_i starvation on growth, and PEPC and APase activities of *B. napus* suspension cells

B. napus suspension cells cultured for 8 days in the absence of exogenous P_i had only about 50% of the fresh weight of the 8-day-old $+P_i$ cells (approximately 20 and 10 g of cells were obtained per 500 mL culture of 8-day-old $+P_i$ and $-P_i$ cells, respectively). The time-courses for PEPC and APase activities of $+P_i$ and $-P_i$ *B. napus* cells are shown in Fig. 1. PEPC and APase specific activities were increased by about 2.5-fold and fourfold, respectively, in the $-P_i$ cells, whereas the activities of the two enzymes remained relatively low and constant in the $+P_i$ cells. Within 24 h of resupplying 2.5 mM P_i to the 8-day-old $-P_i$ cells, extractable PEPC and APase activities were reduced by at least 50% (Fig. 1). Immunoblotting with rabbit anti-(*B. napus* $+P_i$ PEPC) IgG was used to estimate the subunit molecular mass and relative amount of PEPC in clarified extracts from the 8-day-old $+P_i$ vs. $-P_i$ *B. napus* cells. In each instance, a single immunoreactive 104-kDa polypeptide was observed (Fig. 1A, inset), identical to that obtained with the

respective purified PEPCs (see below). Laser densitometric quantification of the immunoblots revealed that the $-P_i$ *B. napus* extracts contained approximately twofold more of the immunoreactive 104-kDa PEPC subunit, relative to extracts of the $+P_i$ cells.

PEPC purification

As shown in Table 1, PEPC was purified about 715-fold from 220 g of 8-day-old $+P_i$ *B. napus* cells to a final specific activity of 20 U·mg⁻¹ and an overall recovery of 14%. Using an identical protocol, the PEPC from 147 g of 8-day-old $-P_i$ cells was purified 410-fold to a final specific activity of 20.9 U·mg⁻¹ and an overall yield of 12% (results not shown). As previously documented for banana fruit PEPC [6], the protein concentration of the purified *B. napus* $+P_i$ and $-P_i$ PEPCs as determined using the bicinchoninic acid-based protein assay was almost 50% of that determined with the Coomassie Blue G-250 dye binding assay (Table 1). Thus, with the bicinchoninic acid protein assay the specific activities of the final $+P_i$ and $-P_i$ PEPC preparations were, respectively, increased to 37.2 and 39.7 U·mg⁻¹ (Table 1, and results not

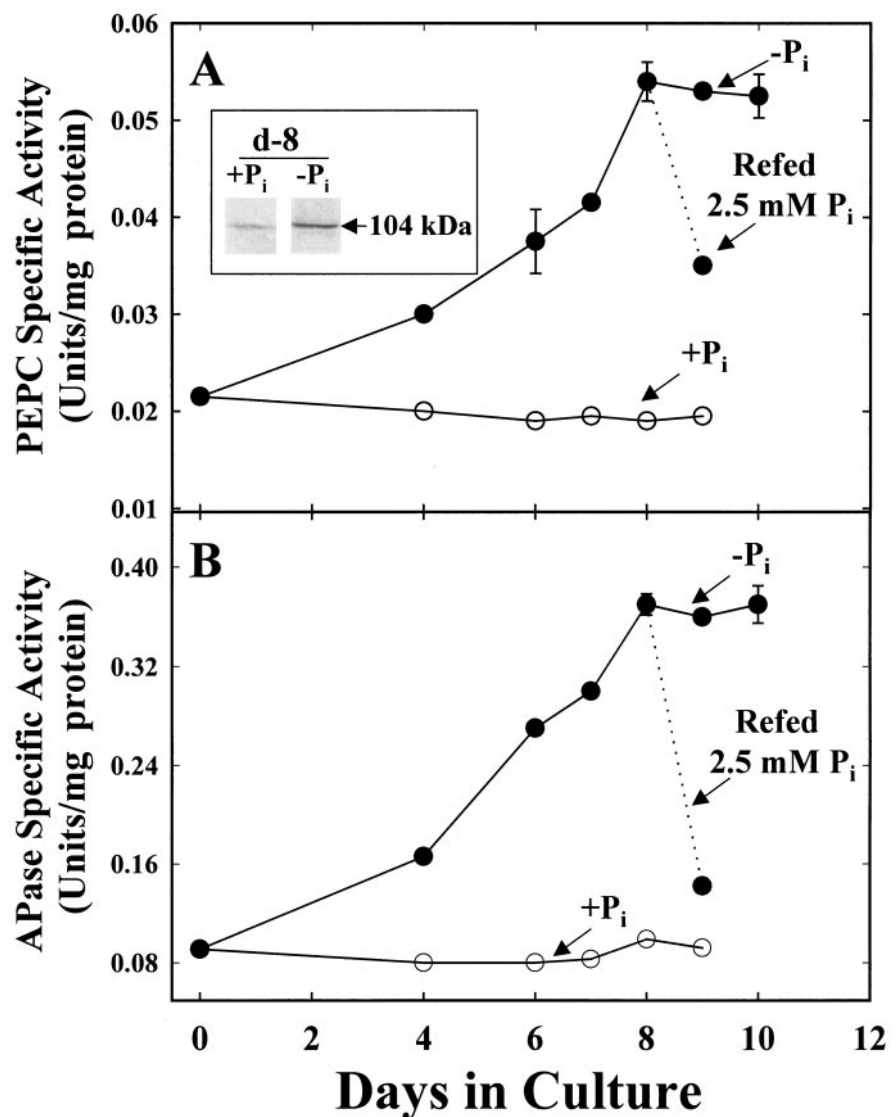


Fig. 1. Time-course for extractable activities of PEPC (A) and APase (B) in *B. napus* suspension cells cultured in 0 or 2.5 mM P_i . Values for the 6-, 8- and 10-day-old $-P_i$ cells represent means \pm SE for replicate assays of separate clarified extracts from $n = 3$ different 500 mL cultures. All other values represent the mean activities of replicate determinations of a single extract. An 8-day-old $-P_i$ culture was resupplied with 2.5 mM P_i and cultured for an additional 1 day as indicated (---). Inset to (A): immunological detection of PEPC from 8-day-old $+P_i$ or $-P_i$ *B. napus* suspension cells. Clarified extracts (each containing 50 μ g of protein) were subjected to SDS/PAGE and blot-transferred to a poly(vinylidene difluoride) membrane. Blots were probed with 20-fold diluted affinity-purified anti-(*B. napus* $+P_i$ PEPC) IgG and immunoreactive polypeptides were detected using an alkaline-phosphatase-linked secondary antibody followed by chromogenic staining as previously described [29].

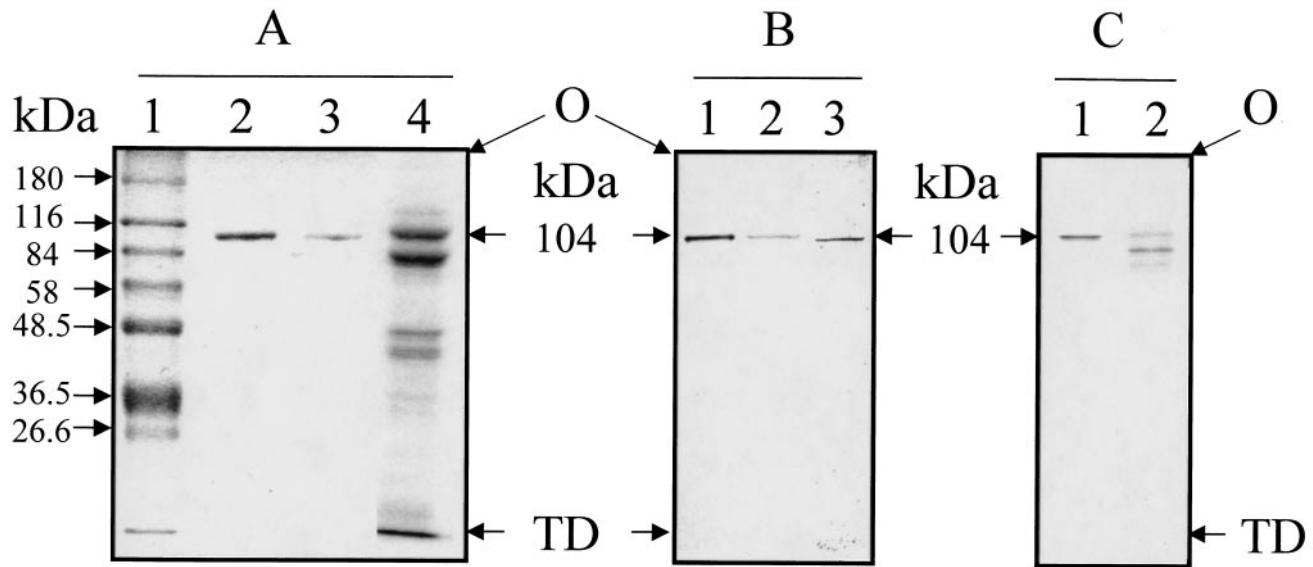


Fig. 2. SDS/PAGE and immunoblot analysis of PEPC from P_i -sufficient (+ P_i) *B. napus* suspension cells and developing seeds. (A) SDS/PAGE (9% separating gel) of PEPC purified from 8-day-old + P_i *B. napus* cells. Lane 1 contains 4 μ g of various molecular mass standards. Lanes 2 and 3 contain 2.5 and 1 μ g, respectively, of the pooled peak fractions from the final purification step (Mono-Q FPLC). Lane 4 contains 5 μ g of the + P_i PEPC that was partially purified in the absence of chymostatin. Protein staining was performed with Coomassie Blue R-250. (B) Immunoblot analysis was performed using 20-fold diluted affinity-purified rabbit anti-(*B. napus* + P_i PEPC) IgG. Lane 1 contains 15 ng of the final preparation of + P_i PEPC. Lane 2 contains 25 μ g of protein from a clarified extract prepared from + P_i *B. napus* suspension cells. Lane 3 contains 15 μ g of protein from an extract prepared from developing *B. napus* seed cotyledons. (C) Immunoblot analysis was performed using 20-fold diluted affinity-purified rabbit anti-(banana fruit PEPC) IgG [6]. Lane 1 contains 15 ng of the final preparation of + P_i PEPC. Lane 2 contains 50 ng of + P_i PEPC that was isolated in the absence of chymostatin. Abbreviations: O, origin; TD, tracking dye front.

shown). The anomalous color yield of certain proteins with the Coomassie-Blue dye binding assay has been well established [26].

Gel electrophoresis

Denaturation, followed by SDS/PAGE of the final + P_i PEPC preparation resolved a single protein staining band of approximately 104 kDa (Fig. 2A, lanes 2 and 3) that strongly cross-reacted with affinity-purified anti-(*B. napus* + P_i PEPC) IgG (Fig. 2B, lane 1) or anti-(banana fruit PEPC) IgG (Fig. 2C, lane 1). Identical results were obtained with the final preparation of - P_i PEPC (results not shown). SDS/PAGE and immunoblotting revealed that a polypeptide of approximately 5 kDa was cleaved from the 104-kDa subunit of PEPC during the enzyme's purification from + P_i cells in the absence of added chymostatin (Fig. 2A, lane 4 and Fig. 2C, lane 2) (final specific activity of proteolyzed + P_i PEPC = 11.4 U \cdot mg $^{-1}$). The repeated

inclusion of 5 μ g \cdot mL $^{-1}$ chymostatin at various stages of the purification prevented partial degradation of the enzyme during its purification (Fig. 2).

Determination of native molecular mass via gel filtration FPLC

The native molecular mass of the purified + P_i and - P_i PEPCs was determined to be 440 \pm 20 kDa (mean \pm SE; $n = 3$) as estimated by gel filtration FPLC on a calibrated Superdex 200 column. Thus, the native PEPCs appear to be homotetrameric.

Absorption coefficient

The molar absorption coefficient of *B. napus* + P_i PEPC is 4.32 \times 10 5 M $^{-1}$ \cdot cm $^{-1}$ at 280 nm ($A_{280}^{0.1\%} = 0.918$). This value

Table 1. Purification of PEPC from 220 g of 8-day-old P_i -sufficient (+ P_i) *B. napus* suspension cells.

Step	Volume (mL)	Activity (U)	Protein (mg)	Specific activity (U \cdot mg $^{-1}$)	Purification (fold)	Yield (%)
Clarified extract	645	113	4000 ^a	0.028	1	100
PEG fractionation	258	75	1340 ^a	0.055	2.0	66
Butyl Sepharose	34	65	240 ^a	0.27	9.6	58
DEAE Fractogel ^c	1.0	45	16 ^a	3.0	107	40
Superdex 200	9.4	25	3.7 ^a	6.9	246	22
Mono-Q ^c	0.65	16	0.80 ^a 0.43 ^b	20.0 37.2	714	14

^a Protein determined with Coomassie Blue R-250 dye-binding assay according to the method of Bollag and Edelstein [26]. ^b Protein determined with the bicinchoninic acid reagent according to the method of Hill and Straka [27]. ^c Concentrated pooled fractions.

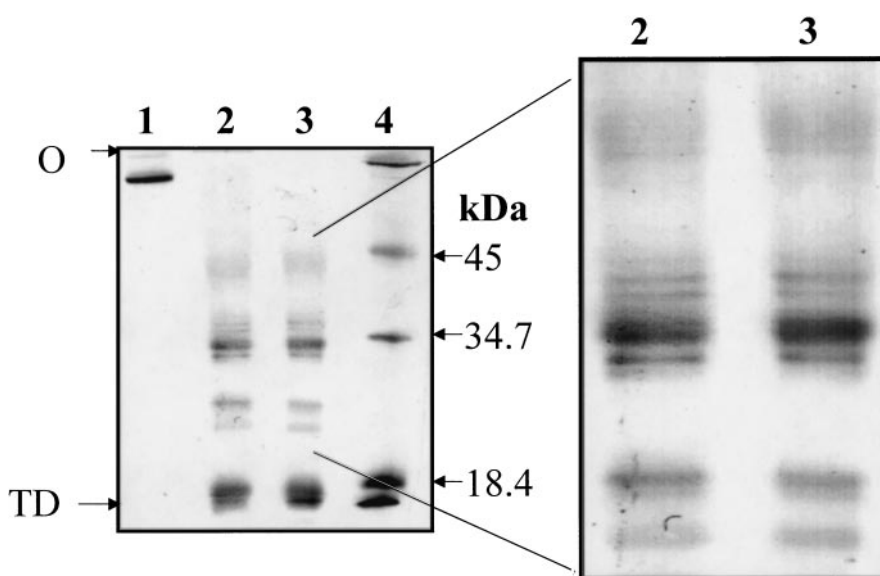


Fig. 3. Electrophoretic patterns of CNBr cleavage fragments of the 104 kDa subunit of *B. napus* +P_i and -P_i PEPCs. CNBr cleavage fragments were prepared from gel slices containing 8 µg of the 104-kDa polypeptide of +P_i (lane 2) or -P_i (lane 3) PEPCs and analyzed on an SDS/14% PAGE mini-gel as previously described [32]. Lane 1 contains 4 µg of the 104-kDa +P_i PEPC polypeptide incubated in the absence of CNBr. Lane 4 contains 4 µg of various molecular mass standards. The gel was stained with silver [33]. O, origin; TD, tracking dye front.

was calculated using the bicinchoninic acid determination of protein concentration (Table 1) and assuming a native molecular mass of 440 kDa.

Immunological characterization

Increasing amounts of rabbit anti-(*B. napus* +P_i PEPC) immune serum immunoprecipitated 100% of the activity of the purified *B. napus* +P_i PEPC. Complete immunoremoval of activity occurred at about 900 µL of immune serum per unit of PEPC activity. By contrast, preimmune serum had no effect on the PEPC activity. The affinity-purified anti-(*B. napus* +P_i PEPC) IgG could detect as little as 5 ng of denatured homogeneous +P_i or -P_i PEPC. Immunoblotting of clarified crude extracts prepared from *B. napus* suspension cells or developing seed embryos (at mid-cotyledonary stage of development) demonstrated monospecificity of the anti-(*B. napus* +P_i PEPC) IgG for the 104-kDa PEPC subunit (Fig. 2B, lanes 2 and 3). Likewise, nondenaturing PAGE of clarified extracts from +P_i *B. napus* cells generated a band of PEPC activity that comigrated with a single anti-(*B. napus* +P_i PEPC) IgG immunoreactive band (results not shown).

Peptide mapping

The structural relationship between the 104 kDa subunits of the +P_i and -P_i PEPCs was examined by peptide mapping of their respective CNBr cleavage fragments (Fig. 3). Indistinguishable peptide maps were obtained indicating that they are likely identical polypeptides.

Kinetic properties

Effect of pH. Similar to other plant PEPCs [1–6,23], the +P_i and -P_i PEPCs exhibited identical and broad pH/activity profiles with a maximum between 8.0 and 9.5 (results not shown). PEPC activity at pH 7.3 was almost 80% of that occurring at pH 8.4 (Table 2), but became almost undetectable below pH 7.0.

Cofactor requirements. The activity of *B. napus* +P_i PEPC showed an absolute dependence for a bivalent metal cation. At pH 8.4, the enzyme's $K_m(\text{Mg}^{2+})$ was determined to be 0.084 mM. Mn^{2+} (12 mM, added as MnCl_2) yielded the same

Table 2. Influence of glycerol, pH and various metabolites on V_{\max} and $K_m(\text{PEP})$ of PEPC purified from 8-day-old P_i-sufficient (+P_i) and P_i-deprived (-P_i) *B. napus* suspension cell cultures. The standard spectrophotometric assay [$\pm 10\%$ (v/v) glycerol] was used except that the PEP concentration was varied. Effectors were added individually to +P_i PEPC assays [pH 7.3, 10% (v/v) glycerol] as follows: 0.1 mM Glc-6-P, 0.1 mM L-malate, 1.25 mM DL-isocitrate, 1.5 mM L-Asp, or 5 mM L-Glu. ND, not determined.

	pH 7.3				pH 8.4			
	V_{\max}		$K_m(\text{PEP})$		$V_{\max} (\text{U}\cdot\text{mg}^{-1})$		$K_m(\text{PEP}) (\text{mM})$	
	- Glycerol	+ Glycerol	- Glycerol	+ Glycerol	- Glycerol	+ Glycerol	- Glycerol	+ Glycerol
+ P _i PEPC	15.7	15.7	0.41	0.17	20.0	20.0	0.15	0.08
+ Glc-6-P	ND	17.0	ND	0.08	ND	ND	ND	ND
+ L-malate	ND	14.5	ND	0.43	ND	ND	ND	ND
+ DL-isocitrate	ND	13.7	ND	0.30	ND	ND	ND	ND
+ L-Asp	ND	14.7	ND	1.0	ND	ND	ND	ND
+ L-Glu	ND	14.7	ND	0.67	ND	ND	ND	ND
- P _i PEPC	15.3	15.4	0.42	0.15	20.9	21.0	0.16	0.08

Table 3. Influence of various metabolites on the activity of PEPC purified from 8-day-old P_i -sufficient (+ P_i) *B. napus* suspension cell cultures. Assays were conducted at pH 7.3 or 8.4 in the presence of 10% (v/v) glycerol using a subsaturating (0.15 mM) concentration of PEP. Enzymatic activity in the presence of effectors is expressed relative to the respective control set at 100%. ND, not determined.

Addition	Concentration (mM)	Relative activity	
		pH 7.3	pH 8.4
Glc-6-P	2	230	122
Glc-1-P	2	143	108
Fru-6-P	2	174	113
Fru-1-P	2	130	102
Fru-1,6- P_2	5	129	92
Glycerol-3-P	5	171	110
3-P-Glycerate	2	140	100
Dihydroxyacetone-P	0.5	127	99
L-Glu	5	52	101
L-Asp	5	30	91
L-malate	5	5	97
DL-isocitrate	5	35	26
Succinate	5	67	66
Rutin	0.1	30	ND
Quercetin	0.1	5	ND

V_{\max} value achieved with saturating Mg^{2+} . These results are analogous to those obtained with other plant PEPCs [1–6].

PEP saturation kinetics. Table 2 summarizes the V_{\max} and apparent K_m (PEP) values of PEPC at pH 7.3 and 8.4 in the presence and absence of 10% (v/v) glycerol. In all instances, identical PEP saturation kinetics were obtained with the purified + P_i and – P_i *B. napus* PEPCs (Table 2). In common with most other plant PEPCs, *B. napus* PEPC exhibited hyperbolic PEP saturation kinetics. Decreasing the assay pH from 8.4 to 7.3 increased the enzyme's K_m (PEP) by more than twofold, whereas the addition of 10% (v/v) glycerol to the assay medium decreased the enzyme's K_m (PEP) by about 60 and 50% at pH 7.3 and pH 8.4, respectively (Table 2). Previous workers have cited stabilization of the enzyme's quaternary structure, due to exclusion of solvent molecules, as the rationale for the favorable influence of glycerol on the affinity of plant PEPC for PEP [35]. Glycerol (10%, v/v) was routinely added to the PEPC assay mixture.

Metabolite effects

A wide variety of compounds were tested as possible effectors of + P_i PEPC at pH 7.3 and 8.4 with subsaturating concentrations of PEP (0.15 mM). The following compounds had little or no influence on PEPC activity ($\pm 20\%$ of the control rate) at either pH 7.3 or 8.4: NH_4Cl , KP_i (10 mM each); 2-oxoglutarate, citrate, L-His, L-Arg, Gly, L-Ala, L-Lys, L-Gln, L-Asn, and sucrose (5 mM each); 2-P-glycerate, and L-Phe (2 mM each); MgADP, MgATP, and shikimic acid (1 mM each); acetyl-CoA, MgPP $_i$, fructose, and NAD^+ (0.5 mM each); L-Trp (0.25 mM); fructose 2,6- P_2 (20 μ M); Triton X-100, Nonidet P-40, and dimethylsulfoxide (2%, v/v, each). Table 3 lists those compounds that were found to significantly activate or inhibit the activity of the purified enzyme.

PEPC displayed pH-dependent modulation by several of the metabolites such that they were generally far more effective at

Table 4. Kinetic constants for several effectors of PEPC purified from 8-day-old P_i -sufficient (+ P_i) or P_i -starved (– P_i) *B. napus* suspension cell cultures. The standard spectrophotometric PEPC assay was used except that the assay pH and PEP concentration were suboptimal (pH 7.3; 0.34 mM PEP). I_{50} values for several inhibitors were determined ± 0.1 mM Glc 6-P. Similarly, K_a (Glc-6-P) values were determined in the presence and absence of approximate I_{50} concentrations of several inhibitors. 'Proteolyzed + P_i PEPC' refers to the partially degraded enzyme isolated in the absence of chymostatin (see Fig. 2A, lane 4; Fig. 2C, lane 2).

Effector	I_{50} (mM)	K_a (mM)
+ P_i PEPC		
Glc-6-P	–	0.066
+ 0.1 mM L-malate	–	0.17
+ 1.5 mM L-Asp	–	0.26
+ 5 mM L-Glu	–	0.22
L-malate	0.085	–
+ 0.1 mM Glc-6-P	0.37	–
L-Asp	1.5	–
+ 0.1 mM Glc-6-P	4.7	–
L-Glu	5.0	–
+ 0.1 mM Glc-6-P	13.0	–
DL-isocitrate	2.5	–
+ 0.1 mM Glc-6-P	2.5	–
Succinate	13.0	–
Quercetin	0.026	–
+ 0.1 mM Glc-6-P	0.028	–
Rutin	0.046	–
+ 0.1 mM Glc-6-P	0.050	–
Proteolyzed + P_i PEPC		
L-malate	0.86	–
– P_i PEPC		
L-malate	0.081	–

pH 7.3 than pH 8.4 (Table 3). Similar observations have been noted for other PEPCs from various plant sources [1,2,4–6,8,23].

Activators. Significant activators of *B. napus* + P_i PEPC at pH 7.3 were the hexose- P s (particularly Glc-6-P), and glycerol-3-P (Table 3). Synergistic or additive effects of activators at pH 7.3 were not observed, suggesting that they all interact at a common allosteric site. At 0.1 mM, Glc-6-P significantly decreased the enzyme's K_m (PEP) (by about 50%) and slightly increased its V_{\max} at pH 7.3 (Table 2). Glc-6-P also functions as an activator by effectively relieving the inhibition of PEPC by L-malate, L-Asp, and L-Glu (Table 4; Fig. 4). The addition of 0.1 mM Glc-6-P increased the I_{50} values of PEPC for these inhibitors by threefold to fourfold (Table 4). Furthermore, the enzyme's fold-activation by saturating Glc-6-P was increased from about twofold to over sixfold in the presence of 0.1 mM L-malate, 5 mM L-Glu, or 1.5 mM L-Asp (Fig. 4).

Inhibitors. At pH 7.3, the *B. napus* + P_i PEPC was potently inhibited by L-malate, DL-isocitrate, L-Asp, and L-Glu (Tables 3 and 4). Increasing the assay pH to 8.4 nullified the enzyme's inhibition by 5 mM L-malate, L-Asp, or L-Glu (Table 3). These metabolites function as inhibitors at pH 7.3 by markedly reducing the affinity of PEPC for its substrate, PEP, and its activator, Glc-6-P (Tables 2 and 4, Fig. 4). This is reflected by the addition of approximate I_{50} concentrations of DL-isocitrate and/or L-malate, L-Asp, or L-Glu causing an increase in the K_m (PEP) of PEPC by up to sixfold (Table 2), and K_a (Glc-6-P) by at least twofold (Table 4). Of interest is the unique inhibition

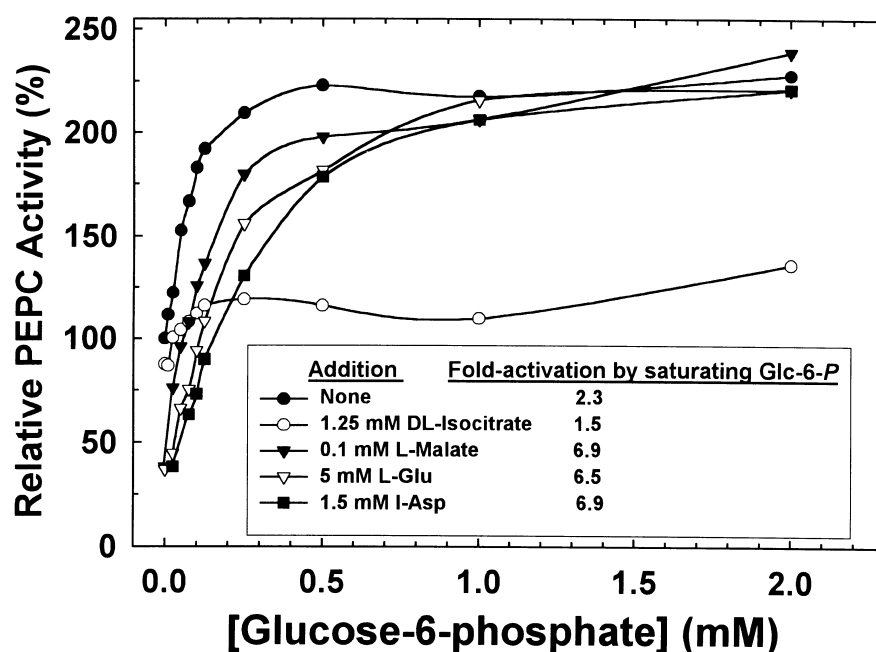


Fig. 4. Relationship between +P_i PEPC activity and the concentration of glucose-6-P in the presence and absence of L-Asp, L-Glu, L-malate, and DL-isocitrate. Assays were conducted at pH 7.3 with subsaturating (0.34 mM) PEP in the absence (●) and presence of 1.25 mM DL-isocitrate (○), 0.1 mM L-malate (▼), 5 mM L-Glu (▽), or 1.5 mM L-Asp (■).

of *B. napus* PEPC by DL-isocitrate. In contrast to L-malate, L-Asp, and L-Glu: (a) inhibition by DL-isocitrate was comparable at both pH 7.3 and 8.4, and was not relieved by the addition of 0.1 mM Glc-6-P (Tables 3 and 4), and (b) the presence of 1.25 mM DL-isocitrate almost completely negated PEPC activation by Glc-6-P at pH 7.3 (Fig. 4). As reported for PEPC from leaves of *Amaranthus viridis* [36], the *B. napus* PEPC also demonstrated potent inhibition by the flavonoids quercetin and rutin (Table 3). *I*₅₀ values for both flavonoids were less than 50 μM, and were not influenced by the addition of 0.1 mM Glc-6-P to the reaction mixture (Table 4).

Phosphorylation status of PEPC from +P_i and -P_i *B. napus* suspension cells

Clarified extracts of 8-day-old -P_i and +P_i *B. napus* cells were prepared and desalted in the presence and absence of the phosphatase inhibitors 20 mM NaF and 50 nM microcystin-LR. The desalted extracts were incubated in the presence and absence of 1 U·mL⁻¹ of bovine heart PP2A or 2 U·mL⁻¹ of bovine alkaline phosphatase for 1 h at 23 °C and assayed for PEPC activity at pH 7.3 with subsaturating (0.4 mM) PEP. The subsequent addition of 0.1 mM L-malate resulted in an approximate 50% inhibition of PEPC activity in the +P_i and -P_i cell extracts, irrespective of the treatment. Likewise, an identical incubation of the purified +P_i and -P_i PEPCs with PP2A or alkaline phosphatase failed to influence their sensitivity to L-malate inhibition when assayed at pH 7.3 with subsaturating PEP. Together with the fact that the purified -P_i and +P_i PEPCs exhibited identical *I*₅₀(L-malate) values at pH 7.3 (Table 4), these results suggest that PEPC mainly exists in its dephosphorylated (L-malate sensitive) form in both +P_i and -P_i *B. napus* cells.

DISCUSSION

Influence of P_i starvation on PEPC

Like lupin, *B. napus* is a 'nonmycotrophic' plant whose roots do not form symbiotic associations with mycorrhizal fungi to

facilitate P_i uptake from the soil [37]. Recent evidence suggests that, relative to mycorrhizal associating or 'mycotrophic' plants (which account for about 90% of terrestrial plant species), the endogenous metabolism of nonmycotrophic plants is geared to allow a more efficient acclimatization to P_i deficiency [11,19,37]. For example, unlike many mycotrophs, roots of P_i-deprived lupin and *B. napus* plants are highly efficient users of rock P_i [16–19]. This has been ascribed to the PEPC-mediated synthesis and subsequent excretion of malic and citric acids from the roots during P_i deficiency, resulting in decreased rhizosphere pH and the solubilization of mineral-bound P_i. However, until now there has been no report describing the detailed comparison of the physical, immunological and kinetic properties of PEPC purified from the same +P_i and -P_i plant source.

Fresh weight of the 8-day-old -P_i *B. napus* suspension cells was about 50% that of the +P_i cells, indicating that the 8-day-old -P_i and +P_i cells were in fact P_i starved and P_i sufficient, respectively, at the time of harvest. This is corroborated by: (a) the marked reduction in intracellular P_i concentration of the -P_i, but not +P_i, *B. napus* cells (the P_i concentration of 8-day-old -P_i and +P_i *B. napus* cells was previously determined to be about 0.7 and 3.8 μmol·g⁻¹ fresh wt, respectively) [25], and (b) the significant induction of APase activity, a universal biochemical indicator of plant P_i stress [11], in the -P_i *B. napus* cells relative to the +P_i controls (Fig. 1B). As anticipated, the extractable PEPC specific activity of the -P_i *B. napus* cells significantly increased (by 250%), relative to that of the +P_i cells, which remained low and constant throughout the 9-day time course (Fig. 1A). PEPC and APase were induced in parallel in response to P_i stress, and this was at least partially reversed 1-day after 2.5 mM P_i was resupplied to 8-day-old -P_i cells (Fig. 1A,B). Laser densitometric quantification of PEPC immunoblots revealed a close correlation between extractable PEPC activity and the relative amount of the immunoreactive 104-kDa PEPC subunit in clarified extracts of 8-day-old +P_i and -P_i cells (Fig. 1A). Analogous results have been described for the PEPC activity, concentration and mRNA levels in -P_i proteoid lupin roots relative to +P_i controls [18,19]. Therefore, in both *B. napus* cell cultures and proteoid lupin roots, the

increased PEPC activity that accompanies P_i stress at least partially arises from an increased expression of PEPC protein.

In particular, we wished to determine whether P_i deprivation of *B. napus* also induced any alteration in the phosphorylation status of PEPC and/or the synthesis of a different PEPC isozyme (having the same subunit size). These goals were provoked by: (a) inhibition studies of partially purified PEPC from proteoid lupin roots indicating that PEPC may be phosphorylated by an endogenous protein kinase during P_i starvation (as reflected by a decreased sensitivity of the enzyme to L-malate inhibition), and (b) the isolation of a PEPC cDNA from proteoid roots of P_i starved lupin plants [19]. Whether this cDNA encodes a separate PEPC isozyme (relative to that expressed in $+P_i$ lupin) has not been reported.

PEPC purification

The specific PP1/PP2A inhibitor microcystin-LR (50 nM) was added to the extraction buffer, and the general phosphatase inhibitor NaF (20 mM) was included in all purification buffers to prevent potential alterations in the phosphorylation status of PEPC during its extraction and isolation from the $+P_i$ and $-P_i$ cells. The protease inhibitor chymostatin was also included in purification buffers to prevent N-terminal truncation and consequent loss of the enzyme's phosphorylation domain, as previously documented for a variety of plant PEPCs [1,2]. Indeed, isolation of the *B. napus* $+P_i$ PEPC in the absence of chymostatin resulted in a proteolytically clipped enzyme (Fig. 2A, lane 4, and Fig. 2C, lane 2) that was an order of magnitude less sensitive to L-malate inhibition relative to the nondegraded $+P_i$ PEPC isolated in the presence of chymostatin (Table 4). Loss of a \approx 4-kDa N-terminal phosphorylation domain of maize or sorghum C_4 PEPC during their purification in the absence of chymostatin kinetically mimics the effect of phosphorylation of the nonproteolyzed enzymes [e.g. N-terminal proteolysis or phosphorylation of the intact PEPC both elicit a similar increase in the enzyme's $I_{50}(\text{L-malate})$ without affecting V_{max}] [1].

The final specific activities of the purified $+P_i$ and $-P_i$ *B. napus* PEPCs were about $20 \text{ U}\cdot\text{mg}^{-1}$ and compare favorably to the values reported previously for homogeneous PEPCs from various plant sources [1,5,6]. Analysis by SDS/PAGE confirmed that both PEPCs had been purified to apparent homogeneity (Fig. 2A, and results not shown). Similar to most other plant PEPCs, the native $+P_i$ and $-P_i$ PEPCs exist as 440-kDa homotetramers.

Immunological properties

Rabbit anti-(*B. napus* $+P_i$ PEPC) immune serum immunoprecipitated up to 100% of the activity of the purified $+P_i$ PEPC. Monospecificity of the antibody preparation for PEPC is indicated by the observation that only the 104-kDa PEPC subunit showed a significant cross-reaction when immunoblots of clarified extracts from *B. napus* developing seed (zygotic) cotyledons and/or suspension cells were probed with the affinity-purified anti-(*B. napus* $+P_i$ PEPC) IgG (Fig. 2B). An immunoblot of the purified $-P_i$ *B. napus* PEPC cross-reacted with the anti-(*B. napus* $+P_i$ PEPC) IgG to a similar extent as the purified $+P_i$ PEPC. Similarly, antibodies to banana fruit PEPC cross-reacted strongly with the $+P_i$ *B. napus* PEPC (Fig. 2C). These results are consistent with those of previous studies [4,6,38] and indicate a high degree of structural similarity between PEPCs of vascular plants. In contrast, the anti-(banana or *B. napus* PEPC) IgGs fail to recognize purified

PEPC from green algae or cyanobacteria [38] (J. Rivoal, W. C. Plaxton, and D. H. Turpin, unpublished data).

Peptide mapping

Peptide mapping is a powerful technique for evaluating the structural relationship between polypeptides [32]. We therefore analyzed the fragments generated by CNBr cleavage of the subunits of the purified $+P_i$ and $-P_i$ *B. napus* PEPCs. The cleavage patterns were identical (Fig. 3), demonstrating that the 104-kDa subunit of the $+P_i$ and $-P_i$ PEPCs is probably the same polypeptide.

Kinetic studies

It has been amply demonstrated that phosphorylation of plant PEPCs significantly decreases the enzyme's sensitivity to L-malate inhibition when assayed at subsaturating PEP and suboptimal, but physiological, pH values ranging from about pH 7–7.4 [1–3,5,7–10]. In addition, phosphorylation of plant PEPC may result in an increased V_{max} at suboptimal pH (e.g. pH 7.3) and/or a reduced $K_m(\text{PEP})$ [7,10]. However, kinetic comparisons of the purified nonproteolyzed $+P_i$ and $-P_i$ *B. napus* PEPCs revealed identical pH–activity profiles, PEP saturation kinetics (Table 2), and sensitivity to L-malate inhibition at pH 7.3 with subsaturating PEP (Table 4). The relatively low $I_{50}(\text{L-malate})$ value of about 0.1 mM obtained for both purified PEPCs (Table 4) suggests that PEPC mainly exists in its dephosphorylated, L-malate sensitive form in the $+P_i$ and $-P_i$ *B. napus* cells. This conclusion was corroborated by the failure of PP2A or alkaline phosphatase treatment of the respective clarified cell extracts or purified PEPCs to alter the enzyme's sensitivity to L-malate inhibition when assayed at pH 7.3 with subsaturating PEP. Thus, phosphorylation does not appear to play a role in regulating *B. napus* PEPC during P_i deprivation. Although phosphorylation of plant PEPC invariably relieves the inhibitory action of L-malate (and L-Asp [1,7]), this could be unnecessary during severe P_i stress when cellular biosynthetic processes are minimal and the bulk of organic acids produced via PEPC (e.g. L-malate and citrate) do not accumulate within the cytosol, but may either be respired by the mitochondria, sequestered in the vacuole, or excreted from the cell. Overall, our results indicate that the up-regulation of PEPC activity during P_i deprivation of *B. napus* suspension cells arises solely from the accumulation of the same PEPC isoform as exists in the $+P_i$ cells. Whether this is due to the increased synthesis and/or reduced proteolytic turnover of the enzyme remains to be determined.

Metabolite effectors of *B. napus* PEPC

PEP is one of the initial substrates for the shikimic acid (aromatic) pathway, and is thus at a major branchpoint between plant primary and secondary metabolism. PEPC from C_4 leaves was recently reported to be potently inhibited by several shikimic acid pathway endproducts, notably the flavonoids quercetin and rutin [36]. This was suggested as a possible modulator of the partitioning of PEP between primary and secondary metabolism. *B. napus* PEPC also displayed potent inhibition by rutin and quercetin (Tables 3 and 4), indicating that this may be a universal response of plant PEPCs. Both flavonoids have been implicated in a number of plant functions, including defense and plant-microbe signaling. However, the precise physiological relevance of rutin and quercetin inhibition of *B. napus* PEPC (and PK_c [20]) will remain obscure until

information is obtained as to their respective concentrations in the plant cytosol. Interestingly, shikimic acid (1 mM), a potent inhibitor of *A. viridis* leaf PEPC [39,40], exerted no influence on the activity of the *B. napus* PEPC in the presence of 10% (v/v) glycerol.

B. napus PEPC was highly responsive to a number of metabolites involved in carbon and nitrogen metabolism. Notably, this PEPC was activated by Glc-6-P and potently inhibited by L-malate, DL-isocitrate, L-Asp and L-Glu at pH 7.3 (Tables 2–4, Fig. 4), whereas sensitivity to these compounds (with the exception of DL-isocitrate) was considerably diminished at pH 8.4 (Table 3). Cytosolic concentrations of Glc-6-P, L-malate, L-Asp and L-Glu in spinach leaves have been estimated to be about 6, 1, 23 and 21 mM, respectively [41]. Thus, the K_a (Glc-6-P) and I_{50} values for the organic and amino-acid inhibitors of *B. napus* PEPC are generally well within their probable physiological concentration range, suggesting that these metabolites are important regulators of PEPC activity *in vivo*. Potent inhibition by L-malate, L-Asp, and L-Glu has also been reported for PEPCs from unicellular green algae [38,42], *C₃* leaves [8,43], as well as several nonphotosynthetic tissues including soybean root nodules [5,44], germinated castor seeds [4], banana fruit [6], and developing seeds of *V. faba* [23]. To our knowledge, marked inhibition of a plant PEPC by DL-isocitrate has only been reported for the germinating castor cotyledon enzyme ($I_{50} = 0.7$ mM at pH 7.0, 0.1 mM PEP) [4]. In the companion paper [20], the kinetic and regulatory features of the purified *B. napus* PEPC and PK_C are compared and contrasted. A model is presented which highlights the critical role played by L-Asp and L-Glu in the coordinate control of these two key enzymes, particularly as pertains to the regulation of glycolysis and PEP partitioning during nitrogen assimilation.

The activation of *C₃*-leaf PEPCs by protein kinase-mediated phosphorylation in response to nitrogen resupply of nitrogen-limited tissues [1,8,45] raises the possibility that the *B. napus* PEPC is also subject to this additional form of metabolic control. However, there are other reports that reversible phosphorylation of *C₃*-plant PEPC is of minor importance for its regulation (relative to its control by allosteric effectors) [2,43,46]. Results discussed above and in the following paper clearly emphasize the fundamental role of allosteric effectors in the coordinate control of *B. napus* PEPC and PK_C.

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REFERENCES

- Chollet, R., Vidal, J. & O'Leary, M.H. (1996) Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 273–298.
- Rajagoplan, A.V., Devi, M.T. & Raghavendra, A.S. (1994) Molecular biology of *C₄* phosphoenolpyruvate carboxylase: Structure, regulation and genetic engineering. *Photosynth. Res.* **39**, 115–135.
- Nimmo, H.G. (1993) The regulation of phosphoenolpyruvate carboxylase by reversible phosphorylation. In *Society for Experimental Biology Seminar Series 53: Post-Translational Modifications in Plants* (Battey, N.H., Dickinson, H.G. & Hetherington, S.M., eds), pp. 161–170. Cambridge University Press, Cambridge, UK.
- Podestá, F.E. & Plaxton, W.C. (1994) Regulation of cytosolic carbon metabolism in germinating *Ricinus communis* cotyledons. II. Properties of phosphoenolpyruvate carboxylase and cytosolic pyruvate kinase associated with the regulation of glycolysis and nitrogen assimilation. *Planta* **194**, 381–387.
- Zhang, X.-Q., Li, B. & Chollet, R. (1995) *In vivo* regulatory phosphorylation of soybean nodule phosphoenolpyruvate carboxylase. *Plant Physiol.* **108**, 1561–1568.
- Law, R.D. & Plaxton, W.C. (1995) Purification and characterization of a novel phosphoenolpyruvate carboxylase from banana fruit. *Biochem. J.* **307**, 807–816.
- Law, R.D. & Plaxton, W.C. (1997) Regulatory phosphorylation of banana fruit phosphoenolpyruvate carboxylase by a copurifying phosphoenolpyruvate carboxylase-kinase. *Eur. J. Biochem.* **247**, 642–651.
- Duff, S.M.G. & Chollet, R. (1995) *In vivo* regulation of wheat-leaf phosphoenolpyruvate carboxylase by reversible phosphorylation. *Plant Physiol.* **107**, 775–782.
- Du, Z., Aghoram, K. & Outlaw, W.H. (1997) *In vivo* phosphorylation of phosphoenolpyruvate carboxylase in guard cells of *Vicia faba* L. is enhanced by fusicoccin and suppressed by abscisic acid. *Arch. Biochem. Biophys.* **337**, 345–350.
- Osuna, L., González, C., Cejudo, F.J., Vidal, J. & Echevarria, C. (1996) *In vivo* and *in vitro* phosphorylation of the phosphoenolpyruvate carboxylase from wheat seeds during germination. *Plant Physiol.* **111**, 551–558.
- Plaxton, W.C. & Carswell, M.C. (1999) Metabolic aspects of the phosphate starvation response in plants. In *Plant Responses to Environmental Stresses: from Phytohormones to Genome Reorganization* (Lerner, H.R., ed.), pp. 349–372. Marcel Dekker, Inc., New York.
- Duff, S.M.G., Moorhead, G.B.G., Lefebvre, D.D. & Plaxton, W.C. (1989) Phosphate starvation inducible 'bypasses' of adenylate and phosphate dependent glycolytic enzymes in *Brassica nigra* suspension cells. *Plant Physiol.* **90**, 1275–1278.
- Nagano, M. & Ashihara, H. (1994) Phosphate starvation and a glycolytic bypass catalyzed by phosphoenolpyruvate carboxylase in suspension cultured *Catharanthus roseus* cells. *Z. Naturforsch.* **49c**, 742–750.
- Neumann, G. & Römheld, V. (1999) Root excretion of carboxylic acids and protons in phosphorus-deficient plants. *Plant Soil* **211**, 121–130.
- Pilbeam, D.J., Cakmak, I., Marschner, H. & Kirkby, E.A. (1993) Effect of phosphorus withdrawal on nitrate assimilation and PEP carboxylase activity in tomato. *Plant Soil* **154**, 111–117.
- Hoffland, E., Van Den Boogaard, R., Nelemans, J. & Findenegg, G. (1992) Biosynthesis and root exudation of citric and malic acids in phosphate-starved rape plants. *New Phytol.* **122**, 675–680.
- Johnson, J.F., Allan, D.L., Vance, C.P. & Weiblen, G. (1996) Root carbon dioxide fixation by phosphorus-deficient *Lupinus albus*. *Plant Physiol.* **112**, 19–30.
- Johnson, J.F., Vance, C.P. & Allan, D.L. (1996) Phosphorus deficiency in *Lupinus albus*. Altered lateral root development and enhanced expression of phosphoenolpyruvate carboxylase. *Plant Physiol.* **112**, 31–41.
- Gilbert, G.A., Vance, C.P. & Allan, D.L. (1998) Regulation of white lupin root metabolism by phosphorus availability. In *Phosphorus in Plant Biology: Regulatory Roles in Molecular, Cellular, Organismic, and Ecosystem Processes* (Lynch, J.P. & Deikman, J., eds), pp. 157–167. American Society of Plant Physiologists, Rockville, MD.
- Smith, C.R., Knowles, V.L. & Plaxton, W.C. (2000) Purification and characterization of cytosolic pyruvate kinase from *Brassica napus* (rapeseed) suspension cell cultures. Implications for the integration of glycolysis with nitrogen assimilation. *Eur. J. Biochem.* **267**, 4477–4485.
- Huppe, H.C. & Turpin, D.H. (1994) Integration of carbon and nitrogen metabolism in plant and algal cells. *Annu. Rev. Plant Physiol. Mol. Biol.* **45**, 577–607.
- Plaxton, W.C. (1996) The organization and regulation of plant glycolysis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 185–214.
- Golombek, S., Heim, U., Horstmann, C., Wobus, U. & Weber, H. (1999) phosphoenolpyruvate carboxylase in developing seeds of

- Vicia faba* L. gene expression and metabolic regulation. *Planta* **208**, 66–72.
24. Orr, W., Keller, W.A. & Singh, J. (1986) Induction of freezing tolerance in an embryogenic cell suspension culture of *Brassica napus* by abscisic acid at room temperature. *J. Plant Physiol.* **126**, 23–32.
 25. Carswell, M.C., Grant, B.R. & Plaxton, W.C. (1997) Disruption of the phosphate-starvation response of oilseed rape suspension cells by the fungicide phosphonate. *Planta* **203**, 67–74.
 26. Bollag, D.M. & Edelman, S.J. (1991) Protein concentration determination. II. The Bradford assay. In *Protein Methods*, pp. 50–55. Wiley-Liss, New York.
 27. Hill, H.D. & Straka, J.G. (1988) Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. *Anal. Biochem.* **170**, 203–208.
 28. Brooks, S.P.G. (1992) A simple computer program with statistical tests for the analysis of enzyme kinetics. *Biotechniques* **13**, 906–911.
 29. Plaxton, W.C. (1989) Molecular and immunological characterization of plastid and cytosolic pyruvate kinase isozymes from castor-oil-plant endosperm and leaf. *Eur. J. Biochem.* **181**, 443–451.
 30. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
 31. Doucet, J.-P. & Trifaró, J.M. (1988) A discontinuous and highly porous sodium dodecyl sulphate polyacrylamide slab gel system of high resolution. *Anal. Biochem.* **168**, 265–271.
 32. Plaxton, W.C. & Moorhead, G.B.G. (1989) Peptide mapping by CNBr fragmentation using a sodium dodecyl sulfate-polyacrylamide minigel system. *Anal. Biochem.* **178**, 391–393.
 33. Wray, W., Boulikas, T., Wray, V.P. & Hancock, R. (1981) Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**, 197–203.
 34. Penefsky, H. (1977) Reversible binding of P_i by beef heart mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **252**, 2891–2899.
 35. Podestá, F.E. & Andreo, C.S. (1989) Maize leaf phosphoenolpyruvate carboxylase. Oligomeric state and activity in the presence of glycerol. *Plant Physiol.* **90**, 427–433.
 36. Pairoba, C.F., Colombo, S.L. & Andreo, C.S. (1996) Flavonoids as inhibitors of NADP-malic enzyme and PEP carboxylase from two C_4 plants. *Biosci. Biotech. Biochem.* **60**, 779–783.
 37. Murley, V.R., Theodorou, M.E. & Plaxton, W.C. (1998) Phosphate starvation-inducible pyrophosphate-dependent phosphofructokinase occurs in plants whose roots do not form symbiotic associations with mycorrhizal fungi. *Physiol. Plant.* **103**, 405–414.
 38. Rivoal, J., Plaxton, W.C. & Turpin, D.H. (1998) Purification and characterization of high- and low-molecular-mass isoforms of phosphoenolpyruvate carboxylase from *Chlamydomonas reinhardtii*. Kinetic, structural and immunological evidence that the green algal enzyme is distinct from the prokaryotic and higher plant enzymes. *Biochem. J.* **331**, 201–209.
 39. Colombo, S.L., Pairoba, C.F. & Andreo, C.S. (1996) Inhibitory effect of shikimic acid on PEP carboxylase activity. *Plant Cell Physiol.* **37**, 870–872.
 40. Colombo, S.L., Andreo, C.S. & Chollet, R. (1998) The interaction of shikimic acid and protein phosphorylation with PEP carboxylase from the C_4 dicot *Amaranthus viridis*. *Phytochemistry* **48**, 55–59.
 41. Winter, H., Robinson, D.G. & Heldt, H.W. (1994) Subcellular volumes and metabolite concentrations in spinach leaves. *Planta* **193**, 530–535.
 42. Rivoal, J., Dunford, R., Plaxton, W.C. & Turpin, D.H. (1996) Purification and properties of four phosphoenolpyruvate carboxylase isoforms from the green alga *Selenastrum minutum*: evidence that association of the 102-kDa catalytic subunit with unrelated polypeptides may modify the physical and kinetic properties of the enzyme. *Arch. Biochem. Biophys.* **332**, 47–57.
 43. Lepoint, L., Kandlbinder, A., Baur, B. & Werner, M.K. (1996) Diurnal modulation of phosphoenolpyruvate carboxylation in pea leaves and roots as related to tissue malate concentrations and to the nitrogen source. *Planta* **198**, 4695–4501.
 44. Schuller, K.A., Turpin, D.H. & Plaxton, W.C. (1990) Metabolite regulation of partially purified soybean nodule phosphoenolpyruvate carboxylase. *Plant Physiol.* **94**, 1429–1435.
 45. Champigny, M.-L. & Foyer, C. (1992) Nitrate activation of cytosolic protein kinases diverts photosynthetic carbon from sucrose to amino acid biosynthesis. Basis for a new concept. *Plant Physiol.* **101**, 7–12.
 46. Gupta, S.K., Lu, M.S.B., Lin, J.H., Zhang, D. & Edwards, G.E. (1994) Light/dark modulation of phosphoenolpyruvate carboxylase in C_3 and C_4 species. *Photosynth. Res.* **42**, 133–143.