Purification and Characterization of Phospho*enol*pyruvate Carboxylase from *Brassica napus* (Canola) suspension cell cultures

By

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ABSTRACT

Regulation of phospho*enol*pyruvate carboxylase (PEPC) during phosphate (Pi) deprivation of *Brassica napus* (canola) suspension cell cultures was examined. PEPC and acid phosphatase specific activities reversibly increased by 2.5- and 4-fold, respectively, following 8- to 10-d of Pi starvation. Densitometric scanning of PEPC immunoblots revealed a close correlation between extractable PEPC activity and relative amount of the 104 kDa PEPC subunit. To determine whether Pi deprivation also induced any alteration in PEPC's phosphorylation status and/or synthesis of a different PEPC isozyme, PEPC was purified to apparent homogeneity from Pi-sufficient (+Pi) and Pi-starved (-Pi) cells. Chymostatin was required in purification buffers to prevent partial *in vitro* proteolysis of PEPC. Final specific activities of the +Pi and -Pi PEPCs were 20-21 (μmol phospho*enol*pyruvate utilized/min)/mg protein. SDS/PAGE of the final preparations resolved single 104 kDa protein-staining polypeptides; the native molecular mass of +Pi or -Pi PEPC was approximately 440 kDa, indicating homotetrameric quaternary structures. Monospecific rabbit anti-(*B. napus* PEPC) immune serum was produced that effectively immunoprecipitated PEPC activity. CNBr peptide mapping demonstrated the 104 kDa +Pi and -Pi PEPC subunits to be identical polypeptides. Respective pH-activity profiles, PEP saturation kinetics, and sensitivity to malate inhibition were also indistinguishable. Kinetic studies and protein phosphatase incubations suggested that PEPC exists in its dephosphorylated, malate sensitive form, in +Pi and -Pi cells. Thus, upregulation of PEPC activity in -Pi *B. napus* cells arises via increased synthesis of the same PEPC isoform being expressed in +Pi cells. PEPC was

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allosterically regulated by several metabolites involved in carbon and nitrogen metabolism. Marked activation by glucose-6-phosphate and inhibition by malate, isocitrate, aspartate and glutamate occurred. Potent inhibition by the flavonoids rutin and quercitin was also observed. Allosteric features of *B. napus* PEPC are compared with those of *B. napus* cytosolic pyruvate kinase. A model is presented which highlights the critical role played by aspartate and glutamate in the coordinate regulation of these PEP utilizing enzymes, particularly as it pertains to the integration of carbohydrate partitioning with the generation of Krebs' cycle intermediates required during nitrogen assimilation.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

A_{280}	absorbance at 280 nm
Apase	Acid phosphatase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CAM	Crassulacean acid metabolism
C_3 - plant	plant that initially fixes CO_2 via the reductive pentose-phosphate (Calvin) cycle
C_4 - plant	plant that initially fixes CO_2 via PEP carboxylase
DHAP	dihydroxyacetone phosphate
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetate
EGTA	(ethylene-dioxy)diethylenedinitrilotetraacetate
$F-1,6-P_2$	fructose-1,6-bisphosphate
$F-2,6-P_2$	fructose-2,6-bisphosphate
Fru-6-P	fructose-6-phosphate
FPLC	fast protein liquid chromatography
FW	fresh weight
G3P	glyceraldehyde 3-phosphate
Glc-1-P	glucose 1-phosphate
Glc-6-P	glucose-6-phosphate
Gly-3-P	glycerol 3-phosphate
Hepes	N-2-hydroxyethylpiperizine-N'-2-ethanesulphonic acid
IgG	immunoglobulin G

I_{50}	concentration of inhibitor producing a 50% reduction in enzyme activity
K_a	concentration of activator causing half-maximal activation of enzyme activity
kDa	kilodalton
K_m	Michaelis-Menten constant
MDH	malate dehydrogenase
ME	NAD-dependent malic enzyme
Mes	2-(N-morpholino)ethanesulfonic acid
min	minute(s)
M_r	relative molecular mass
NADH	nicotinamide adenine dinucleotide (reduced)
NAD^-	nicotinamide adenine dinucleotide (oxidized)
n.d.	not determined
-Pi	cultured in the absence of phosphate
+Pi	cultured in the presence of phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PEP	phospho*enol*pyruvate
PEPC	phospho*enol*pyruvate carboxylase
PFK	ATP-dependent phosphofructokinase
PFP	PPi-dependent phosphofructokinase
3-PGA	3-phosphoglycerate
Pi	inorganic orthophosphate
PK	pyruvate kinase
PMSF	phenylmethylsulphonyl fluoride

PPi	inorganic pyrophosphate
PP2A	protein phosphatase type 2A1
PVDF	polyvinylidne difluoride
PVP	polyvinylpyrrolidone
PVPP	polyvinyl(polypyrrolidone)
RBV	resin bed volume
rpm	revolutions per minute
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulfate
s.e.m	Standard error of the mean
Tris	tris(hydroxymethyl)methylamine
V_{max}	maximal velocity

PREFACE

This thesis was written in accordance with regulation 8.6a of the Queen's

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CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Comparative Biochemistry of the Glycolytic Pathway.

The universality of glycolysis, in which hexoses are sequentially oxidized to the three-carbon compound pyruvate, has been demonstrated by its initial discovery in yeast and subsequent analysis in animal, bacterial and plant systems. An intense study of glycolysis in yeast and animal systems has defined a linear metabolic sequence of 10 cytosolic localized enzymes from hexokinase to pyruvate kinase (PK) (Fig. 1.1 A). In contrast, plants can complete this process through modified glycolytic pathways and also in two subcellular compartments, the cytosol and plastid (Fig. 1.1 B). Additionally, plants possess alternative cytosolic glycolytic enzymes that can serve to bypass or supplement the activity of 'classical' enzymes when the activity of the latter are limited by environmental factors [1]. It is necessary for plants to be adaptive in this area of metabolism because their immobility leaves them few options other than acclimation. Animal glycolysis, by contrast, often occurs within the rigidly controlled intervals of temperature, pH and substrate concentration.

Not only do plants possess alternate means to effectuate glycolysis, but the manner in which this pathway is regulated differs significantly from animal systems. The pathway in organisms from both kingdoms is controlled through allosteric and covalent modification of key enzymes involved with two metabolite interconversion points, that of fructose-6-phosphate (Fru-6-P) / fructose-1,6-bisphosphate (Fru-1,6-P_2) and phospho*enol*pyruvate (PEP)/pyruvate [1]. In animal systems, control of hexose catalysis is 'topdown': primary allosteric control rests at the level of phosphofructokinase

Figure 1.1. A comparison of the organization of nonplant (A) vs plant (B) glycolysis and associated pathways. The enzymes that catalyze the numbered reactions are as follows: 1. hexokinase; 2. phosphorylase; 3. phosphoglucomutase; 4. phosphoglucose isomerase; 5. PFK; 6. aldolase; 7. triose phosphate isomerase: 8. NAD-dependent GAPDH (phosphorylating); 9, 3-PGA kinase; 10, phosphoglyceromutase; 11, enolase: 12, PK; 13. invertase; 14. sucrose synthase; 15. UDP-glucose pyrophosphorylase: 16. nucleoside diphosphate kinase; 17. α- and β-amylases; 18. PFP; 19. NADP-dependent GAPDH (nonphosphorylating); 20. PEPase; 21, PEPC; 22, MDH; 23. ME. Abbreviations are in the text or as follows: Glu-1-P, glucose-1-phosphate: DHAP. dihydroxyacetone phosphate: G3P, glyceraldehyde-3-phosphate; 1,3-DPGA. 1,3-diphosphoglycerate; 2-PGA. 2-phosphoglycerate; OAA, oxaloacetate. (From Plaxton [1])

A. NONPLANT GLYCOLYSIS

GLUCOSE   GLYCOGEN

① ATP / ADP   ② Pi

Glu-6-P ③ → Glu-1-P

④

Fru-6-P

⑤ ATP / ADP

Fru-1,6-P_2

⑥

DHAP ⑦ → G3P

⑧ NAD^++Pi / NADH

1,3-DPGA

⑨ ADP / ATP

3-PGA

⑩

2-PGA

⑪

PEP

⑫ ADP / ATP

Pyruvate

Respiration ← → *Biosynthesis*

B. PLANT GLYCOLYSIS

SUCROSE

⑬   ⑭

Glucose Fructose UDP / UDP-Glucose / PPI

① ATP/ADP ① ATP/ADP ⑯ ⑮ UTP

Glu-6-P   Glu-1-P

④ Fru-6-P ← ④ → Glu-6-P

⑤ ATP/ADP ⑱ PPI / Pi

Fru-1,6-P_2

⑥ → DHAP

⑦

G3P

⑲ $NADP^+$ / NADPH ⑧ NAD^++Pi / NADH

1,3-DPGA

⑨ ADP / ATP

3-PGA

⑩

2-PGA

⑪

PEP HCO_3^- Pi ㉑ → OAA

⑫ ADP / ATP ㉒ NADH / NAD^+

Pyruvate Malate

CYTOSOL

STARCH

② Pi ⑰

Glu-1-P Glucose

③ ① ATP / ADP

Glu-6-P

④

Fru-6-P

⑤ ATP / ADP

Fru-1,6-P_2

⑥

DHAP ⑦ → G3P

⑧ NAD^++Pi / NADH

1,3-DPGA

⑨ ADP / ATP

3-PGA

⑩

2-PGA

⑪

PEP ⑫ ADP/ATP → Pyruvate

㉑ NADPH / $NADP^+$

Malate

PLASTID

Fatty acid & other biosynthetic pathways

VACUOLE

PEP ⑳ H_2O / Pi → Pi

Pyruvate

MITOCHONDRION

Pyruvate ㉓ Malate CO_2 NADH NAD^+

Respiration ← → *Biosynthesis*

(PFK) while secondary control is at the level of PK. Activation of PFK leads to an increase in its product Fru-1,6-P_2, which is a potent feed-forward allosteric activator of most nonplant PKs (Fig. 1.2 A). In nonplant systems, PK is the only route by which PEP can be transformed into subsequent substrates required for the Krebs cycle and oxidative phosphorylation. Animal PKs and PFKs are generally inhibited by ATP, allowing regulation of glycolysis to be correlated to energy charge and adenylate recycling. In contrast, in plants, quantification of changes in levels of glycolytic intermediates that occur following stimulation of glycolytic flux in green algae, ripening fruit, aged storage root slices, *R. communis* cotyledons, and *Chenopodium rubrum* suspension cell cultures consistently demonstrate that plant glycolysis is controlled from the 'bottom up' with primary and secondary regulation exerted at the levels of PEP and Fru-6-P utilization, respectively (Fig. 1.2 B)[1]. Plant PFK is potently inhibited by PEP and activation of PEP utilizing enzymes such as PK and phospho*enol*pyruvate carboxylase (PEPC) enables relief of this inhibition, allowing glycolysis to proceed [2]. Many plant PKs possess allosteric effectors. For example, several plant PKs are potently inhibited by glutamate [3,4], which could balance the activity of PK with the provision of carbon skeletons required for active biosynthesis such as N-assimilation [5].

These observations are consistent with assigning PEP metabolism as the central control point in plant glycolysis. Furthermore, it has been suggested that the importance of PEP stems from its ability to generate a large amount of energy for metabolism because it possesses the largest amount of potential energy of all phosphorylated intermediates [6]. PEP can be cleaved on either side of its enol oxygen atom and thus can

A. MAMMALIAN LIVER

B. PLANT CYTOSOL
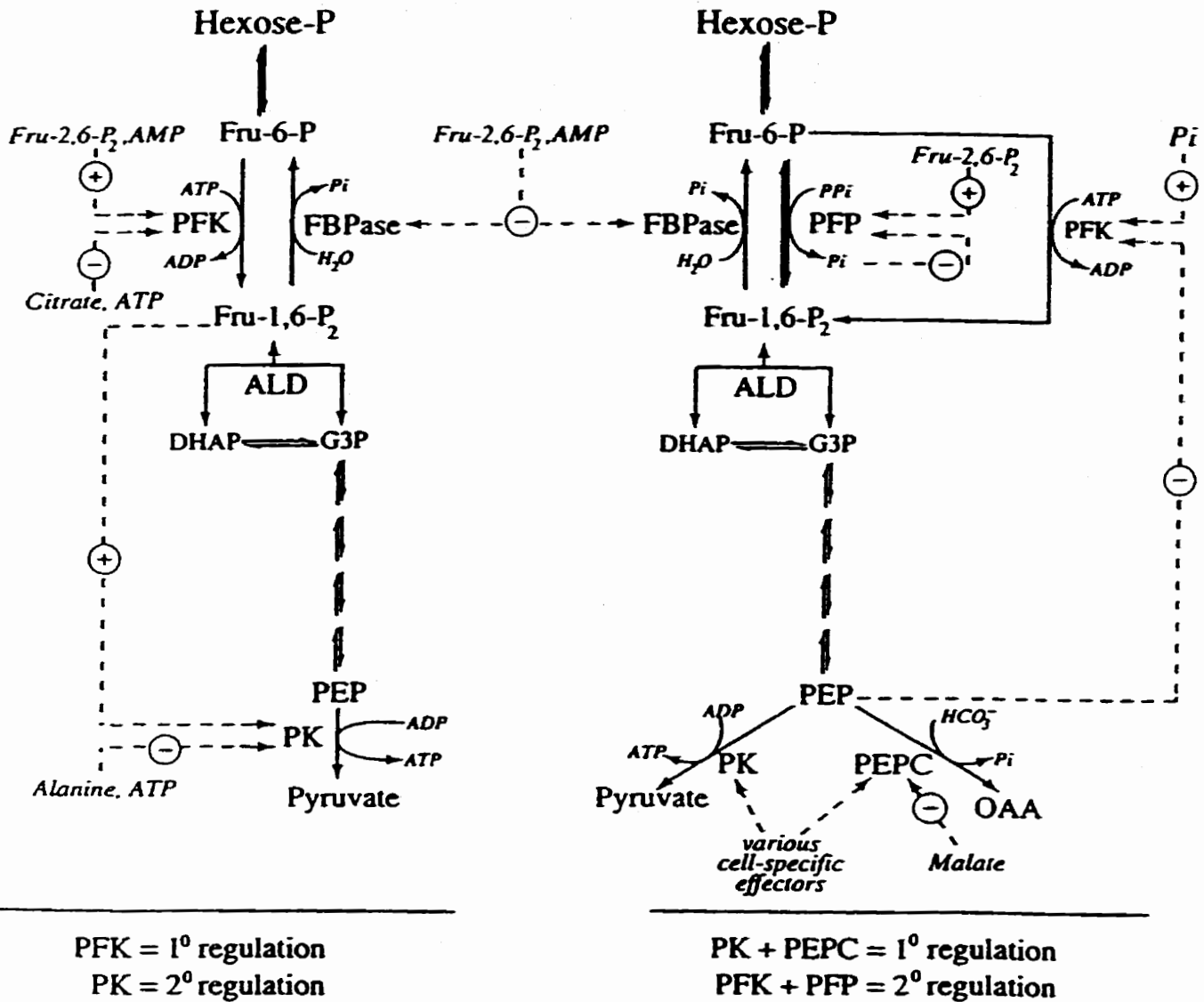
Figure 1.2. A comparison of the metabolite regulation of glycolytic flux from

hexose-monophosphates to pyruvate in mammalian liver (A) vs the plant cytosol (B).

⊕ and ⊖ denote activation and inhibition, respectively. (From Plaxton [1])

participate in a wide variety of enzymatic reactions by virtue of its position as a carbon source for many different catabolic and anabolic pathways of primary and secondary metabolism [7,8].

1.2 The Unique Flexibility of Plant PEP metabolism.

There are a number of different enzymatic reactions that may utilize PEP. The 'classical' glycolytic route of PEP metabolism utilizes PEP and ADP as substrates in the production of pyruvate and ATP via PK. The absence of PK in nonplant species causes detrimental effects. In humans. a genetic defect that results in the lowered PK activity in the red blood cells give rise to the serious condition of hemolytic anemia [9]. However, transgenic tobacco plants (*Nicotiana tacacum* L.) deficient in leaf PK_c grew from seed to seed. demonstrating the remarkable flexibility of plant PEP metabolism [10]. Plant cells can use a variety of alternative metabolic routes to directly or indirectly circumvent the reaction catalyzed by cytosolic PK (PK_c). PEP can be metabolized to create pyruvate by PEP phosphatase or via PEPC, malate dehydrogenase (MDH). and NAD-malic enzyme (ME) by route of malate synthesis (Fig. 1.1). PEP is a substrate not only for the production of organic acids used in the TCA cycle. but also for the shikimic acid pathway by combining with the oxidative pentose phosphate pathway intermediate erythrose-4-P (Fig. 1.3). This pathway synthesizes chorismate as an intermediate in the production of the aromatic amino acids. flavonoids. alkaloids and lignin [11]. In order to compete for this common substrate. it is critical that plant PEP utilizing enzymes be sufficiently regulated in order to provide for optimal partitioning of PEP. PEP utilizing enzymes may be regulated by pH. allosteric effectors and reversible covalent modification.

1.3 Multiple functions of PEP carboxylase in plants.

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a ubiquitous plant cytosolic enzyme that catalyzes the irreversible β-carboxylation of PEP to yield oxaloacetate (OAA) and inorganic phosphate (Pi)[12]. PEPC also occurs in prokaryotes, but not in animals or yeast[13]. Vascular plants appear to express tissue-specific isozymes of PEPC having kinetic and regulatory properties geared to each tissue's function. The photosynthetic form of the enzyme from C_4 and CAM leaves is a homotetramer with subunits of M_r ~100-110 kDa[14]. High PEPC activity in these tissues is associated with its role in primary CO_2 fixation, where it is involved in the CO_2 concentration mechanism as part of the C_4 cycle. The role of the enzyme in these tissues is thus well defined[15]. In contrast, PEPC's function in C_3 leaves and non-photosynthetic tissues[13] has yet to be fully explained but may include: (i) the anaplerotic replenishment of TCA cycle intermediates consumed, for example in the synthesis of amino acids[12,16,17] (~40% of protein bound Asp is synthesized via PEPC[17]), (ii) a source of organic acids required for maintenance of cytosolic charge balance and pH[18], (iii) a metabolic bypass of PK_c and a Pi recycling mechanism during Pi deprivation[19] and, (iv) reassimilation of respired CO_2 in bulky storage organs[20]. Purification and characterization of the enzyme from various tissues may aid in explaining its physiological role. It may be that the kinetic and structural properties of PEPC depend on the functional nature of the parent tissue, which is supported by evidence of 3 to 4 families of nuclear genes that encode different isoforms of PEPC: C_4-specific, C_3 or etiolated, CAM and root forms[21].

PEPC has been purified from a variety of C_4 (maize, sorghum), CAM and C_3 photosynthetic tissues. In nonphotosynthetic tissue the enzyme has been partially purified and characterized from potato tubers [22], from dark-brown germinating peanut cotyledons [23], from germinating *Ricinus communis* [24] and soybean nodule [25]. Law and Plaxton [33;100] have fully purified and characterized PEPC from banana fruit. However, the regulatory properties of PEPC in mediating glycolytic flux during Pi nutritional deprivation have not been addressed.

The isolation of PEPC from many plant tissues is generally problematic because the *N*-terminus of PEPC is subject to *in vitro* truncation by endogenous proteases, which can only be prevented by the addition of protease inhibitors such as PMSF and chymostatin [14]. Employing accelerated isolation protocols such as FPLC and HPLC is also thought to limit the activity of these proteases [26-28]. The cleavage of the *N*-terminus does not effect PEPC's V_{max} but removes the critical serine residue necessary for phosphorylation by PEPC-kinase and has been shown to decrease sensitivity to malate, a potent inhibitor of PEPCs [15].

Purified PEPC has a specific activity of approximately 25 (μmol OAA synthesized/ min)/mg protein [12]. The enzyme has an absolute requirement for divalent cation cofactor (presumably Mg^{2+} *in vivo*), with a $K_m(Mg^{2+})$ around 0.1 to 1 mM. Hyperbolic PEP saturation kinetics are generally observed, but a decrease in assay pH from 8 to 7 for the maize enzyme can promote positive cooperativity with respect to PEP binding [29;30]. The enzyme's $K_m(PEP)$ is up to an order of magnitude lower for the C_3 as compared to the C_4 leaf enzyme [12]. The concentration of HCO_3^- in an aqueous solution at

equilibrium with air is 1 mM at pH 8, about 5 times higher than the $K_m(HCO_3^-)$ of maize PEPC [31], suggesting this co-substrate is near saturating.

1.4 PEPC Regulation.

Early reports suggested that C_3 PEPCs possess few regulatory properties [18] but the elucidation of the modulation of the activity of C_4 leaf PEPC led to investigations with the C_3 form of the enzyme to determine its regulatory features. There are many similarities between the control of activity of the various molecular forms of PEPC. In 1996, Plaxton lists six methods by which enzymatic activity may be modulated via 'fine control' as opposed to 'coarse control' which refers to an alteration in the amount of enzyme [1]. Four of these fine control mechanisms have been shown to be of critical importance in the regulation of PEPC activity, both in C_3 and C_4 leaves, and nonphotosynthetic tissues.

1.4.1 Variation in pH.

The pH dependence of PEPC activity has been well documented. While PEPC has a broad pH optimum of between 7.5 and 9 [12], its activity declines substantially below this range, with activity at pH 7 being approximately half that at pH 8 in many C_3 [27,32,33] and C_4 [30] species. pH is known to affect the affinity of maize leaf PEPC for PEP, which declines outside the range of 7.5 to 9.5 [34]. pH effects are also apparent when comparing the ability of allosteric effectors to inhibit or activate the enzyme. Most PEPC effectors only function at suboptimal pH values (i.e., 7 to 7.5) approaching the physiological pH found in the plant cytosol. For example, malate and Glc-6-P have been shown to be ineffective at pH 8 [13,33,35]. In addition, it has been shown that regulation by

phosphorylation of PEPC by PEPC-kinase is dependent on an alkalization of the cytosol [15].

1.4.2 Subunit association-dissociation.

Reversible association of the subunits of regulatory oligomeric enzymes is an alternative method of modulating their activity. The association-dissociation is usually linked to substrate or effector binding and serves to modify the activity of the enzyme. This regulation has been shown *in vitro* in glycolytic non-plant enzymes (hexokinase in yeast) [36] and in plant pyrophosphate dependent phosphofructokinase (PFP) [7] and cytosolic PFK (PFK$_c$) [37]. In CAM PEPC. dimer-tetramer interconversion has been proposed to be of major importance in regulating not only the activity of PEPC but also its susceptibility to inhibition by malate [38]. X-ray crytallography analysis together with functional site-directed mutagenesis on *E. coli* PEPC [39] suggests that its 'dimer of dimers' tetrameric structure is dependent on salt bridges formed between neighbouring subunits. The disruption of these interactions can lead to the formation of dimers. which relates the regulation of subunit composition to cytosolic pH.

1.4.3 Metabolic effectors.

The kinetic effect of metabolite activators or inhibitors on plant PEPC is to change the enzyme's affinity for PEP. The most well known anaplerotic function attributed to PEPC is to replenish TCA cycle intermediates consumed during nitrogen assimilation and amino acid biosynthesis [5,31]. a function thought to be important during periods of protein synthesis. such as during seed formation and germination. and also fruit maturation. Therefore. TCA cycle intermediates such as malate are important inhibitors

of many C_3-plant PEPCs [24;25;27;33]. Feedback inhibition by the allosteric effector malate is universal among plant PEPCs [12;13]. Plant PEPC is also subject to feed-forward activation by Glc-6-P and other hexose monophosphates. This activation by Glc-6-P is antagonistic to the enzyme's inhibition by malate, aspartate and glutamate. The metabolic regulation of PEPC suggests that the metabolic branch point at PEP utilization is subject to tight feedback regulation by carbon skeletons removed from the TCA cycle for anabolism, and the provision of carbohydrates via glycolysis. The kinetic effects of these metabolites are often only seen at sub-optimal pH comparable to that in the plant cytosol [27;33;40]. In addition to allosteric inhibitors controlled by Glc-6-P there are also inhibitors such as rutin and quercitin (flavonoids) which have been shown to potently inhibit C_4-leaf PEPC at very low concentrations [11].

1.4.4 Covalent modification.

Reversible phosphorylation of proteins plays a well documented role in the regulation of cellular activities including glucose and glycogen metabolism in animal systems [41;42]. Over the past 20 years it has become evident that phosphorylation also plays a critical role in metabolic control in plants as well [42]. A number of key plant enzymes involved in primary carbon metabolism are regulated by reversible phosphorylation, including pyruvate dehydrogenase [43], pyruvate phosphate dikinase [44], sucrose phosphate synthase [45] and PEPC [15]. Though many of the components of the signal transduction described in animal cells are present in plants [46], few targets have been identified and information concerning the regulation of these cascades is scarce. For many of these enzymes, the phosphorylation state is light dependent, and this includes photosynthetic

forms of PEPC. Indeed, PEPC represents one of the best examples of *in vivo* regulation by phosphorylation in plants [15]. Early work had determined that CAM and C_4 photosynthetic PEPCs were regulated via a circadian rhythm that served to alter their activity and sensitivity to malate, without affecting V_{max} or PEPC protein amount [47]. Within the C_4 leaf, phosphorylation of PEPC is completed after 1 hour of illumination and causes a gradual reduction in the enzyme's sensitivity to malate inhibition [48]. In contrast, PEPC extracted from CAM leaves during the dark period is more active than that from the light period, and this rhythm parallels the phosphorylation status of the enzyme [49]. The change in PEPC activity and sensitivity to malate is due to reversible phosphorylation by an endogenous PEPC protein kinase [50-53] and protein phosphatase type 2A [51] at a key serine residue located near the *N*-terminus of the 100-110 kDa PEPC subunit. This has been demonstrated for both C_4 and CAM leaf PEPC. There is convincing evidence that the reversible phosphorylation of the *N*-terminal domain of plant PEPC in widespread, if not ubiqituous. *In vivo* studies with [32]Pi have demonstrated the reversible phosphorylation of nonphotosynthetic PEPC in soybean root nodules [26] and C_3-leaf PEPC in wheat leaves excised from N-deficient seedlings [48,74]. In banana fruit an endogenous Ca^{2+}-independent PEPC kinase forms a tight complex with its target enzyme [100]. Interestingly, unlike plant PEPCs, bacterial, green alga and cyanobacterial PEPCs do not possess this *N*-terminal phosphorylation motif [14,40,54], which illustrates an additional form of regulation of higher plant PEPCs.

1.5 Pi: a vital and limiting nutrient.

Phosphorous is an essential element for normal growth and metabolism. It plays a central role in virtually all metabolic processes. Plants preferentially absorb phosphorous from the soil in its fully oxidized anionic form, Pi ($H_2PO_4^-$; orthophosphate). Pi is vital to plant metabolism owing, in part, to its ability to form chemical bonds which, when hydrolyzed, provide energy for essential metabolic reactions. In the cell Pi can be condensed to form phosphoanhydride bonds in compounds such as ATP and pyrophosphate which can be used to transfer chemical energy from catabolic reactions to energy requiring cellular processes. Pi is also important as a regulator of many enzymes of plant metabolism through allosteric effects [55,56] or the reversible phosphorylation of key regulatory enzymes.

The ubiquitous importance of Pi in plant metabolism is increased because Pi is one of the least available nutrients in aquatic and terrestrial environments. Although it is widely distributed in the earth's crust, most Pi exists in insoluble mineral forms and, as such, is unavailable to plants [57]. In many environments Pi deficiency is the rule rather than the exception. Algal blooms resulting from a large addition of Pi to aquatic ecosystems from pollution or up-welling water demonstrate the constitutive limitation of plant growth by this element. Significant amounts (90%) of mineral Pi used worldwide are accounted for by the prolific use of Pi in fertilizers in agriculture.

1.5.1 Effects of Pi deprivation on cytoplasmic pools of Pi, Nucleotide-P and PPi.

Plant cells selectively distribute Pi between cytoplasmic ('metabolic') and vacuolar ('storage') pools, with cytoplasmic Pi being maintained at the expense of large fluctuations in the vacuolar Pi. However, ^{31}P-NMR studies indicate that during extended

Pi starvation the levels of Pi in the cytoplasm decrease from 20 to 50 fold [19]. As a consequence, levels of ATP, CTP, GTP and UTP are reduced by 70-80% in *C. roseus* suspension cells [58,59] and ADP and ATP are reduced by at least 75% in Pi-deprived *B. nigra* suspension cells [60] limiting the available adenylate pools. The dramatic reductions in metabolic Pi and adenylate pools that accompany long-term Pi deprivation have important implications with respect to respiratory metabolism.

Plants have evolved to acclimatize to Pi stress through a number of mechanisms. The induction of acid phosphatase (APase) activity is a universal symptom of Pi stress in higher plants [61]. APases can function as intra- or extracellular Pi salvaging systems that can scavenge Pi from P-esters as well as from insoluble soil minerals. ^{31}P-NMR studies of Pi-starved sycamore suspension cells illustrated the role of secreted APase, whose activity was increased threefold during Pi starvation, in hydrolyzing extracellular P-choline into Pi and choline which are quickly absorbed by Pi-limited cells [62]. The "Pi starvation response" also includes the induction of alternative glycolytic enzymes such as PFP, PEPC and PEP Phosphatase to scavenge and recycle Pi, and to bypass some of the adenylate dependent steps in glycolysis. Apart from circumventing adenylate limited enzymes such as PFK and PK, each of these enzymes may function during Pi stress as part of a Pi recycling system. Pi is a by-product of the reactions catalyzed by each of these enzymes (Fig. 1.3). Upregulation of PEPC has been observed during Pi limitation in numerous C_3 plant species, including *Brassica nigra* [60], *Lupinus albus* [63], *Lycopersicon*

Figure 1.3. A model suggesting several 'adaptive' metabolic processes (indicated by heavy arrows) that may facilitate the survival of higher plants during extended periods of nutritional Pi deprivation. Alternative pathways of cytosolic glycolysis and mitochondrial electron transport, and tonoplast H^+ pumping may facilitate respiration and vacuolar pH maintenance by Pi deficient plant cells because they negate the dependence upon adenylates and Pi, both of which become depressed during severe Pi starvation. Organic acids produced via PEPCase may also be excreted by roots to increase the availability of mineral bound Pi by solubilizing Ca-, Fe- and Al-phosphates. Enhanced flux from PEP into the aromatic (shikimate) pathway leads to the production of 'protective' compounds such as anthocyanins. A key component of this model is the critical secondary role played by 'metabolic Pi recycling systems' during Pi deprivation; these include the putative glycolytic 'bypass' enzymes PFP, PEP phosphatase and PEPCase, the tonoplast H^+-PPiase, and several aromatic pathway enzymes. The enzymes that catalyze the numbered reactions are as follows: 1, invertase; 2, sucrose synthase; 3, HK; 4, fructokinase; 5, UDP-glucose pyrophosphorylase; 6, nucleoside diphosphate kinase; 7, phosphoglucomutase; 8, phosphoglucose isomerase; 9, PFK; 10 PFP; 11, NAD-dependent G3PDH (phosphorylating); 12. 3-PGA kinase; 13, NADP-dependent G3PDH (nonphosphorylating); 14. PK; 15. PEP phosphatase; 16, PEPC; 17, malate dehydrogenase; 18, malic enzyme; 19, DAHP synthase; 20. 3-dehydroquinate dehydratase; 21, EPSP synthase; 22. chorismate synthase; 23, tonoplast H^+-ATPase; 24, tonoplast H^+-PPiase. Abbreviations are as in the text or as follows: Glu-1-P, glucose-1-P; Glu-6-P, glucose-6-P; Fru-6-P, fructose-6-P; Fru-1,6-P_2, fructose-1,6-P_2; G3P, glyceraldehyde-3-P; 1,3-DPGA, 1,3-P_2-glycerate; OAA, oxaloacetate; E4P, erythrose-4-P; DHQ, dehydroquinate; S3P, shikimate-3-P; CHA, chorismate. (From Plaxton [19])

SUCROSE

① ②

Glucose Fructose UDP UDP-Glucose

③ ATP → ADP

④ ATP → ADP ⑥ PPi ⑤ UTP

Glu-6-P

⑦ Glu-1-P

Anthocyanins Flavanoids & Indole Alkakoids

⑧ Fru-6-P ← ⑧ Glu-6-P

CHA

⑨ ATP → ADP ⑩ PPi

Fru-1,6-P₂ → **Pi**

EPSP

②②

②①

CYTOSOL

G3P NAD⁺+ Pi → NADH ⑪

S3P ADP ← ATP

⑬ NADP⁺ → NADPH

1,3-DPGA

⑫ ADP → ATP

⑲ DAHP ⑳ DHQ

E4P

3-PGA HCO₃⁻ **Pi**

VACUOLE

ATP ②③ → H⁺ H⁺ → ADP + Pi

PEP ← PEP E4P Pi ←

⑮ H₂O → Pi → Pi ⑯ ⑭ ADP → ATP OAA

PPi ②④ → H⁺ H⁺ → 2Pi

NADH → NAD⁺ ⑰

Pyruvate -- → Pyruvate Malate → Excretion to Environment

MITOCHONDRION

Pyruvate ← ⑱ ← Malate

CO₂ NADH NAD⁺

Pi + ADP → ATP Pi + ADP → ATP Pi + ADP → ATP

COMPLEX 1 -X- UBIQUINONE → CYTOCHROME a₃

Rotenone Cyanide

2H⁺ + 1/2 O₂ → H₂O

KREB'S CYCLE → 2e⁻

ALTERNATIVE OXIDASE

2CO₂ Biosynthesis

2H⁺ + 1/2 O₂ → H₂O

esculentum [64], *B. napus* [65], and *Catharanthus roseus* [66]. During Pi deprivation PEPC has been proposed to fulfil the following roles: (i) the enzymatic reaction of β-carboxylation and cleavage of a Pi from PEP, which recycles limited Pi back into the cytosol, (ii) the flux of carbon through PEPC in coordination with MDH and ME provides a "bypass" around the ADP-limited PK_c to supply pyruvate to the TCA cycle, and (iii) PEPC in the roots of Pi deficient plants provides as much as 25% of the carbon needed for citrate production and 34% of the carbon for malate exudation [67]. Citrate and malate exudation acidifies the rhizosphere, which therefore increases the Pi available to the plant by mobilizing the sparingly soluble mineral Pi, and possibly organic Pi sources [68]. The precise mechanism by which PEPC activity is increased during Pi starvation has yet to be determined. The expression of a separate isozyme of PEPC has been implicated by northern blots of -Pi proteoid lupin roots [69]. In addition, inhibition studies of partially-purified PEPC from Pi-deficient proteoid roots of lupin indicate that the enzyme may be phosphorylated by an endogenous protein kinase, resulting in decreased sensitivity of the enzyme to inhibition by malate[69].

1.6 The role of PEPC in C/N metabolism.

Nitrogen is mainly absorbed by plants as nitrate which is reduced in leaf cells to NH_4^- by the consecutive action of two enzymes, nitrate reductase (NR), and nitrite reductase (NiR) [70]. The assimilation of NH_4^- is dependent on the joint action of glutamine synthetase (GS) and glutamine 2-oxoglutarate aminotransferase (GOGAT). The net synthesis of glutamate utilizes 2-OG in the GS/GOGAT reaction. This synthesis of amino acids requires carbon skeletons in the form of keto acids (such as the

intermediates 2-OG and OAA [5]) which are generally taken from the TCA cycle. The anaplerotic fixation of CO_2 by PEPC becomes a prerequisite for the redirection of carbon flow to amino-acid synthesis [71]. In coordination with increased rates of NH_4^+ assimilation, it has been shown that there are elevated rates of dark CO_2 fixation in green alga, cyanobacteria, and higher plants [72], and these elevated rates of *in vivo* CO_2 fixation are directly correlated to an increase in PEPC activity [73]. It has also been shown that the increase in PEPC activity is not affected by the addition of cycloheximide [74], suggesting that increase in PEPC activity is through a 'fine control' mechanism.

The regulatory properties responsible for this increase in PEPC activity are still speculative. It has been suggested that the initial steps in nitrogen assimilation result in metabolite changes which activate glycolysis at the level of PEPC and PK_c [5]. Coordinate regulation of PK_c and PEPC plays a critical role in the integration of carbon partitioning with the generation of 2-OG needed for N-assimilation by GS/GOGAT. PEPC has an additional metabolic function during N-assimilation to produce OAA for aspartate production by aspartate aminotransferase (AAT). The activation of PK_c and PEPC which occurs following N-resupply to N-limited plant tissues [5] not only provides the necessary carbon skeletons for GS/GOGAT and AAT, but also serves to reduce PEP levels thereby relieving PEP inhibition of PFK and stimulating overall glycolytic flux.

Despite the tremendous importance of PEPC and PK_c in the interaction between carbon and nitrogen metabolism, few workers have simultaneously characterized both enzymes from the same tissue or cell type [24]. This is important since tissue and/or

developmental specific isozymes of PK_c and PEPC have been discovered which display different physical and/or regulatory properties [1].

1.7 Model system: *B. napus* suspension cells.

The conventional method of plant breeding has brought about major improvements in the oil yield and quality of many oilseed crops, including the *Brassicas* [75]. *B. napus* (canola) has recently surpassed wheat as Canada's leading cash crop. Isolated microspore (pollen)-derived embyros and suspension cell cultures of *B. napus* are an effective model system to investigate metabolism in these oilseeds [76,77]. The suspension cell cultures are an attractive system for enzymology because they can be easily scaled up to provide sufficient quantities of material to purify enzymes and to test different nutritional effects on these cells [78,79].

1.8 Rationale and Objective of Research.

The aim of this thesis was to purify and characterize the physical, immunological and kinetic properties of PEPC from *B . napus* suspension cell cultures. The regulatory mechanisms that are responsible for the increased PEPC activity during Pi starvation were addressed by depriving cultured cells of nutritional Pi and testing for induction of PEPC activity. Immunoblots provided a screen to determine if the amount of PEPC was altered during Pi stress. A complete purification and characterization of PEPC from Pi-sufficient and Pi-starved *B. napus* cells was also undertaken. In addition, studies were conducted to test the hypothesis that PEPC is phosphorylated into its less malate sensitive state during Pi-starvation (as has been suggested for PEPC in Pi deprived lupin roots [69]). Further, coupled with the simultaneous purification and characterization of the *B. napus*

PK_c (performed by Chris Smith [80]), the combined results provide insights into the critical role played by the amino acids aspartate and glutamate in the allosteric control of higher plant PK_cs and PEPCs.

Chapter 2: Materials and Methods

2.1 Chemicals and Plant material.

Bis-Tris-propane, PEP, Coomassie Blue R-250, Mes, and DTT were from

Research Organics, Inc. (Cleveland, OH, U.S.A). Tris base and SDS were from

Schwartz/Mann Biotech (Cambridge, MA, U.S.A.). Ribi adjuvant (product code R-730)

was obtained from Ribi Immunochemical Research (Hamilton, MT, U.S.A.). Poly-

(vinylidene difluoride) membranes (Immobilon, 0.45 μm pore size) were obtained from

Millipore and all solutions were prepared using Milli-Q-processed water (Millipore;

Mississauga, ON, Canada). Other biochemicals, coupling enzymes, SDS M_r standards,

cell culture reagents and alkaline phosphatase-tagged goat anti-(rabbit IgG) IgG were

obtained from Sigma Chemical Co. (Oakville, ON, Canada). All other reagents were of

analytical grade obtained from BDH Chemicals (Toronto, ON, Canada). Purified bovine

heart PP2A was a gift of Dr. Greg Moorhead, Univ. of Calgary (1 unit of PP2A

dephosphorylates 1 μmol of bovine glycogen phosphorylase/min at 30°C).

An embryogenic microspore-derived heterotrophic cell suspension of winter

oilseed rape (canola) (*B. napus* L. cv. Jet Neuf) [31] was provided by Dr. Randall Weselake,

Univ. of Lethbridge. Cells were maintained on a rotational shaker (125 rpm) at 22°C in

NLN medium (non-hardy) [82] (pH 7.5) containing 6.5% (w/v) sucrose, 3.3 mM NO_3^-, 5.5

mM Gln, 0.5 mg/L α-naphthalene acetic acid, 0.5 mg/L 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 [79] (see Appendix I for details). Subculturing was performed by

transferring 10 mL of a 7-d-old cell suspension into 40 mL (125 mL flask) of fresh NLN

media containing 2.5 mM K$_2$HPO$_4$. Cells used in time-course studies and PEPC

purification were obtained by scaling up the culture volume. Briefly, two 7-d-old 50 mL

cultures were combined, concentrated to about 60 mL by removing excess media and

used to innoculate 440 mL of fresh NLN media containing either 2.5 mM K$_2$HPO$_4$ (+Pi

cells) or 0 mM K$_2$HPO$_4$ (-Pi cells) in 2 L flasks. +Pi and -Pi 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.

2.2 Enzyme and Protein Assays

The PEPC and acid (PEP) phosphatase (APase) reactions were routinely 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 Bis-Tris-propane/HCl (pH 8.0),

10% (v/v) glycerol, 2 mM PEP, 2.5 mM KHCO$_3$, 12 mM MgCl$_2$, 0.15 mM NADH and 5

units of porcine heart malate. Assay conditions for APase were: 25 mM Bis-Tris-

propane/25 mM Mes (pH 5.7), 2 mM PEP, 0.15 mM NADH and 5 units of rabbit muscle

lactate dehydrogenase. All assays were: (i) initiated by addition of enzyme preparation;

(ii) corrected for NADH oxidase activity; and (iii) linear with respect to time and

concentration of enzyme assayed. One unit of enzyme activity is defined as the amount

of enzyme resulting in the production of 1 μmol of product/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

-20-

Bollag and Edelstein [83]. Protein concentration of the purified PEPCs was also determined using the bicinchoninic acid method of Hill and Straka [84]. Bovine γ-globulin was used as the protein standard.

2.3 Kinetic Studies

Kinetic studies were conducted using a Dynatech MR-5000 Microplate reader and final volume of 0.2 mL for the PEPC reaction mixture. Apparent K_m values were calculated from Michaelis-Menten equation fitted to a non-linear least-squares regression computer kinetics program [85]. K_a and I_{50} values (concentration of activator and inhibitor producing 50% activation and inhibition of PEPC activity, respectively) were determined using the aforementioned computer kinetics program. All kinetic parameters are the means of at least three separate determinations and are reproducible to within $\pm 10\%$ s.e.m.

2.4 Preparation of Clarified Homogenates Used in Time-Course Studies

+Pi or -Pi *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 polyvinylpolypyrrolidone, 1 mM DTT, 1 mM phenylmethyl sulfonyl fluoride, 5 μg/mL 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.

2.5 Buffers used in *B. napus* PEPC purification

All buffers were degassed and adjusted to their respective pH values at 23°C.

Buffer A: Aforementioned extraction buffer containing 0.1% (v/v) Triton X-100 and 4% (w/v) PEG 8,000. Buffer B: 50 mM Imidazole/HCl (pH 7.1) containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 20 mM NaF and 25% (saturation) $(NH_4)_2SO_4$. Buffer C: 50 mM Imidazole/HCl (pH 7.1) containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 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 DTT. 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 DTT.

2.6 Purification of *B. napus* PEPC

All procedures were carried out at 4°C unless otherwise noted. Identical protocols were employed for the purification of PEPC from 8-d-old +Pi and -Pi *B. napus* suspension cells, henceforth referred to as the '+Pi PEPC' and '-Pi PEPC' preparations, respectively.

Clarified Extract and PEG Fractionation

Quick-frozen *B. napus* suspension cells (220 g of +Pi cells or 147 g of -Pi 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 PEG 8,000 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. PEG pellets were stored overnight at -20°C.

Butyl Sepharose Hydrophobic Interaction FPLC

PEG pellets were resuspended in buffer B lacking $(NH_4)_2SO_4$, but containing 5 μg/mL chymostatin, to yield a protein concentration of about 5 mg/mL. Following centrifugation for 20 min at 35,000 g, the extract was adjusted to 20% (saturation) $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$. The solution was stirred for 20 min, centrifuged as above, and adsorbed at 4 mL/min onto a column (3 X 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 eluted with 60% buffer C (40% buffer B) (flow rate = 4 mL/min; fraction size = 5 mL). Pooled peak PEPC activity fractions were diluted with an equal volume of 50% (w/v) PEG 8000, stirred for 30 min, and centrifuged as above. The resultant pellets were stored at -20 °C overnight.

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

The PEG pellets were solubilized in buffer D to which 5 μg/mL chymostatin was added to yield a protein concentration of about 10 mg/mL, centrifuged as above, and loaded at 1.5 mL/min onto a column (1.1 x 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 PEPC eluted with 80 mL of a linear 0 to 300 mM KCl gradient in buffer D (fraction size = 5 mL). Pooled peak activity fractions were adjusted to contain 5 μg/mL chymostatin and concentrated to about 1 mL using an Amicom XM-50 ultrafilter.

Superdex 200 Gel Filtration FPLC

The anion-exchange fractions that were concentrated to 1 mL were passed through a 0.45 μm syringe filter and applied via a 1 mL sample loop at 0.3 mL/min onto a column (1.6 X 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 mL/min onto a Mono-Q HR 5/5 column (Pharmacia) preequilibrated with buffer D. PEPC was eluted using 25 mL of a linear 0 to 300 mM KCl gradient in buffer D (fraction size = 1 mL). Peak activity fractions were pooled, adjusted to 5 μg/mL 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.

2.7 Determination of Native Molecular Mass via Superdex 200 Gel Filtration

Native M_r estimation for the +Pi and -Pi PEPCs was performed during their respective purifications on the Superdex 200 Prep Grade column as described above. Native M_rs were estimated from plots of K_{av} (partition coefficient) versus log M_r 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.

2.8 Antibody Production

Purified +Pi PEPC (500 μg) was dialyzed overnight against Pi-buffered saline (20 mM NaPi, pH 7.4, 150 mM NaCl), filtered through a 0.2 μm membrane, 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) of the same protein emulsified in Ribi adjuvant was

administered subcutaneously after 6 weeks. Ten d 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 1,500 g for 10 min. The crude antiserum was frozen in

liquid N_2 and stored -80 °C in 0.04% (w/v) NaN_3. For immunoblotting, the anti-(*B. napus*

+Pi PEPC) IgG was affinity-purified against 25 μg of purified *B. napus* +Pi PEPC as

previously described [86].

2.9 Immunotitration of PEPC Activity

Immunoremoval of enzyme activity was tested by mixing 0.05 units of

homogeneous +Pi PEPC with 25 mM Hepes/NaOH (pH 7.5), containing 0.1 mg/mL

BSA, 10% glycerol, 1 mM DTT, and various amounts of rabbit preimmune or anti-(*B.

napus* PEPC) immune serum diluted into Pi-buffered saline (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 a 17,000 g. Residual PEPC activity in the supernatant was

determined as described above.

2.10 Electrophoresis and Immunoblotting

SDS/PAGE was performed according to Laemmli [87] using the 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 DTT. Gels were run at a constant voltage of 200 V for 45 min. To determine subunit M_rs by SDS/PAGE, a plot of relative mobility versus the log M_r was constructed using the following protein standards: α_2 macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumerase (48.5 kDa), lactic dehydrogenase (36.5 kDa) and triose phosphate isomerase (26.6 kDa).

Non-denaturing PAGE was conducted at 4°C using the highly porous SDS/PAGE system of Doucet et al. [88] 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 stained for protein with Coomassie Blue R-250, or incubated in a PEPC activity stain, or immunoblotted using affinity-purified anti-($B.$ napus +Pi PEPC) IgG. To detect 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 $KHCO_3$ and 0.15 mM NADH. 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 as previously described [86]. Antigenic polypeptides were visualized using an alkaline phosphatase-tagged secondary antibody (appendix II)[86]. Relative amount of PEPC in clarified extracts from 8-d-old +Pi versus -Pi $B.$ napus cells was

determined by quantification of the antigenic 104 kDa PEPC subunit on immunoblots (in terms of A_{633}) using an LKB Ultroscan XL laser densitometer and Gel Scan software (version 2.1) (Pharmacia LKB Biotech, Montreal, QC, Canada). 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 pre-immune serum was substituted for the affinity-purified anti-(B. napus +Pi PEPC) IgG.

2.11 Peptide Mapping by CNBr Cleavage

Polypeptides corresponding to the 104 kDa subunit of purified +Pi and -Pi PEPCs were excised from SDS/PAGE mini-gels and cleaved *in situ* with CNBr. The degradation products were analyzed on an SDS/14%-PAGE mini-gel according to Plaxton and Moorhead [89]. Following SDS/PAGE, the gel was stained with silver [90].

2.12 Phosphatase Treatment of *B. napus* PEPC

Clarified homogenates of -Pi and +Pi 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 [91] which had been preequilibrated in 50 mM Bis-Tris-propane/HCl (pH 7.3) containing 10% (v/v) glycerol, 5 mM $MgCl_2$, and 1 mM DTT. The desalted extracts as well as the purified +Pi and -Pi PEPCs (dialyzed free of NaF) were incubated for 1 h at 23°C in the presence and absence of 0.5 unit/mL of bovine heart PP2A or 2 units/mL 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.

Chapter 3: Results

3.1 Influence of Pi Starvation on Growth, and PEPC and APase Activities of *B. napus* Suspension Cells
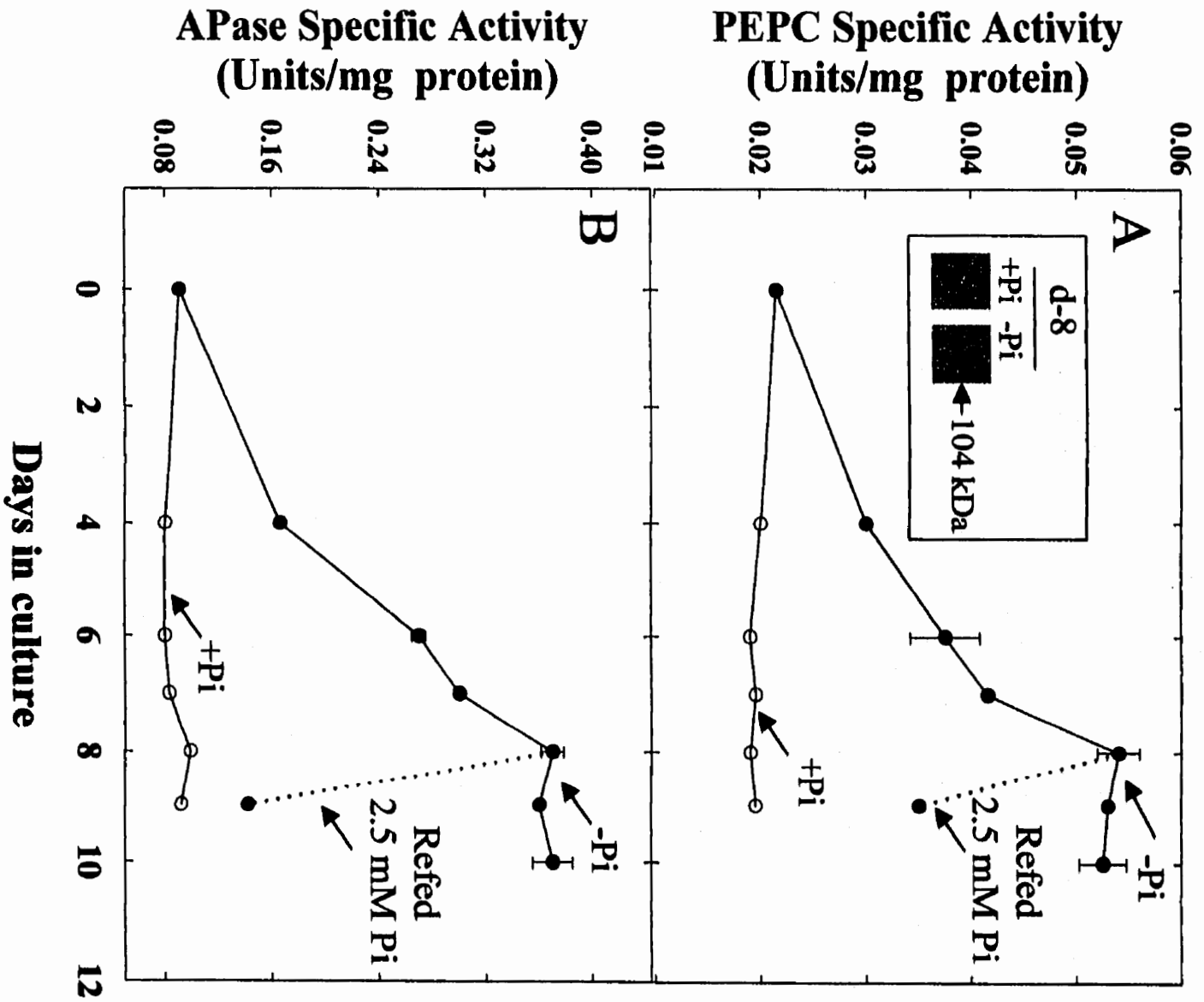
B. napus suspension cells cultured for 8 d in the absence of Pi had only about 50% of the fresh weight of the 8-d-old +Pi cells (approx. 20 and 10 g of cells were obtained per 500 mL culture of 8-d-old +Pi and -Pi cells, respectively). The time-courses for PEPC and APase activities of +Pi and -Pi *B. napus* cells are shown in Fig. 3.1. The specific activities of PEPC and APase were increased by about 2.5-fold and 4-fold, respectively, in the -Pi cells, whereas the activities of the two enzymes remained relatively low and constant in the +Pi cells. Within 24 h of resupply of 2.5 mM Pi to the 8-d-old -Pi cells the extractable PEPC and APase activities were reduced by at least 50% (Fig. 3.1). Immunoblotting with rabbit anti-(*B. napus* +Pi PEPC) IgG was used to examine the subunit M_r and relative amount of PEPC in clarified extracts from the 8-d-old +Pi versus -Pi *B. napus* cells. In each instance, a single immunoreactive 104 kDa polypeptide was observed (Fig. 3.1A, inset), identical to that obtained with the respective purified PEPCs (see below). Laser densitometric quantification of the immunoblots revealed that the -Pi *B. napus* extracts contained approximately 2-fold more of the immunoreactive 104 kDa PEPC subunit, relative to extracts from the +Pi cells.

3.2 PEPC Purification

As shown in Table 3.1, PEPC was purified about 710-fold from 220 g of 8-d-old +Pi *B. napus* cells to a final specific activity of 20 units/mg and an overall recovery of 14%. Using an identical protocol, the PEPC from 8-d-old -Pi cells was purified 410-fold

Figure 3.1. Time-course for extractable activities of PEPC (A) and APase (B) in *B. napus* suspension cells cultured in 500 mL of media containing 0 or 2.5 mM Pi. Values for the 6-, 8- and 10-d-old -Pi cells represent means ± s.e.m. for replicate assays of separate clarified extracts from $n = 3$ different cultures. All other values represent the mean activities of replicate determinations of a single extract. An 8-d-old -Pi culture was resupplied with 2.5 mM Pi and cultured for an additional 1 d as indicated. *Inset to panel A*: Immunological detection of PEPC from 8-d-old +Pi or -Pi *B. napus* suspension cells. Clarified extracts (each containing 50 µg 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* +Pi PEPC) IgG and immunoreactive polypeptides were detected using an alkaline-phosphatase-linked secondary antibody and chromogenic staining as in [36].

APase Specific Activity (Units/mg protein)

PEPC Specific Activity (Units/mg protein)

Days in culture

A

B

+Pi

−Pi

Refed 2.5 mM Pi

d-8
+Pi −Pi

←104 kDa

Table 3.1. Purification of PEPC from 220 g of 8-d-old Pi-sufficient (+Pi) *B. napus* suspension cells

Step	Volume (mL)	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Clarified extract	645	4000*	113	0.028	--	100
PEG fractionation	258	1340*	75	0.055	2.0	66
Butyl Sepharose	34	240*	65	0.27	9.6	58
DEAE Fractogel***	1.0	16*	45	3	107	40
Superdex 200	9.4	3.7*	25	6.9	236	22
Mono-Q***	0.65	0.8*	16	20	714	14
		0.43**		37.2		

* Protein determined with Coomassie Blue R-250 dye-binding assay according to the method of Bollag and Edelstein [83].

** Protein determined with the bicinchoninic acid reagent according to the method of Hill and Straka [84].

*** Concentrated pooled fractions

to a final specific activity of 20.9 units/mg and overall recovery of 12% (Table 3.2). As previously documented for banana fruit PEPC [33], the protein concentration of the purified *B. napus* +Pi and -Pi 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 (Tables 3.1 and 3.2). Thus, with the bicinchoninic acid protein assay the specific activities of the final +Pi and -Pi PEPC preparations were respectively increased to 37.2 and 39.7 units/mg (Tables 3.1 and 3.2). The anomolous color yield of certain proteins with Coomassie-Blue dye binding assay has been well established [92,93].

3.3 Physical and Immunological properties

Gel Electrophoresis

Denaturation, followed by SDS/PAGE of the final +Pi PEPC preparation resolved a single protein staining band of approximately 104 kDa (Fig. 3.2A, lanes 2 and 3) that strongly cross-reacted with affinity-purified anti-(*B. napus* +Pi PEPC) IgG (Fig. 3.2B, lane 1 and 2) or anti-(banana fruit PEPC) IgG (Fig. 3.2C, lane 1). Analogous results were obtained with the final preparation of -Pi PEPC (Fig. 3.3A and B). SDS/PAGE and immunoblotting revealed that an approximately 5 kDa polypeptide was cleaved from PEPC's 104 kDa subunit during the enzyme's purification from the +Pi cells in the absence of added chymostatin (Fig. 3.2A, lane 4; Fig. 3.2C, lane 2)(final specific activity of proteolyzed +Pi PEPC = 11.4 units/mg). The repeated inclusion of 5 µg/mL chymostatin at various stages of the purification prevented partial degradation of the enzyme during its purification from +Pi and -Pi cells (Figs. 3.2 and 3.3).

Table 3.2. Purification of PEPC from 147 g of 8-d-old Pi-starved *B. napus* suspension cells

Step	Volume (mL)	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Clarified extract	390	3720*	191	0.051	--	100
PEG fractionation	230	1300*	175	0.13	2.6	92
Butyl Sepharose	30	470*	165	0.35	6.9	86
DEAE Fractogel***	1.9	16*	51	3.2	63	27
Superdex 200	8.4	8.1*	39	4.8	94	20
Mono-Q***	0.45	1.1*	23	20.9	410	12
		0.58**		39.7		

* Protein determined with Coomassie Blue R-250 dye-binding assay according to the method of Bollag and Edelstein [83].

** Protein determined with the bicinchoninic acid reagent according to the method of Hill and Straka [84].

*** Concentrated pooled fractions

Figure 3.2. SDS/PAGE and immunoblot analysis of PEPC from Pi-sufficient *B.*

***napus* suspension cells and developing seeds. (A)** SDS/PAGE (9% separating gel) of

PEPC purified from 9-d-old +Pi *B. napus* cells. Lane 1 contains 4 µg of various M_r

standards. Lanes 2 and 3 contain 2.5 and 1 µg, respectively, of the pooled peak fractions

from the final purification step (Mono-Q FPLC). Lane 4 contains 5 µg of the +Pi 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* +Pi PEPC) IgG. Lane 1 contains 15 ng of

the final preparation of +Pi PEPC. Lanes 2 contains 25 µg of protein from a clarified

extract prepared from +Pi *B. napus* suspension cells. Lane 3 contains 15 µg 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 [33]. Lane 1 contains 15 ng of the final preparation of +Pi PEPC. Lane 2

contains 50 ng of +Pi PEPC that was isolated in the absence of chymostatin.

Abbreviation: O, origin; TD, tracking dye front.
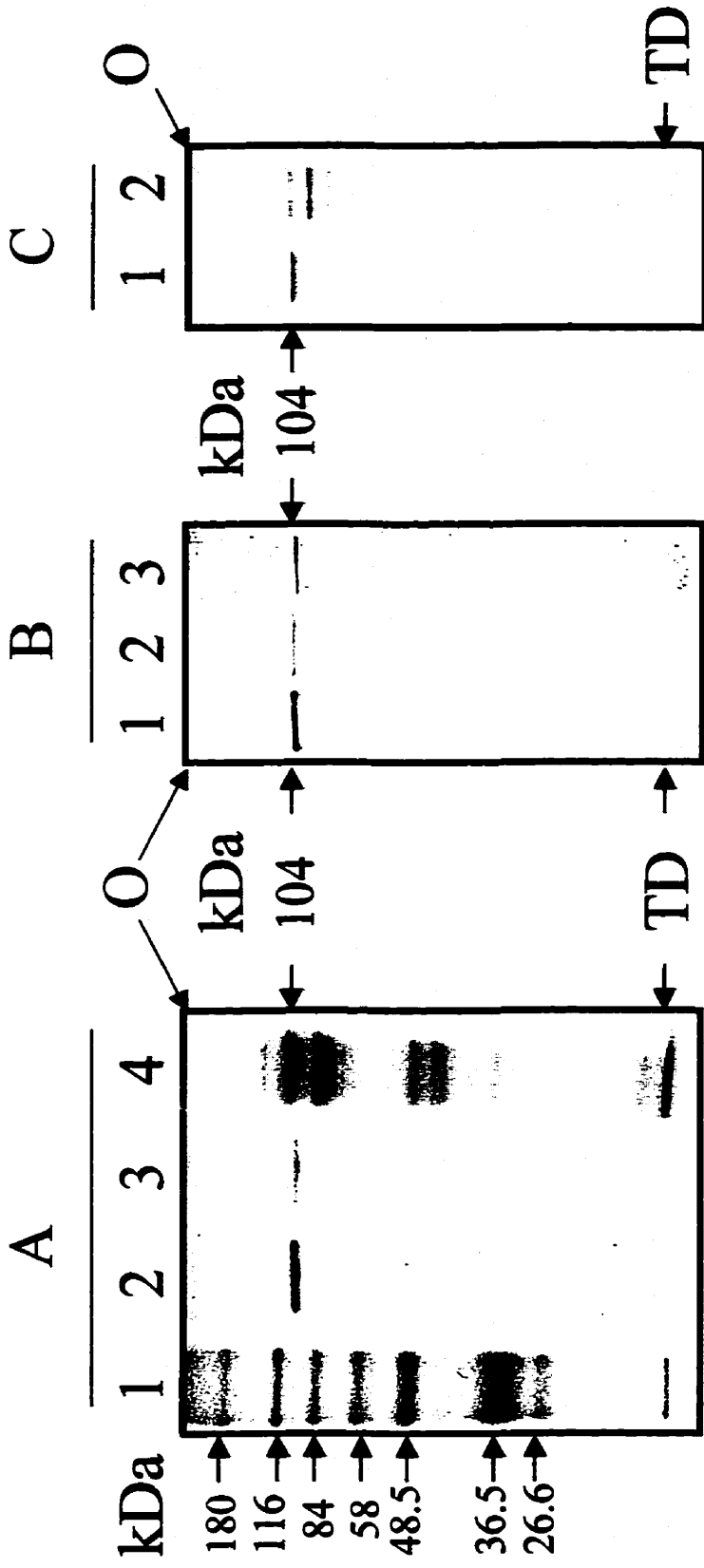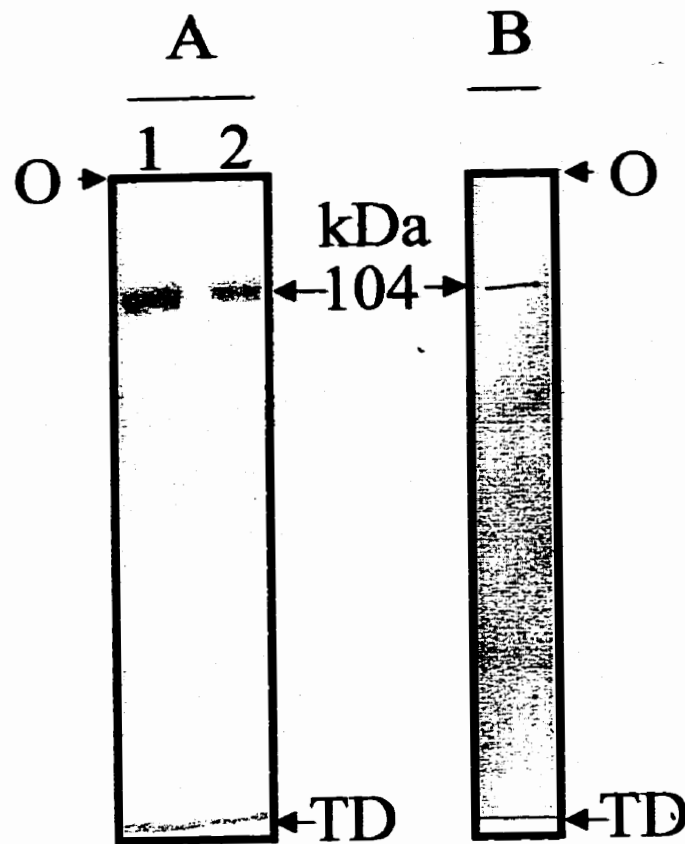
Figure 3.3. SDS/PAGE and immunoblot analysis of PEPC purified from 8-d-old Pi-starved *B. napus* suspension cells. (A) SDS/PAGE (9% separating gel) of purified *B. napus* -Pi PEPC. Lanes 1 and 2 contain 2.5 and 1.25 μg, respectively, of the pooled peak fractions from the final purification step (Mono-Q FPLC). Protein staining was performed with Coomassie Blue R-250. **(B)** Immunoblot analysis was performed using 20-fold diluted affinity-purified rabbit anti-(*B. napus* +Pi PEPC) IgG. The single lane contains 20 ng of the final preparation of -Pi PEPC. Abbreviations: O, origin; TD, tracking dye front.

Determination of Native M_r via Gel Filtration FPLC

The native M_r of the purified +Pi and -Pi PEPCs was determined to be 440 ± 20 kDa (mean $\pm$ s.e.m; $n = 3$) 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* +Pi PEPC was determined to be 4.32 $\times 10^5$ $M^{-1}cm^{-1}$ at 280 nm ($A_{280}^{0.1\%} = 0.918$). This value was based on the bicinchoninic acid determination of protein concentration (Table 3.1) and calculated by assuming a native M_r of 440 kDa.

Immunological Characterization

Increasing amounts of rabbit anti-(*B. napus* +Pi PEPC) immune serum immunoprecipitated up to 100% of the activity of the purified *B. napus* +Pi PEPC; complete immunoremoval of activity occurred at about 900 μL of immune serum per unit of PEPC activity (Fig. 3.4). By contrast, preimmune serum had no effect on the PEPC activity. Rabbit anti-(*B. napus* +Pi PEPC) IgG was affinity purified using 25 μg of homogeneous +Pi PEPC as previously described [86]. The affinity-purified anti-(*B. napus* +Pi PEPC) IgG could detect as little as 5 ng of denatured homogeneous +Pi or -Pi PEPC. Immunoblotting of clarified extracts prepared from *B. napus* suspension cells or developing seed embryos (at mid-cotyledonary stage of development) demonstrated monospecificity of the anti-(*B. napus* +Pi PEPC) IgG for the 104 kDa PEPC subunit (Fig. 3.2B, lanes 3 and 4). Likewise, non-denaturing PAGE of clarified extracts from
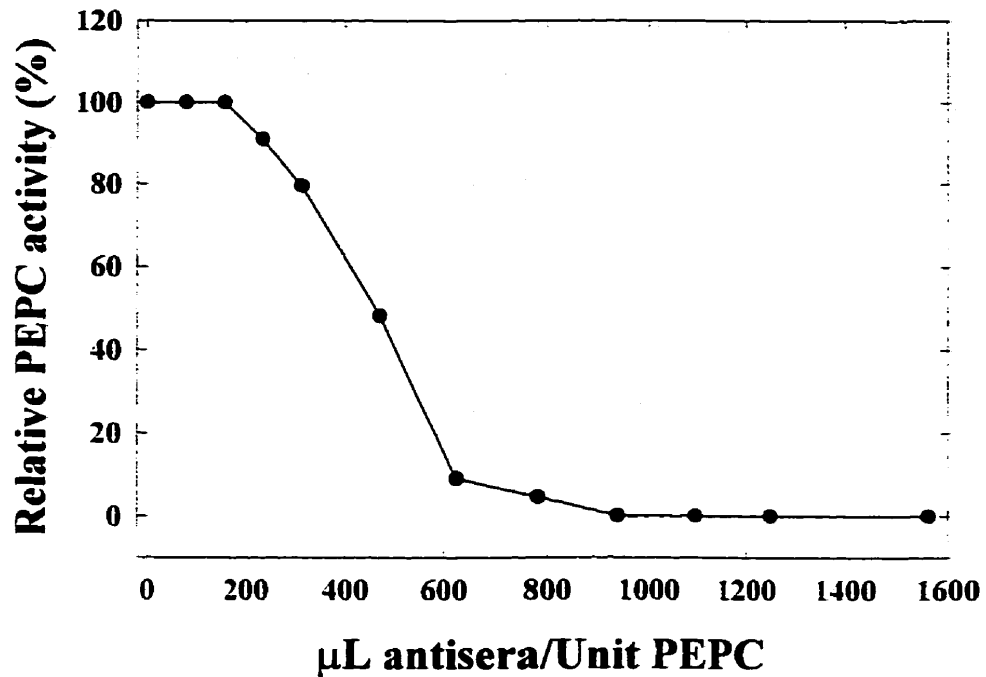
**Figure 3.4. Effect of rabbit anti-(*B.napus* +Pi PEPC) immune serum on the activity

of *B. napus* +Pi PEPC.** Immunoremoval was performed using 0.05 units of +Pi PEPC as

described under Materials and Methods. Figure 2.7. Activity of +Pi and -Pi *B. napus*

PEPCs as a function of pH. PEPC activity was determined as described under the

Materials and Methods except that the assay pH was varied and assays were buffered with

100 mM Bis-tris-propane/HCl.

+Pi *B. napus* cells generated a band of PEPC activity that co-migrated with a single anti-(*B. napus* +Pi PEPC) IgG immunoreactive band (Fig. 3.5).

Peptide Mapping

The structural relationship between the 104 kDa subunits of the +Pi and -Pi PEPCs was examined by peptide mapping of their respective CNBr cleavage fragments (Fig. 3.6). Indistinguishable peptide maps were obtained suggesting that they are identical polypeptides.

3.4 Kinetic Properties

Effect of pH

Similar to other plant PEPCs [24,25,33], the +Pi and -Pi *B. napus* PEPCs exhibited broad pH/activity profiles with a maximum between 8.0 and 9.5 (Fig. 3.7). In each instance, PEPC activity at pH 7.3 was about 65% of that occurring at pH 8.4 (Table 3.3), but became almost undetectable below pH 7.0 (Fig. 3.7).

Cation Requirements

B. napus +Pi PEPC showed an absolute dependence for divalent cation. In the absence of $MgCl_2$ and the presence of 5 mM EDTA PEPC activity was undetectable. At pH 8.4 the enzyme's $K_m(Mg^{2+})$ was determined to be 0.084 mM. Mn^{2+} (12 mM, added as $MnCl_2$) yielded the same V_{max} value achieved with saturating Mg^{2+}. These results are analogous to those obtained with other plant PEPCs [13,33].

PEP Saturation Kinetics

Table 3.3 summarizes PEPCs' V_{max} and apparent $K_m(PEP)$ at pH 7.3 and 8.0 in the presence and absence of 10% (v/v) glycerol. In all instances.
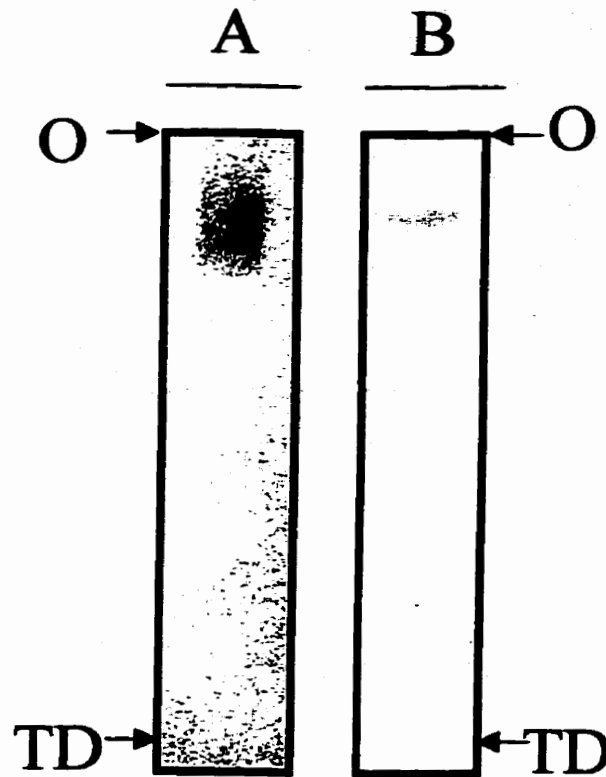
Figure 3.5. Non-denaturing PAGE of clarified extracts from 8-d-old +Pi *B. napus* suspension cells. A clarified extract (containing 3.5 µg of protein or about 0.1 mU of PEPC activity) was subjected to non-denaturing PAGE (6% separating gel) as described under the Materials and Methods. The gel was either stained for PEPC activity (**A**) or blot-transferred to a poly(vinylidene) difluoride membrane (**B**) and probed with 20-fold diluted affinity-purified anti-(*B. napus* +Pi PEPC) IgG. Immunoreactive polypeptides were detected using an alkaline-phosphatase-linked secondary antibody and chromogenic staining as previously described [86]. Abbreviations: O, origin; TD, tracking dye front.

Figure 3.6. Electrophoretic patterns of CNBr-cleavage fragments of the 104 kDa subunit of *B. napus* +Pi and -Pi PEPCs. CNBr-cleavage fragments were prepared from gel slices containing 8 μg of the respective 104 kDa polypeptide of +Pi (lane 2) and -Pi (lane 3) PEPCs and analyzed on an SDS/14% PAGE mini-gel as previously described [89]. Lanes 1 contains 4 μg of the 104 kDa +Pi PEPC polypeptide incubated in the absence of CNBr. Lane 4 contains 4 μg of various M_r standards. The gel was stained with silver according to the method of Wray et al. [90]. Abbreviations: O, origin; TD, tracking dye front.
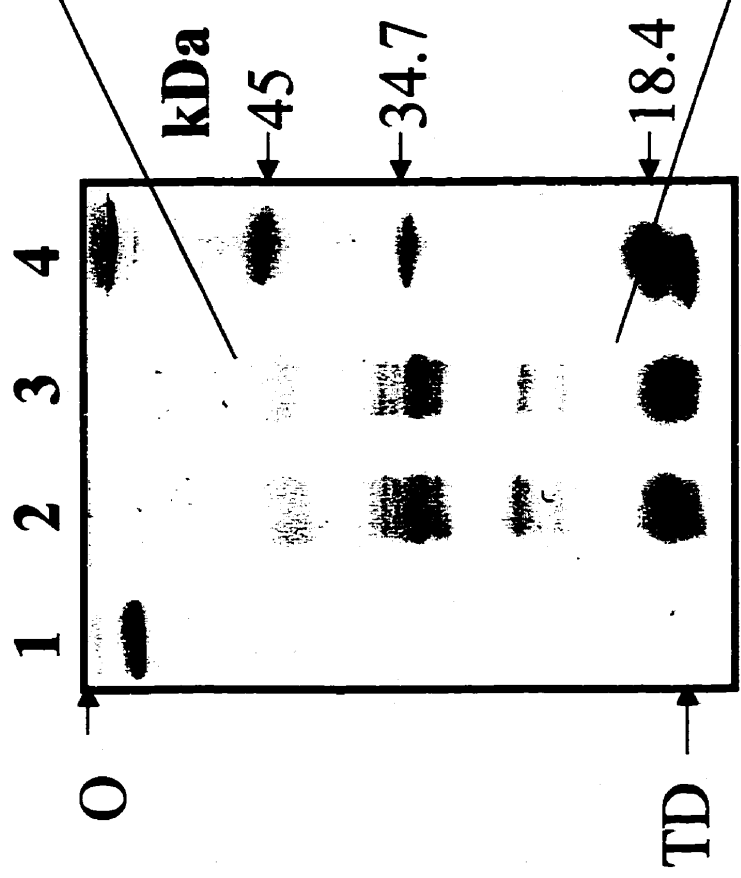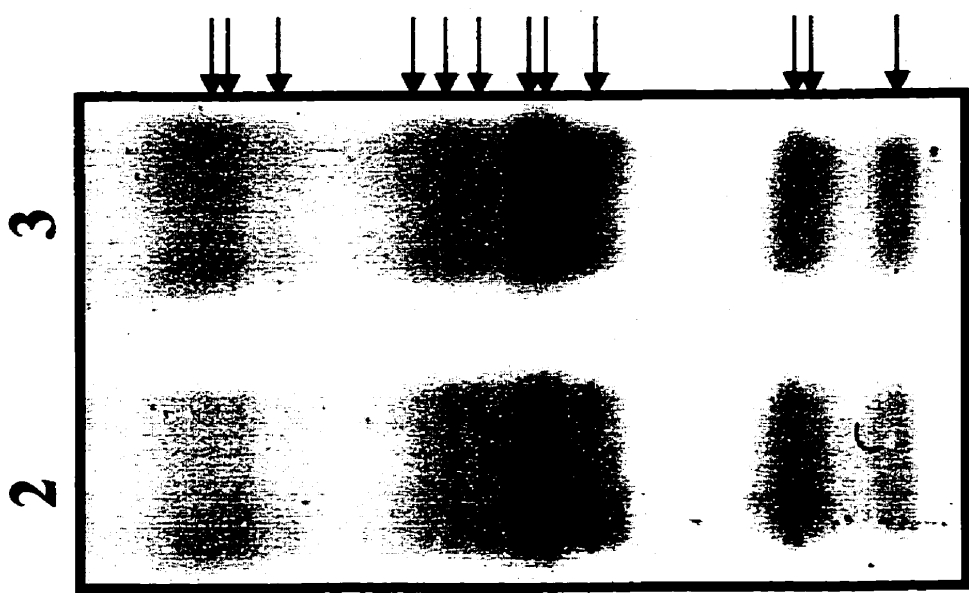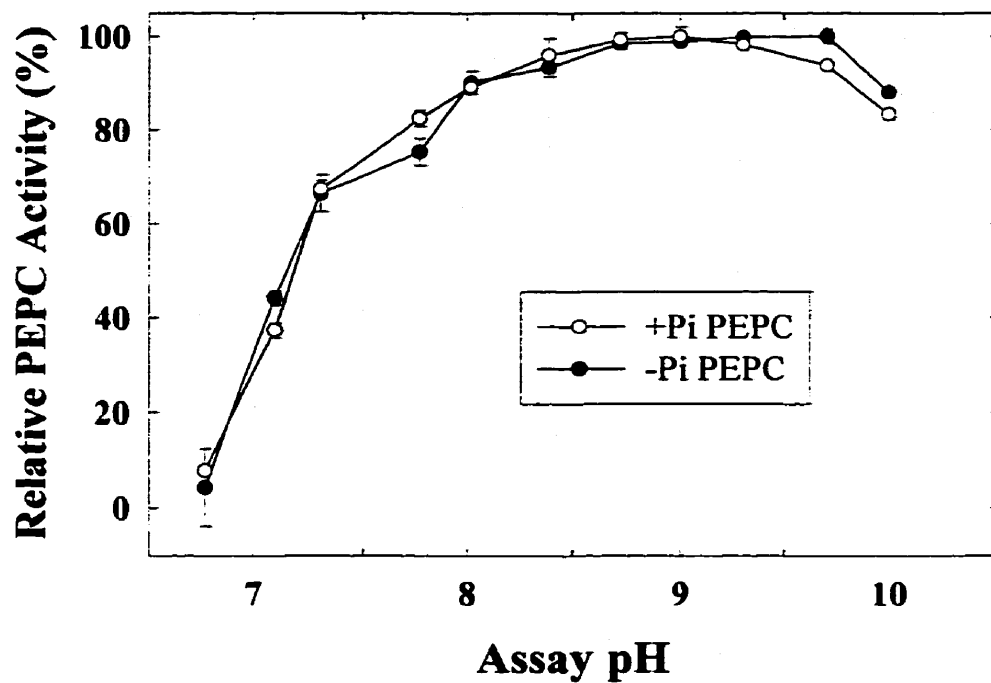
Figure 3.7. Activity of +Pi and -Pi *B. napus* PEPCs as a function of pH. PEPC activity

was determined as described under the Materials and Methods except that the assay pH

was varied and assays were buffered with 100 mM Bis-Tris-propane/HCl.

identical PEP saturation kinetics were obtained with the purified +Pi and -Pi *B. napus*

PEPCs (Table 3.3). In common with most other plant PEPCs [24,33], the *B. napus* PEPCs

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 2-fold, whereas the addition of 10% (v/v)

glycerol to the assay medium decreased the enzyme's K_m(PEP) by 30% and 70% at pH 7.3

and pH 8.4, respectively (Table 3.3). Previous workers have cited stablization of the

quaternary structure of PEPC, due to exclusion of solvent molecules, as the rationale for

the favourable influence of glycerol on the affinity of plant PEPC for PEP [38,94]. Glycerol

(10% v/v) was routinely added to all subsequent PEPC assays.

Metabolite Effects

A wide variety of compounds were tested as possible effectors of +Pi PEPC at pH

7.3 and 8.4 with subsaturating concentrations of PEP (0.15 mM). The following

compounds had no influence on PEPC activity (± 20% of the control rate) at either pH 7.3

or 8.4: NH_4Cl, KPi (10 mM each); 2-oxoglutarate, citrate, His, Arg, Gly, Ala, Lys, Gln,

Asn and sucrose (all 5 mM); 2-P-glycerate and Phe (2 mM each); MgADP, MgATP and

shikimic acid (all 1 mM); acetyl-CoA, MgPPi, fructose and NAD^+ (0.5 mM); Trp (0.25

mM); fructose-2-6-P_2 (20 µM); Triton X-100 and NP-40 (2% (v/v) each). Table 3.4 lists

those compounds which 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 pH 7.3 than pH 8.4 (Table 3.4). Similar

observations have been noted for other PEPCs from various plant sources including

Table 3.3. Influence of glycerol, pH and various metabolites on V_{max} and K_m(PEP) of PEPC purified from 8-d-old Pi-sufficient (+Pi) and Pi-deprived (-Pi) _B. napus_ suspension cell cultures. The standard spectrophotometric assay (±10% (v/v) glycerol) was used except that the PEP concentration was varied. Effectors were added individually to +Pi PEPC assays (pH 7.3, 10% (v/v) glycerol) as follows: 0.1 mM Glc-6-P, 0.1 mM malate, 1.25 mM isocitrate, 1.5 mM Asp, or 5 mM Glu.

	pH 7.3				pH 8.3			
	V_{max} (units/mg)		K_m (PEP) (mM)		V_{max} (units/mg)		K_m (PEP) (mM)	
	-Glycerol	+Glycerol	-Glycerol	+Glycerol	-Glycerol	+Glycerol	-Glycerol	+Glycerol
+Pi PEPC	15.7	15.7	0.41	0.17	20	20	0.15	0.08
+ Glc-6-P	n.d.	17	n.d.	0.08	n.d.	n.d.	n.d.	n.d.
+ Malate	n.d.	14.5	n.d.	0.43	n.d.	n.d.	n.d.	n.d.
+ Isocitrate	n.d.	13.7	n.d.	0.30	n.d.	n.d.	n.d.	n.d.
+ Asp	n.d.	14.7	n.d.	1.0	n.d.	n.d.	n.d.	n.d.
+ Glu	n.d.	14.7	n.d.	0.67	n.d.	n.d.	n.d.	n.d.
-Pi PEPC	15.3	15.4	0.42	0.15	20.9	21	0.16	0.08

*n.d., not determined.

Table 3.4. Effects of various metabolites on the activity of purified *B. napus* +Pi PEPC. 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%.

Addition	Concentration Tested	Relative Activity	
		pH 7.3	pH 8.4
Glucose-6-P	2 mM	230	122
Glucose-1-P	2 mM	143	108
Fructose-6-P	2 mM	174	113
Fructose-1-P	2 mM	130	102
Fructose-1,6-P_2	5 mM	129	92
Glycerol-3-P	5 mM	171	110
3-P-Glycerate	2 mM	140	100
Dihydroxyacetone-P	0.5 mM	127	99
Glutamate	5 mM	52	101
Aspartate	5 mM	30	91
Malate	5 mM	5	97
DL-Isocitrate	5 mM	35	26
Succinate	5 mM	67	66
Rutin	0.1 mM	30	n.d.[*]
Quercitin	0.1 mM	5	n.d.

[*]n.d., not determined.

banana fruit [33], maize leaves [30], soybean nodules [25], cotyledons of germinated castor seeds [24].

Activators

The significant activators of *B. napus* +Pi PEPC at pH 7.3 were the hexose-monophosphates, particularly Glc-6-P, and glycerol-3-P (Table 3.4). 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 60%) and slightly increased its V_{max} (Table 3.3; Fig. 3.8). Glc-6-P also functions as a PEPC activator by effectively relieving the enzyme's inhibition by malate, Asp, and Glu (Table 3.5; Fig. 3.9). The addition of 0.1 mM Glc-6-P increased PEPC's I_{50} values for these inhibitors by 3- to 4-fold (Table 3.5). In addition, the enzyme's overall fold-activation by saturating Glc-6-P was increased from about 2- to over 6-fold in the presence of 0.1 mM malate, 5 mM Glu, or 1.5 mM Asp (Fig. 3.9).

Inhibitors

At pH 7.3, the *B. napus* +Pi PEPC was potently inhibited by malate, isocitrate, Asp, and Glu (Tables 3.4 and 3.5). Increasing the assay pH to 8.4 from 7.3 nullified the enzyme's inhibition by 5 mM malate, Asp, or Glu (Table 3.4). These metabolites function as inhibitors at pH 7.3 by markedly reducing PEPC's affinity for its substrate, PEP, and its activator, Glc 6-P. This is reflected by the addition of approximate I_{50} concentrations of isocitrate and/or malate, Asp, or Glu causing an increase in PEPC's: (i) $K_m(PEP)$ by up to 6-fold (Table 3.3), and (ii) $K_a(Glc-6-P)$ by at least 2-fold (Table 3.5). Of interest is the unique inhibition of *B. napus* PEPC by isocitrate. In contrast to malate,

-44-
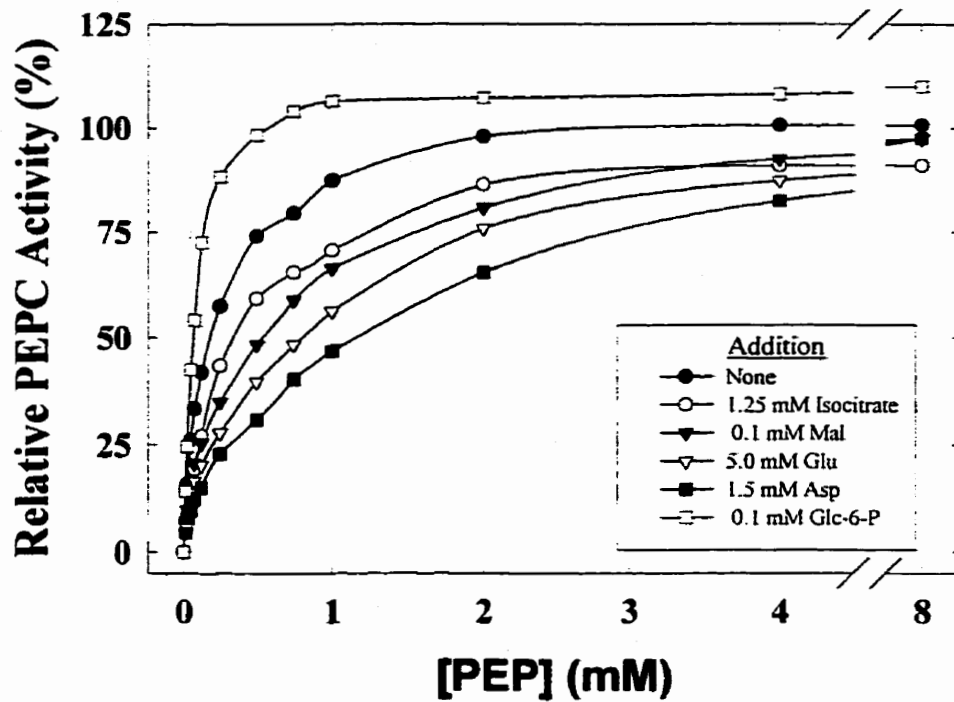
Figure 3.8. Influence of several metabolite effectors on the PEP saturation kinetics of +Pi *B. napus* PEPC. Assays were conducted at pH 7.3 in the absence (•) and presence of 0.1 mM malate (▾), 1.25 mM DL-isocitrate (○), 1.5 mM Asp (■), 5 mM Glu (▽) or 0.1 mM Glc-6-P (□).
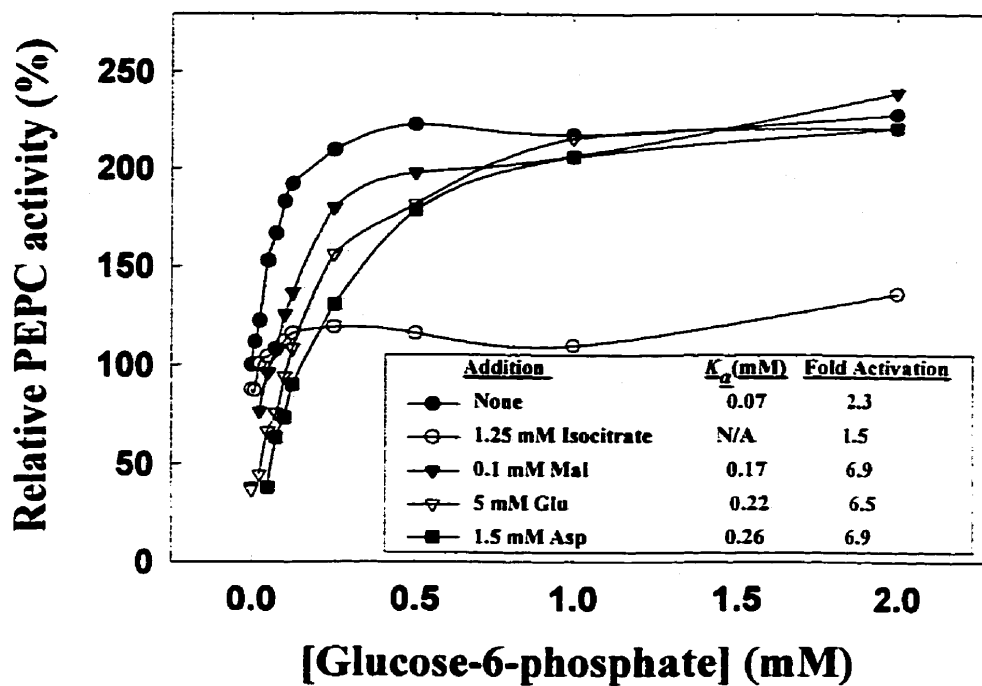
Figure 3.9. Relationship between +Pi PEPC activity and the concentration of Glucose-6-P in the presence and absence of aspartate, glutamate, malate, and isocitrate. Assays were conducted at pH 7.3 with subsaturating (0.34 mM) PEP in the absence (•) and presence of 0.1 mM malate (▾), 1.25 mM DL-isocitrate (○), 1.5 mM Asp (■), or 5 mM Glu (▽).

Table 3.5. Kinetic constants for several effectors of *B. napus* +Pi PEPC. The standard spectrophotometric PEPC assay was used except that the assay pH and PEP concentration were sub-optimal (pH 7.3; 0.34 mM PEP). The I_{50}s were determined in the presence and absence of 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 the various inhibitors. Proteolyzed '+Pi PEPC' refers to the partially degraded enzyme isolated in the absence of chymostatin.

Effector	I_{50} (mM)	K_a (mM)
+Pi PEPC		
Glucose-6-P	-	0.066
+ 0.1 mM Malate	-	0.17
+ 1.5 mM Aspartate	-	0.26
+ 5 mM Glutamate	-	0.22
Malate	0.085	-
+ 0.1 mM Glucose-6-P	0.37	-
Aspartate	1.5	-
+ 0.1 mM Glucose-6-P	4.7	-
Glutamate	5.0	-
+ 0.1 mM Glucose-6-P	13.0	-
Isocitrate	2.5	-
+ 0.1 mM Glucose-6-P	2.5	-
Succinate	13	-
Quercitin	0.026	-
+ 0.1 mM Glucose-6-P	0.028	-
Rutin	0.046	-
+ 0.1 mM Glucose-6-P	0.050	-
Proteolyzed +Pi PEPC		
Malate	0.86	-
-Pi PEPC		
Malate	0.081	-

Asp, and Glu: (i) inhibition by 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 3.4 and 3.5), and (ii) the presence of 1.25 mM isocitrate almost completely negated PEPC activation by Glc-6-P at pH 7.3 (Fig. 3.9). As reported for PEPC from leaves of *Amaranthus viridus* [95,96], the *B. napus* PEPC also demonstrated potent inhibition by the flavonoids quercitin and rutin (Table 3.3). I_{50} values for the two 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 3.5).

Phosphorylation Status of PEPC from Pi-sufficient *versus* Pi-starved *B. napus* Suspension Cell Cultures

Clarified extracts of 8-d-old -Pi and +Pi *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 unit/mL of bovine heart PP2A or 2 units/mL 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 malate uniformly resulted in an approximate 50% inhibition of PEPC activity in the +Pi and -Pi cell extracts, irrespective of the treatment. Likewise, an identical incubation of the purified +Pi and -Pi PEPCs with PP2A or alkaline phosphatase had no influence on their sensitivity to malate inhibition when assayed at pH 7.3 with 0.34 mM PEP. Together with the fact that the purified -Pi and +Pi PEPCs exhibited identical pH activity profile, PEP saturation kinetics, and I_{50}(malate) values (Tables 3.3 and 3.5), these results suggest that PEPC exists mainly in its dephosphorylated (malate sensitive) form in both +Pi and -Pi *B. napus* cells.

Chapter 4: Discussion

The initial goal of this thesis was to investigate the influence of Pi starvation on the PEPC of *B. napus* suspension cell cultures. Suspension cell cultures represent an ideal model system for studies concerning the influence of Pi nutrition on plant metabolism, since they contain a homogeneous population of cells, with each cell in direct contact with the culture media. Moreover, relatively large quantities of cells at a precise nutritional and developmental state can be amassed for use in enzyme purification. A second goal of this thesis was to compare the kinetic and regulatory properties of the purified +Pi PEPC with those being simultaneously characterized by Mr. Chris Smith [80] for homogeneous cytosolic pyruvate kinase (PK_c) from the same cells. Coordinate regulation of these two enzymes plays a critical role in the regulation of plant cytosolic glycolytic flux, particularly as pertains to the interactions between C- and N-metabolism [1,5]. However, the simultaneous complete purification and thorough comparative study of both PEP utilizing enzymes from the same plant tissue or cell type has not been described. This is important since tissue and/or developmental specific isozymes of PK_c and PEPC have been discovered which may display very different physical and/or kinetic/regulatory properties [1,26]. Moreover, a number studies describing the regulation of plant PEPC by allosteric effectors and/or reversible phosphorylation fail to consider the need for coordinate control of PK_c during N-assimilation.

4.1 Influence of Pi starvation on PEPC of *B. napus* Suspension Cell Cultures

Like lupin, *B. napus* is a 'non-mycotrophic' plant whose roots do not form symbiotic associations with mycorrhizal fungi to facilitate Pi uptake from the soil [78].

Recent evidence suggests that, relative to mycorrhizal associating or 'mycotrophic' plants (which account for about 90% of terrestrial plant species), the endogenous metabolism of non-mycotrophic plants is geared to allow a more efficient acclimation to Pi deficiency [78]. Unlike many mycotrophs, Pi-deprived lupin and *B. napus* plants are efficient users of rock Pi [63;65;69]. This has been ascribed to the PEPC-mediated synthesis and consequent excretion of malic and citric acids from the roots during Pi deficiency, resulting in a decrease of rhizosphere pH and the solubilization of the mineral-bound Pi [65;69]. Increased PEPC activity following Pi deprivation has been documented for several plants, including *B. napus* seedlings [69], proteoid lupin roots [68], and *B. nigra* [60] and *Catharanthus roseus* cell cultures [66]. This increase has been correlated with the Pi starvation-dependent elevation of *in vivo* dark $^{14}CO_2$ fixation and/or levels of excreted PEPC-derived organic acids [59;65;66;69]. However, until now there has been no detailed comparison of the physical, immunological and kinetic/regulatory properties of PEPC purified from the same +Pi and -Pi plant source.

Fresh weight of the -Pi 8-d-old *B. napus* suspension cells was about 50% that of the +Pi cells, indicating that the 8-d-old +Pi and -Pi cells were in fact Pi starved and Pi sufficient at the time of harvest. This is corroborated by: (i) the marked reduction in intracellular Pi concentration of the -Pi, but not +Pi, *B. napus* cells (the Pi concentration of 8-d-old +Pi and -Pi *B. napus* cells was previously determined to be about 0.7 and 3.8 μmol/gFW, respectively) [97], and (ii) the significant induction of APase activity, a well established biochemical indicator of plant Pi stress [61], in the -Pi *B. napus* cells relative to the +Pi controls (Fig. 3.1B). As anticipated, the extractable PEPC specific activity of the *B. napus* cells significantly increased (by about 2.5-fold), relative to that of the +Pi cells

which remained low and constant throughout the 10-d time course (Fig. 3.1A). *B. napus* PEPC and APase were induced in parallel in response to Pi stress, and this was reversed when Pi was resupplied to Pi-deficient cells (Fig. 3.1A and B). As previously documented for *B. napus* APase [97], laser densitometric quantification of PEPC immunoblots revealed a close correlation between extractable PEPC activity and relative amount of the immunoreactive 104 kDa PEPC subunit in clarified extracts of 8-d-old +Pi and -Pi cells (Fig. 3.1A). Analogous results have been described for the PEPC activity, concentration and mRNA levels in -Pi proteoid lupin roots relative to +Pi controls [68]. Therefore, in both *B. napus* cell cultures and proteoid lupin roots the increased PEPC activity that accompanies Pi stress at least partially arises from an increased expression of PEPC protein.

In particular, we wished to determine whether Pi deprivation of *B. napus* also induced any alteration in PEPC phosphorylation status, and/or the synthesis of a different PEPC isozyme (having the same subunit size). These goals were provoked by: (i) inhibition studies of partially-purified PEPC from Pi-deficient proteoid lupin roots indicating that PEPC may be phosphorylated by an endogenous protein kinase during Pi starvation (as reflected by a decreased sensitivity of the enzyme to malate inhibition) [69], and (ii) the isolation of a lupin root Pi-starvation inducible PEPC cDNA [69]; whether this cDNA encodes a separate PEPC isozyme (relative to that expressed in +Pi lupin) was not clearly addressed.

PEPC Purification. As a first step to obtaining a definitive answer for both of the aforementioned questions, PEPC from 8-d-old +Pi and -Pi *B. napus* cells was purified to

homogeneity (Tables 3.1 and 3.2; Figs. 3.2 and 3.3). The specific 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 PEPC's phosphorylation status during its extraction and isolation from the +Pi and -Pi 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 including the enzyme from sorghum and maize leaves [26,98]. Indeed, isolation of the B. napus +Pi PEPC in the absence of chymostatin resulted in a proteolytically clipped enzyme (Fig. 3.2A and C) which was an order of magnitude less sensitive to malate inhibition relative to the non-degraded +Pi PEPC isolated in the presence of chymostatin (Table 3.6). Loss of an approximate 4 kDa N-terminal phosphorylation domain of maize or sorghum PEPC during their purification in the absence of chymostatin kinetically mimics the effect of phosphorylation of the non-proteolyzed enzymes (i.e., N-terminal proteolytic clipping or phosphoryation of the intact PEPC both elicit a similar increase in the enzyme's I_{50}(malate) without affecting V_{max}) [26,99].

The final specific activities of the +Pi and -Pi B. napus PEPCs were about 20-21 units/mg (Tables 3.1 and 3.2.) and compare favourably to the values reported for homogeneous PEPCs from various C_3- and C_4-metabolizing plant sources [12,33,99]. Analysis by SDS/PAGE confirmed that both PEPCs had been purified to apparent homogeneity (Figs. 3.2A and 3.3A). Similar to most other plant PEPCs, the native +Pi and -Pi B. napus PEPCs exist as 440 kDa homotetramers.

Immunological Properties. Rabbit anti-(*B. napus* +Pi PEPC) immune serum immunoprecipitated up to 100% of the activity of the purified +Pi PEPC (Fig. 3.4). Monospecificity of the antibody preparation for PEPC is indicated by the observation that only the 104 kDa PEPC subunit shows any significant cross-reaction when denaturing and non-denaturing immunoblots of clarified extracts from *B. napus* developing seed (zygotic) cotyledons and/or suspension cells were probed with the affinity-purified anti-(*B. napus* +Pi PEPC) IgG (Fig. 3.2B; Fig. 3.5B). An immunoblot of the purified -Pi *B. napus* PEPC cross-reacted with the anti-(*B. napus* +Pi PEPC) IgG to a similar extent as the purified +Pi PEPC (Fig. 3.3B). Similarly, antibodies to banana fruit PEPC cross-reacted strongly with the +Pi *B. napus* PEPC (Fig. 3.2C). These results are consistent with those of previous studies [27,33,40] and indicate a high degree of structural similarity between PEPCs of higher plants. By contrast, the anti-(banana or *B. napus* PEPC) IgGs fail to recognize purified PEPC from green algae or cyanobacteria [40](J. Rivoal, W. Plaxton. and D. Turpin, unpublished data).

Peptide Mapping. Peptide mapping is a powerful technique for evaluating the structural relationship between polypeptides [89]. We therefore analyzed the fragments generated by CNBr cleavage of the subunits of the purified +Pi and -Pi *B. napus* PEPCs. The cleavage patterns were identical (Fig. 3.6), which demonstrates that the 104 kDa subunit of the +Pi and -Pi PEPCs is the same polypeptide. Thus, the increased PEPC specific activity and protein that accompanies Pi deprivation of *B. napus* (Fig. 1A), appears to arise from increased synthesis of the same PEPC isoform as exists in the +Pi cells.

Kinetic Studies. It has been amply demonstrated that phosphorylation of plant and algal PEPCs significantly decreases the enzyme's sensitivity to malate inhibition when assayed at subsaturating PEP and suboptimal, but physiological, pH values ranging from about pH 7 to 7.4 [15,33,100]. In addition, phosphorylation of plant PEPC may result in an increased V_{max} at suboptimal pH (*i.e.*, pH 7.3) and/or a reduced K_m(PEP) [100,101]. However, absolutely no kinetic differences between the purified non-proteolyzed +Pi and -Pi *B. napus* PEPCs were noted. Their respective pH activity profiles (Fig. 3.6), PEP saturation kinetics (Table 3.3), and sensitivity to malate inhibition at pH 7.3 with subsaturating PEP (Table 3.5) were identical. The relatively low I_{50}(malate) value of about 0.1 mM obtained for the purified PEPCs (Table 3.6) suggests that PEPC mainly exists in its dephosphorylated, malate sensitive form, in the +Pi and -Pi *B. napus* cells. This conclusion is corroborated by the failure of PP2A or alkaline phosphatase treatment of the respective clarified extracts or purified PEPCs to alter PEPC's sensitivity to malate inhibition when assayed at pH 7.3 with 0.34 mM PEP. Thus, phosphorylation does not appear to play a role in regulating *B. napus* PEPC during Pi deprivation. Although phosphorylation of plant PEPC invariably overcomes the inhibitory action of malate, this could be unnecessary during Pi stress when cellular biosynthetic processes are minimal and the bulk of organic acids produced via PEPC (*i.e.*, malate and citrate) do not accumulate within the cytosol, but may either be respired by the mitochondria or excreted from the cell to solubilize otherwise inaccessible sources of mineral Pi [65]. The results indicate that the upregulation of PEPC activity during Pi deprivation of *B. napus*

suspension cells solely arises via an increase in the expression of the same PEPC isoform as exists in +Pi cells.

4.2. Metabolite Effectors of *B. napus* PEPC.

The activity of PEPC from +Pi *B. napus* suspension cells is highly responsive to a number of metabolites involved in C- and N-metabolism (Tables 3.4 and 3.5). PEPC was activated by Glc-6-P and potently inhibited by malate, isocitrate, Asp and Glu at pH 7.3, whereas sensitivity to these compounds (with the exception of isocitrate) was considerably diminished at pH 8.4. Concentrations of Glc-6-P, malate, Asp and Glu in the plant cytosol have been reported to be up to 1, 2.5, 23 and 21 mM, respectively [102;103;104]. 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 physiological concentration range, suggesting that these metabolites are important regulators of PEPC *in vivo* [104]. Potent inhibition by malate, Asp, isocitrate and/or Glu has been reported for PEPCs from unicellular green algae [27;40;70], as well as several non-photosynthetic plant tissues including banana fruit [33], soybean root nodules [25], and cotyledons of 5-d germinated castor seeds [24].

A PEPC and PEPC kinase from a C_4 leaf (*A. viridis*) was recently shown to be potently inhibited by several shikimic pathway intermediates, particularly the flavonoids quercitin and rutin [11;95;96]. This was suggested to potentially play a role in modulating the partitioning of PEP between primary (*i.e.*, PEPC and PK) and secondary (*i.e.*, shikimic pathway) metabolism. *B. napus* PEPC also displayed potent inhibition by rutin and quercitin (Tables 3.4 and 3.5), suggesting that this may be a universal response of plant PEPCs. Both flavonoids have been implicated in a number of plant functions, including

plant-microbe signalling and defense. However, the precise physiological relevance of

rutin and quercitin inhibition of *B. napus* PEPC (and PK_c [80]) will remain obscure until

information is obtained as to their respective concentrations in the plant cytosol.

4.3 Coordinate Regulation of *B. napus* PEPC and PK_c by Allosteric Effectors During N-Assimilation

The stimulation of respiration that occurs in vascular plants and green algae during

periods of active N-uptake, has been ascribed to an initial activation of PEPC and PK_c

leading to increased production of ATP and Krebs' cycle C-skeletons needed for NH_4^--

assimilation and transamination reactions [5;24;105;106]. 2-OG is required as an acceptor for

NH_4^- in the plastid-localized GS/GOGAT pathway, OAA is required for Asp production

via AAT. and malate and citrate are required as counterions to replace nitrate and prevent

alkalinization [107]. Malate and OAA are synthesized from PEP via PEPC, whereas citrate

and 2-OG are synthesized from PEP via the concerted action of PEPC and PK_c (Fig. 4.1).

The conversion of isocitrate to 2-OG occurs in the cytosol. catalyzed by NADP-ICDH.

Following incorporation of NH_4^- into Glu and Asp, amino acids such as Ala. Gly and Ser

are generated via transaminations with Glu or Asp, and Asn is formed from Asp and Gln

via Asn synthetase. The minor amino acids are synthesized in longer biosynthetic

pathways in which Glu (and occasionally Gln or Asp) act as the NH_4^--donor [108].

Fig. 4.1 presents a model summarizing the allosteric mechanisms of PEPC and

PK_c that may be most important in coordinating C- and N-metabolism in *B. napus*

suspension cells. The regulatory features of both enzymes appear to be well suited to their

Figure 4.1. A model for the regulation of PEPC and PK$_c$ in *B. napus* suspension cells. The coordinate regulation of PEPC and PK$_c$ by the amino acids Glu and Asp provide a mechanism for the control of cytosolic glycolytic carbon flux during N-assimilation as discussed in the text. *B. napus* PEPC is inhibited by malate. isocitrate. Glu and Asp and PK$_c$ is inhibited by Glu as indicated by ····▶ in the figure. Glc-6-P activates PEPC and Asp activates PK$_c$ as indicated by ····▶ in the figure. Abbreviations: MDH, malate dehydrogenase; PDC. pyruvate dehydrogenase complex; CS. citrate synthase; NADP-ICDH. NADP-dependent isocitrate dehydrogenase; NR. nitrate reductase; GS. glutamine synthase; GOGAT, glutamate 2-oxoglutarate aminotransferase; AAT. aspartate aminotransferase; Pyr. pyruvate; Cit; citrate; 2-OG. 2-oxoglutarate; IC. isocitrate.

Sucrose

Glc-6-P

ATP ADP

HCO_3^-

PEP

Pyr

P_i

OAA

MDH

Mal

Asp

Glu

AAT

2-OG

NADP-ICDH

CO_2

IC

other amino acids

Mal

NO_3^- *NR* NH_4^+
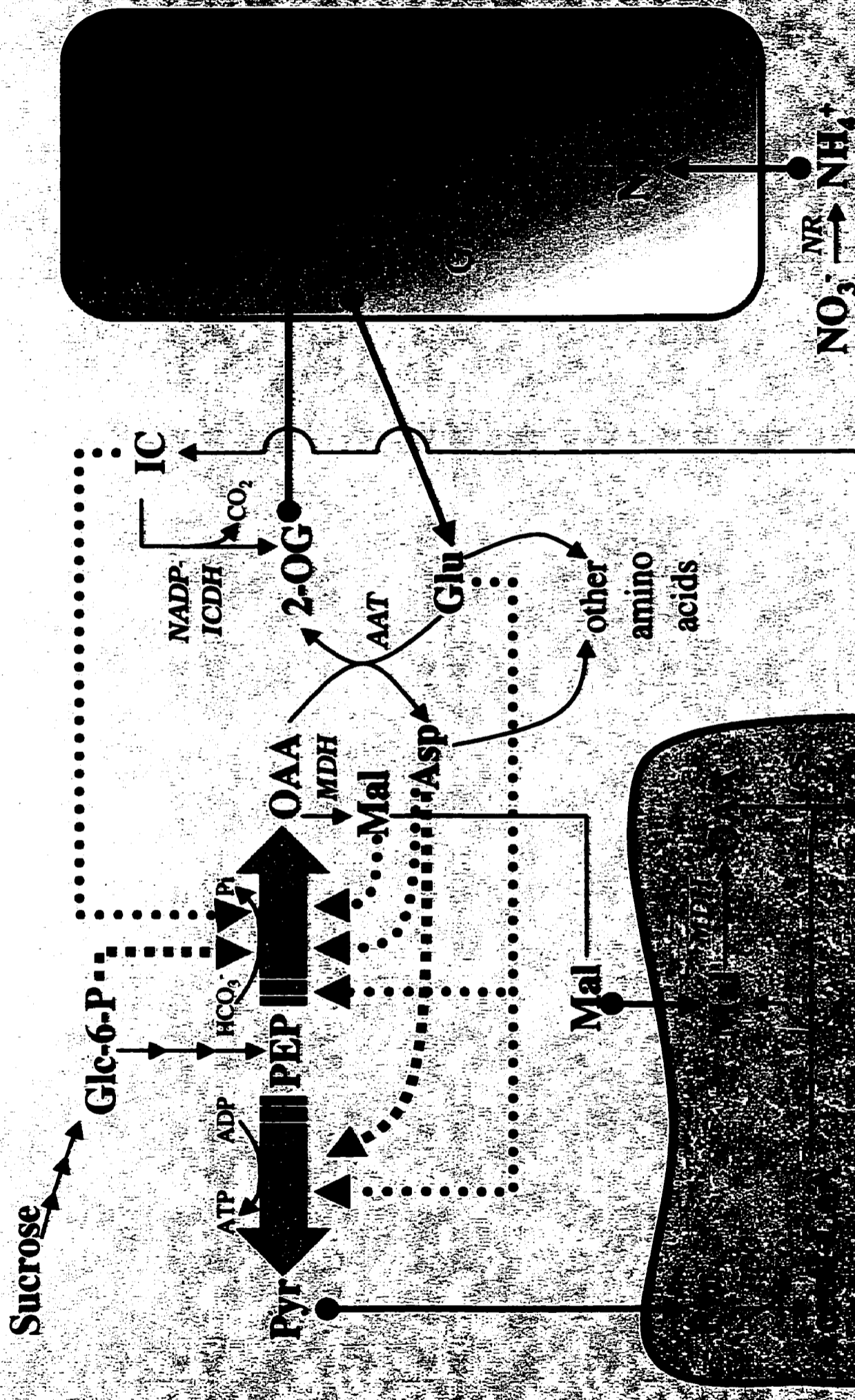
central role in providing the mitochondria with respiratory substrates as well as for generating C-skeletons for NH_4^+ assimilation via GS/GOGAT and AAT. Hexose-6-P activation of PEPC could coordinate sucrose availability with the flux of PEP carboxylation to dicarboxylic acids via PEPC. Malate inhibition of PEPC may provide a tight feedback regulation which closely balances PEPC activity with the overall rate of malate metabolism (Fig. 4.1). PEPC inhibition by Asp, isocitrate and Glu provides additional feedback controls that may coordinate overall PEPC activity with the production of C-skeletons (i.e. 2-OG and OAA) required for NH_4^- assimilation by GS/GOGAT and transamination reactions (i.e. AAT) in *B. napus*. In particular, however, the combined results of the M.Sc. thesis research of myself and Mr. Chris Smith [80] provide important insights into the critical role played by Asp and Glu in the coordinate 'allosteric regulation of higher plant PEPC and PK_c. Feedback inhibition of *B. napus* PK_c and PEPC by Glu provides a rationale for known activation of the two enzymes that occurs *in vivo* during periods of enhanced N-assimilation (when cellular Glu concentrations are reduced) [51,73,105]. In contrast to PEPC, Asp functions as an *allosteric activator* of the *B. napus* PK_c by effectively relieving the enzyme's inhibition by Glu [80]. Reciprocal control of *B. napus* PK_c and PEPC by Asp provides an intriguing mechanism for decreasing flux from PEP to Asp (via PEPC & AAT) while promoting PK_c activity when cytosolic Asp levels are elevated (Fig. 4.1).

The activation of C_3-leaf PEPCs by protein kinase-mediated phosphorylation in response to N-resupply of N-limited tissues [16,74] raises the possibility that the *B. napus* PEPC is also subject to this additional form of fine metabolic control. However, there are

other reports that reversible phosphorylation of the C_3-plant PEPC is of minor importance for its regulation (relative to its control by allosteric effectors)[21;109;110]. Results discussed above and summarized in Fig. 4.1 clearly emphasize the cardinal role of allosteric effectors in the coordinate regulation of plant PEPC and PK_c.

4.4 Future Directions

PEPC turnover is one of the projects that is beyond the scope of this thesis. The mechanism by which PEPC specific activity rapidly decreases in -Pi cells after they have been refed Pi could be approached by monitoring PEPC via immunoblotting and enzyme activity assays. Preliminary immunoblots indicated a decrease in the PEPC protein levels corresponding to the decrease in PEPC specific activity. It would be of interest to determine the role, if any, ubiquitination of PEPC may play in this process. A preliminary study suggested that ubiquitin-dependent proteolysis may be involved in the turnover of PEPC in *Vicia faba* [111]. PEPC kinetic studies together with the use of *B. napus* PEPC-specific antibodies. phosphoserine specific antibodies and *in vivo* ^{32}P-labelling would determine if phosphorylation of PEPC occurs during the initial burst of biosynthetic activity that follows Pi resupply to -Pi cells. In addition, the complete sequence of *B. napus* PEPC has not been reported. The isolation of a *B. napus* PEPC cDNA would facilitate Northern blot analysis of PEPC mRNA levels during a Pi starvation and subsequent Pi refeeding time courses. PEPC genomic DNA and promoter analyses could eventually help to elucidate signal transduction mechanisms linking cellular Pi status to the transcriptional control of PEPC expression.

Moreover, the characterization of PEPC from *B. napus* leaves, roots and germinating as compared to developing seeds would determine if tissue-specific PEPC isozymes exist. Preliminary examination of developing and germinating endosperm and germinating cotyledons of castor oil seeds demonstrated the expression of different isoforms of PEPC [112;113].

Another problem that needs to be examined is the possible role of flavonoids in regulating partitioning of PEP between primary and secondary metabolism. The cytosolic concentrations of quercitin and rutin need to be monitored to determine if their concentration changes sufficiently to modulate PEPC and PK_c *in vivo*.

4.5 PERSPECTIVES

As plant enzymologists/biochemists one of our primary objectives is to understand the structure, regulation and function of metabolic pathways that allow plants to acclimate to different environmental stresses. However, this is sometimes driven by the economic demand for increases in plant growth and yield. Increases in crop yield have been addressed through both environmental (fertilizers and herbicides/fungicides) and genetic manipulation. Plant glycolysis is controlled at the level of PEP metabolism [1], and 40% of protein bound Asp in C_3 -plants is generated by PEPC reiterating the importance of this enzyme in plant cells [17]. Transgenic tobacco has been transformed with maize C_4 PEPC genes under the control of the constitutive cauliflower mosaic virus 35S promoter [26]. These tobacco plants contained maize PEPC transcripts of the correct size and about twice as much PEPC protein. However, their growth was retarded relative to that of non-transformed plants [26]. In addition, crops that have been treated with

-60-

phosphonate (a fungicide) have been shown to markedly decrease root development

during Pi starvation[97]. To improve plant growth through genetic or environmental

manipulation we must first understand the regulatory properties of enzymes such as

PEPC in response to perturbations such as Pi- or N-limitation and how they function in

coordination with other enzymes.

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NLN MEDIUM FOR B. NAPUS (CV. JET NEUF) SUSPENSION CULTURE
(REF: Johnson-Flanagan and Singh 1987 Plant Physiol 85: 699-705)

	1 L
NN 5X stock	200 mL
AA 10X stock	100 mL
BA stock (0.1 mg/ml)	5.0 mL
NAA stock (0.1 mg/ml)	0.5 mL
2,4 D stock (0.1 mg/ml)	5.0 mL
Sucrose	65 g

Adjust pH to 7.5 with KOH divide into 40ml (small 125ml flasks) or 440 ml (large 2 L flasks) autoclave for 20 min at 121 °C (liquid setting; AMSCO model 2021).

NN 5X stock

	1 L
10X macronutrient stock	500 mL
100X Vit stock	50 mL
100X micronutrient stock	50 mL

Divide into 100 mL and/or 200 mL stocks and store at -20°C

To make NN stock we make up these stock solutions

10X macronutrient stock — 2 L

KNO_3	2.5 g
$MgSO_4\ 7H_2O$	2.5 g
$Ca(NO_3)_2\ 4H_2O$	10.0 g
Fe EDTA Na Salt	0.8 g (0.4 g/L)

Divide into 250 and/or 500 ml stocks and store at -20°C

-normally add KH_2PO_4 (Pi nutrient) into this stock. for ÷Pi cultures we add 2.5 mM Pi after autoclaving, from a pre-autoclaved stock of 0.2 M Pi (pH 6.0 made from mono- and di- basic KPi)

100X Vit stock

	1 L
Glycine	200 mg
myo-inositol	10 g
Nicotinic acid	500 mg
Pyridoxine HCl	50 mg
Thiamine HCl	50 mg
Folic acid	50 mg
Biotin	5.0 mg

divide into 50 and/or 100 mL stocks and store at -20°C

Micronutrient 100X stock

	1 L
$MnSO_4 \, 4H_2O$	2.23 g
H_3BO_3	0.62 g
$ZnSO_4 \, 7H_2O$	0.86 g
$Na_2MoO_4 \, 2H_2O$	25.0 mg
$CuSO_4 \, 5H_2O$	2.5 mg
$CoCl_2 \, 6H_2O$	2.5 mg

divide into 50 and/or 100 mL stocks and store at -20°C

AA 10X stock

	500 mL
Glutathione (reduced)	0.15 g
L-Glutamine	4.0 g
L-serine	0.5 g

divide into 50, 100 and/or 250 ml stocks and store at -20°C

Hormone stocks:

NAA (α-naphthalene acetic acid) (0.1 mg/mL): dissolve 10 mg in 5 mL of ethanol, then make up to 100 mL with distilled water

BA (3-bromopyruvic acid)(0.1 mg/mL): dissolve 10 mg of BA in 2 mL 0.5 N HCl by heating slightly and make up to 100 mL with distilled water.

2,4 D (2,4-dichlorophenoxyacetic acid) (0.1 mg/mL): same as for BA

Appendix II

Immunological detection of phospho*enol*pyruvate carboxlyase via Western Blotting
(REF: Plaxton 1989 *Eur. J. Biochem* 181, 443-451)

Polypeptides were separated by SDS/PAGE and electroblotted onto PVDF as described in the methods and materials. PVDF membranes were blocked for 1 hour at 24 °C with blocking buffer (Buffer X + 3% Carnation-skim milk) and were subsequently probed with affinity-purified rabbit anti-(*B. napus* PEPC) IgG (diluted 15-fold in Buffer X + 0.3% BSA) for 1 hour at 24 °C. Blots were washed 3 times with 10 mL of Buffer X for 5 min to remove unbound antibodies and then incubated with alkaline-phosphatase-linked-goat anti-(rabbit IgG) antibodies "2° antibodies" (diluted 10000-fold in Buffer X + 0.3% BSA) for 1 hour at 24 °C. Blots were washed 3 times with 10 mL of Buffer X for 5 min to remove unbound 2° antibodies. Immuoreactive polypeptides were revealed by histochemical staining with 0.165 mg/mL 5-bromo-4-chloro-3-indoyl phosphate and 0.33 mg/mL nitro blue tetrazolium in Buffer Y.

Buffer X

50 mM Tris/HCl pH 7.5
150 mM NaCl
0.05% (v/v) Tween-20
0.04% (v/v) NaN_3

Buffer Y

100 mM Tris-HCl pH 9.5
100 mM NaCl
2 mM $MgCl_2$
0.05% Tween-20