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Does phosphoenolpyruvate carboxykinase have a role in both amino acid and carbohydrate metabolism?

Review Article

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Summary. Phosphoenolpyruvate carboxykinase (PEPCK) catalyses the reversible decarboxylation of oxaloacetate to yield phosphoenolpyruvate and CO_2. The role of the enzyme in gluconeogenesis and anaplerotic reactions in a range of organisms is discussed, along with the important function in C_4 and CAM photosynthesis in higher plants. In addition, new data are presented indicating that PEPCK may play a key role in amino acid metabolism. It is proposed that PEPCK is involved in the conversion of the carbon skeleton of asparagine/aspartate (oxaloacetate) to that of glutamate/glutamine (2-oxoglutarate). This metabolism is particularly important in the transport system, seeds and fruits of higher plants.

Keywords: Amino acid metabolism – Asparagine – Glutamate – Higher plants – Oxaloacetate – Phosphoenolpyruvate carboxykinase

1 Introduction

Phosphoenolpyruvate carboxykinase (PEPCK) catalyses the ATP dependent (EC 4.1.1.49) or GTP dependent (EC 4.1.1.32) reversible decarboxylation of oxaloactetate (OAA) to yield phosphoenolpyruvate (PEP) and CO_2, in a reaction that requires Mn^{2+}:

$$OAA + A(G)TP \leftrightarrow PEP + A(G)DP + CO_2$$

The equilibrium position favours decarboxylation and the enzyme is therefore in potential conflict with phosphoenolpyruvate carboxylase (PEP carboxylase, EC 4.1.1.31) which catalyses the synthesis of OAA, with the possibility of the occurrence of an ATP hydrolysing futile cycle:

$$PEP + HCO_3^- \rightarrow OAA + P_i$$

As will be seen later, PEPCK can be involved in both gluconeogenesis and anaplerotic reactions in a wide range of different organisms, as well a playing a major role in photosynthesis in some higher plants. However it also obvious to those of us with an interest in nitrogen metabolism, that both PEPCK and PEP carboxylase can form a bridge between the OAA/aspartate family of amino acids, asparagine, lysine, threonine, methionine and isoleucine (Azevedo et al., 1997) and the PEP family of amino acids, phenylalanine, tyrosine and tryptophan synthesised via the shikimate pathway (Schmid and Amrhein, 1999), plus of course alanine which is derived from pyruvate (Lea and Ireland, 1999; Ireland and Lea, 1999).

Whilst significant amino acid sequence homology (40–80%) has been detected within the ATP and GTP dependent PEPCK proteins, initial studies indicated that there is no significant overall sequence homology between the two forms of PEPCK (Reymond et al., 1992). However following X-ray structural analysis of crystals of ATP-dependent enzyme from *E. coli* (Matte et al., 1996), Matte et al. (1997) concluded that there were similarities between the active sites of the two enzyme classes. Homologous sequences associated with OAA binding (the PEPCK specific domain) and the NTP-Mg^{2+} binding (Kinase-1a and Kinase-2a motives, respectively), suggest that the two enzymes operate in a similar fashion. Matte et al. (1997) argued that the conversion of OAA to PEP is a two step process involving the decarboxylation of OAA to form the pyruvyl enolate anion intermediate, followed by the transfer of the γ-phosphoryl group from either ATP or GTP. A key arginine group (Arg-333 in *E. coli*) has been shown to stabilise the enolate anion (Matte et al., 1997) and a lysine group (Lys-256 in *Saccharomyces cereviiae*) is involved in the phosphoryl transfer reaction (Krautwurst et al., 1998).

2 Animals

In animals, PEPCK is considered to be the first commited step in gluconeogensis in the liver and kidney, although enzyme activity has also been detected in a range of other organs including the small intestine, mammary glands and adipose tissue (Hanson and Reshef, 1997). Two distinct isoenzymic forms of GTP dependent PEPCK are present in animals, one located in the mitochondria and one in the cytosol, the proportions of which vary greatly between species. Both isoenzymes are monomeric and have similar molecular mass (67–69 kDa) and kinetic properties (Hanson and Patel, 1994). OAA generated in the mitochondria can be converted directly to PEP, or alternatively exported from the mitochondria as malate and reoxidised to OAA prior to conversion to PEP in the cytosol. There is little evidence to suggest that the activity of animal PEPCK is regulated by allosteric effects, or by protein modification (e.g. by phosphorylation).

The synthesis of cytosolic PEPCK in the liver is regulated by gene transcription and the rate of turnover of the mRNA and can be induced by starvation and reduced by a high carbohydrate diet. In the kidney, acid/base status also has an effect. Overexpression of PEPCK in transgenic animals

causes a diabetes-like condition, emphasising the importance of the enzyme in maintaining the concentration of glucose (Valera et al., 1994). Hormones including glucagon (acting via cAMP), glucocorticoids, retinoic acid, adrenaline and thyroid hormone all stimulate PEPCK synthesis, whilst insulin can depress synthesis within 30 minutes of administration. The metabolism of glucose also represses PEPCK synthesis, independent of the effect of insulin (Scott et al., 1998). In contrast, glutamine (which is a good substrate for gluconeogenesis) has been shown to cause an increase in the transcription of PEPCK mRNA in rat liver (Newshome et al., 1994). In a more detailed study, Lavoinne et al. (1996) showed that the glutamine stimulation required high concentrations and subsequent metabolism, but that glutamine was able to completely block the repressive effects of glucose. At least twelve regulatory elements have been identified in the 5′-flanking region of the cytosolic PEPCK gene, which are responsible for its complex pattern of regulation. However, the complexity of the cytosolic PEPCK gene promoter and the large number and combination of transcription factors that regulate expression, have made the synthesis of the enzyme very difficult to study (Hanson and Reshef, 1997; Yamada et al., 1999).

3 Microorganisms

3.1 Bacteria

In the majority of the bacteria that have been studied, PEPCK has been shown to be a key enzyme in gluconeogesis and that it is required when carbon sources are metabolised through the Krebs tricarboxylic acid cycle (Gottschalk, 1986). A PEPCK-deficient mutant of *Rhizobium* lacked the capacity to grow on succinate or other tricarboxylic acid intermediates, but was able to utilise glucose or glycerol as sole carbon sources, this mutant was unable to induce the formation of nitrogen fixing nodules (Osterås et al., 1991). Analysis of *E. coli* mutants has however indicated that PEPCK can be bypassed by the joint action of malic enzyme and PEP synthase that converts pyruvate to PEP (Goldie and Sanwal 1980), similar results have also been obtained in *Rhodopseudomonas palustris* (Inui et al., 1999). Using metabolic flux ratio analysis, Sauer et al. (1999) have recently provided "fluxome" information concerning central carbon metabolism in *E. coli.* In glucose limited cultures there strong evidence that there was a futile cycle operating involving PEPCK and PEPC, which was reduced in ammonia limited cultures. *E. coli* strains that overexpressed PEPCK, exhibited increased glucose consumption and contained higher concentrations of PEP, which may favour aromatic amino acid synthesis (Chao and Liao, 1993). Initial studies showed that the expression of the PEPCK encoding gene (*pckA*) was induced 100-fold in the stationary cells of *E. coli* (Goldie, 1984). Expression of *pckA* was shown to be induced by gluconeogenic carbon sources such as succinate, arabinose and glycerol in *E. coli* (Goldie, 1984) and succinate and arabinose, but not glycerol in *Rhizobium meliloti* (Osterås et al., 1995). In contrast, glucose and sucrose are strong repressors of the transcription of *pckA* in *E. coli* (Goldie,

1984), *R. Meliloti* (Osterås et al., 1995) and *Staphylococcus aureus* (Scovill et al., 1996).

Evidence has also been presented that PEPCK can operate in the direction of OAA synthesis and thus act as an anaplerotic enzyme in a similar manner to PEPC, when the tricarboxylic acid cycle is being used for anabolic purposes, e.g. the synthesis of organic and amino acids. In the anaerobic bacterium *Anaerobiospirillum succiniciproducens*, glucose is metabolised to succinate at high CO_2 concentrations using PEPCK (Samuelov et al., 1991). Similar results have been obtained with *Alcaligenes eutrophus* (Schobert and Bowien, 1984) and *Ruminococcus flavefaciens* (Schocke and Weimer, 1997). However, utilising a mutant of the aerobic organism, *Corynebacterium glutamicum*, lacking PEP carboxylase activity, Peters-Wendlisch et al. (1997) argued strongly that pyruvate carboxylase (which catalyses the direct ATP dependent conversion of pyruvate to OAA), but not PEPCK, was able to fulfil an anaplerotic function. Thus the role of PEPCK in bacteria requires further detailed study in individual species.

3.2 Yeast

In the yeast *Saccharomyces cerevisiae*, the synthesis of PEPCK is strictly regulated by the available carbon sources and is repressed by the presence of glucose and is derepressed by the presence of gluconeogenic substrates such as acetate, organic acids or ethanol. Two upstream activation sites and one upstream repression site have been determined by a detailed deletion analysis of the *PCK1* gene (Proft et al., 1995). The derepression mechanism requires a transcriptional activator CAT8 zinc cluster protein that is subject to extensive phosphorylation by a Snf1 protein kinase, which is also regulated by the available carbon source (Randez-Gil et al., 1997; Rahner et al., 1999).

4 Plants

4.1 Seed germination

During the germination of seeds lipids are metabolised via β-oxidation and the glyoxylate cycle to form malate and hence OAA. Sucrose is then synthesised from OAA by the enzymes of the gluconeogenic pathway which has been shown to be located in the cytosol of major lipid containing seeds (Leegood and ap Rees 1978; Nishimura and Beevers 1979). Using 3-mecaptopicolinic acid as an inhibitor of PEPCK, Trevanion et al. (1995) followed the incorporation of ^{14}C-acetate into soluble sugars in germinating marrow. They were able to demonstrate that flux control coefficient of PEPCK for gluconeogenisis in darkened cotyledons was high, (0.7–1.0) at all temperatures studied, perhaps indicating that the enzyme is inactive in the dark. Considering the importance of PEPCK in regulating gluconeogenesis, it is somewhat surprising that early studies indicated that the enzyme activity was not subject to regulation.

A cDNA clone, isolated from a senescing cucumber cotyledon library, was shown to encode a PEPCK protein of molecular mass 74 kDa, the amino acid sequence of which was 43%, 49% and 57% identical to bacterial, trypanosome and yeast enzymes respectively. Expression of the PEPCK gene increased 2–3 days after the onset of germination, in line with enzymes of the glyoxylate cycle, however there was no evidence of any dark stimulation of mRNA transcription (Kim and Smith, 1994). The somewhat surprising feature of the predicted cucumber PEPCK polypeptide, was it that it appeared to be larger than previously purified enzymes from plant sources due to the presence of an N-terminal extension of 12 kDa (Kim and Smith, 1994). When Walker et al. (1995) purified PEPCK from cucumber cotyledons, the molecular mass of the protein subunit was 62 kDa. However if cotyledons of cucumber and other seedlings were extracted in SDS and at high pH, a 74 kDa protein was visible after Western blotting that was susceptible to rapid proteolytic cleavage to a 62 kDa form, a process that had no effect on enzyme activity. Walker and Leegood (1995) then devised a rapid purification procedure for cucumber PEPCK that allowed the isolation of a native enzyme with subunits of molecular mass of 74 kDa. Incubation of the purified intact enzyme protein from cucumber with mammalian cAMP-dependent protein kinase, or maize PEP carboxylase kinase and [γ-^{32}P] ATP led to the incorporation of ^{32}P into a part of the polypeptide which was cleaved during proteolysis. This phosphorylation was reversed by incubation with protein phosphatase 2A. When cucumber cotyledons were supplied with $^{32}P_i$ *in vivo*, PEPCK was one of the five major polypeptides in darkened cotyledons and this labelling was reversed by ilumination. Walker and Leegood (1996) went on to show that PEPCK isolated from the cotyledons of a range of other gluconeogenic seedlings was also susceptible to *in vivo* phosphorylation.

Inspection of the sequence of cucumber PEPCK (Kim and Smith, 1994), reveals two potential phosphorylation sites in the N-terminal region. The first, (residues 42–51, ICHDDSTTPM) forms a consensus sequence for recognition by SNF-1 related protein kinases. These latter kinases are thought to be global regulators of key enzymes in plants, including sucrose phosphate synthase and nitrate reductase (Halford and Hardie, 1998). The second potential phosphorylation site (residues 64–69, KKRSTP) shows homology with 14-3-3 binding sites (Muslin et al., 1996). The 14-3-3 inhibitory proteins have been shown to regulate a range of plant enzymes, including sucrose phosphate synthase, nitrate reductase and glutamine synthetase (Moorhead et al., 1999). It should be noted that PEPCK phosphorylation is unique to plants and the additional N-terminal sequence is not found in the enzyme protein isolated from other organisms. The related enzyme PEP carboxylase is also subject to phosphorylation in plants and there is clear evidence that the kinetic properties of the enzyme are altered by this process (Chollet et al., 1996).

PEPCK has an absolute requirement for Mn^{2+} and has previously been shown to be inhibited by millimolar concentrations of Mg^{2+} (Burnell, 1986). Walker et al. (2000), have optimised assay conditions so that it is possible, for the first time, to measure the activity of PEPCK under conditions approximating to the cytosol (i.e. micromolar Mn^{2+} and millimolar Mg^{2+}). These assay

conditions have allowed the detection of new effectors and the kinetic effects of phosphorylation and dephosphorylation. The response of PEPCK to its substrates PEP and OAA, strongly depends upon the available adenylate ratio and changes with phosphorylation state. Light-dark changes in activity are maximised at high ratios of ATP/ADP, even in the carboxylation assay for which ADP is the substrate. These changes in the properties of PEPCK have been used to follow the activation state of PEPCK in crude extracts of leaves of the C_4 plants, maize, *Panicum maximum* and the CAM plant, pineapple.

4.2 Photosynthesis

4.2.1 C_4 plants

An important element of C_4 photosynthesis is the decarboxylation of a C_4 acid in a separate compartment close to the site of Rubisco activity, in order to increase the concentration of CO_2, with respect to O_2 (Leegood, 1997). Until recently, C_4 plants could be divided into three types based on this decarboxylation reaction, (1) NADP-malic enzyme, (2) NAD-malic enzyme and (3) PEPCK. As can be seen in Fig. 1, in the PEPCK plants there are two mechanisms of decarboxylation of C_4 acids, one utilising NAD-malate enzyme in the mitochondria yielding NADH, which subsequently gives rise to the ATP required for the second, conversion of OAA to PEP catalysed by PEPCK (Agostino et al., 1996; Leegood, 1997). Aspartate is transported to the bundle sheath cells, prior to transamination to yield OAA and the amino group is transported back to the mesophyll cells as alanine.

PEPCK has been purified to homogeneity from a range of C_4 grasses (Burnell, 1986; Arnelle and O'Leary, 1992). The K_m for OAA is 12–25 μM and for ATP, 16–25 μM. The enzyme activity of the PEPCK protein that has been subject to proteolysis, was shown to be inhibited by glycerate-3-P, fructose-6-P, fructose-1,6-bisphosphate and dihydroxyacteone phosphate at concentrations between 1–5 mM (Burnell 1986). It is possible that the accumulation of these metabolites could have a physiological role in the regulation of C_4 photosynthesis (Leegood and Walker, 1999a). However, none of these properties indicate how PEPCK activity could be subject to diurnal regulation and be switched off in the dark. (Carnal et al., 1993), which is much more likely to be regulated by change in phosphorylation status.

Initial studies with PEPCK isolated from C_4 plants had indicated that the purifed active enzyme had a subunit molecular mass of 62–64 kDa and was present as a hexamer (Burnell, 1986). However in a careful series of experiments using high pH, protease inhibitors and 1% SDS in the extraction buffer, Walker et al. (1997) were able to show that the subunit of the enzyme isolated from a range of PEPCK C_4 species had a molecular mass of 67–71 kDa and that a N-terminal sequence was normally lost during isolation and purification. The presence of this N-terminal sequence allowed the enzyme isolated from two of the five C_4 species tested, to be phosphorylated in the dark but not

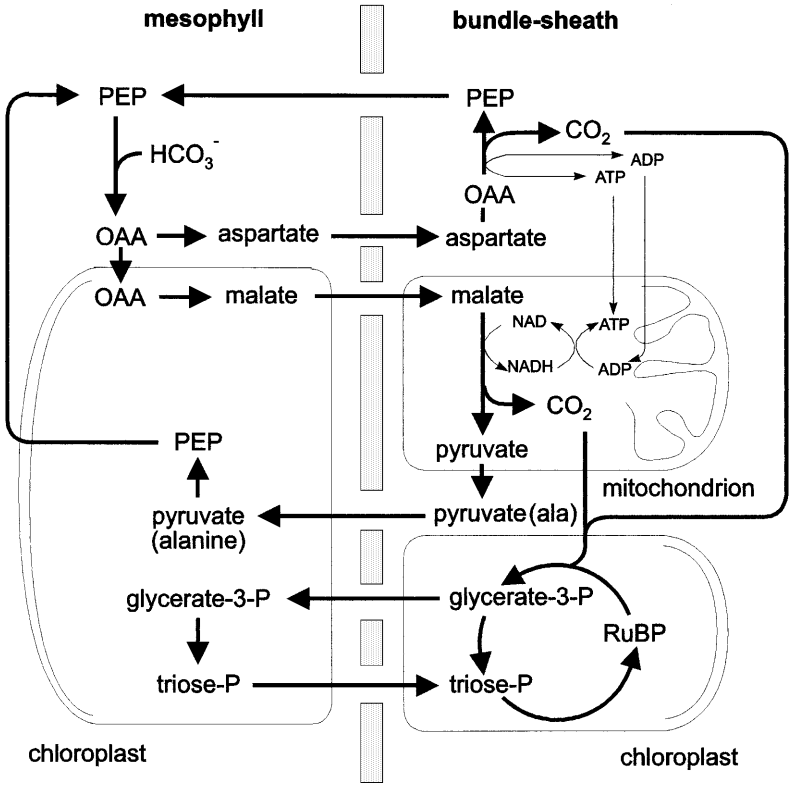

Fig. 1. The intracellular compartmentation of the PEP-carboxykinase type pathway of C_4 photosynthesis. Note that both PEPCK and NAD-ME carry out the decarboxylation reactions. NADH formed by NAD-malic enzyme is used to generate the ATP required for PEPCK. Alanine and aspartate are shuttled between the mesophyll and bundle sheath cells, in order to maintain a balance of amino groups between the two compartments (Leegood, 1997)

in the light (Walker and Leegood, 1996, 1997). The reason for the differences in the phosphorylation of PEPCK in plants apparently carrying out the same meachanism of C_4 photosynthesis is not clear, however non-phosphorylated enzyme proteins lacked the putative phosphorylation motif KKRST. It is possible that as PEPCK and PEPC are located in different cell types in C_4 plants, there is not a strong evolutionary pressure to maintain the phosphorylation site (Walker and Leegood, 1996; Leegood et al., 1999).

There is now an indication that the classification of the three types of C_4 photosynthesis is not rigid. It was originally thought that maize did not contain PEPCK, and that all of the C_4 acids were decarboxylated via NADP-malic enzyme. However using the extraction procedures described above, Walker et al. (1997) were able to detect, by Western blot analysis the presence of PEPCK in maize and *Digitaria sanguinalis*, which had a molecular mass of 74 kDa, but which was not phosphorylated. In contrast, PEPCK protein was not detectable in some other NADP-malic enzyme plants (e.g. sorghum and sugar cane). In a more detailed analysis, Wingler et al. (1999) confirmed the

presence of PEPCK in the bundle sheath cells of maize and showed that PEPCK catalysed the decarboxylation of OAA derived from aspartate, whilst NADP-malic enzyme was required for the decarboxylation of malate. Kellog (1999), in an interesting discussion of the evolution of photosynthesis, has argued that there are only two biochemical types of C_4 photosynthesis, requiring either NAD-malic enzyme or NADP-malic enzyme. Both decarboxylating enzyme activities can however be supplemented by the presence of PEPCK.

The complete DNA sequence of a gene encoding PEPCK from the C_4 grass *Urochloa panicoides* was originally published by Finnegan and Burnell (1995) and evidence was presented for the occurrence of a mulitigene family. In a more recent study, Finnegan et al. (1999) have indicated that there are at least four genes encoding PEPCK in *U. panicoides*. Two of the genes, *PCK1* and *PCK2* were shown to encode subunits of with a 96% amino acid sequence identity and were expressed in a light dependent manner in the leaves, suggesting that they were involved in C_4 photosynthesis. The two additional genes *PCK3* and *PCK4* were expressed predominantly in the roots (Finnegan et al., 1999). Interestingly, in cucumber there appears to be only one copy of a gene encoding PEPCK (Kim and Smith, 1994). A full length cDNA clone encoding PEPCK has also been isolated and sequenced from maize (Furomoto et al., 1999). The gene was expressed in the bundle sheath cells, confirming the presence of the enzyme protein described above (Walker et al., 1997; Wingler et al., 1999). The level of mRNA transcription was higher during the day in young seedlings, but the diurnal variation decreased in older plants. Following expresion of the maize PEPCK gene in *E. coli*, both truncated and full length proteins were isolated, the latter was shown to be subject to phosphorylation by a mammalian cAMP kinase and to be less sensitive to inhibition by 3-phosphoglycerate (Furomoto et al., 1999).

4.2.2 Crassulacean acid (CAM) metabolism

CAM metabolism involves the uptake of CO_2 though the stomata at night and assimilation through the action of PEP carboxylase into C_4 organic acids, of which malate predominates. The photosynthetic pathway is often induced by drought stress (Cushman and Bohnert, 1997). During the day the C_4 acids are decarboxylated to yield CO_2 which is assimilated by Rubisco and the normal Calvin photosynthetic carbon reduction cycle (Osmond et al., 1999). CAM plants can be subidivided in a manner analagous to C_4 plants, dependent on whether they utilise PEPCK or NADP- and NAD-malic enzymes in the decarboxylation reaction. Futher division is dependent upon the compounds used to store carbohydrate during the day, which may be starch and glucans in the chloroplasts, or soluble sugars and polysaccharides in extrachloroplastic compartments (Christopher and Holtum, 1996, 1998; Osmond et al., 1999). In different species of *Clusia*, PEPCK activity increased in response to drought in both young and mature leaves. Immunohistochemical techniques indicated that in the leaves of well watered *Clusia aripoensis*, PEPCK was localised solely around latex producing ducts. However in *Clusia* species that were

carrying out CAM photosynthesis, the PEPCK protein was located (along with Rubisco and PEP carboxylase) in the palisade and spongy parenchyma cells (Borland et al., 1998).

PEPCK isolated from the leaves of all four species of CAM plants tested was shown to be phosphorylated *in vivo*, following the application of $^{32}P_i$ (Walker and Leegood, 1996), this is in contrast to the variation in phosphorylation detected with plants carrying out C_4 photosynthesis (see above). CAM plants in which PEPCK and PEP carboxylase are located in the cytoplasm of the same cells (Borland et al., 1998) have maintained the requirement for a phosphorylation sequence, which is again located on the N-terminal proteolytically cleaved portion of the protein. In *Tillandsia fasciculata* leaves, the PEPCK protein was dephosphorylated during the afternoon and phosphorylated again during the night, in a similar manner to C_4 plants (Walker and Leegood, 1996), which is consistent with the enzyme being active as a decarboxylase during the daytime.

In both C_4 and CAM photosynthesis, the enzyme responsible for the initial assimilation of CO_2 is PEP carboxylase. In quite a revolutionary paper, Reiskind and Bowes (1991) demonstrated that in the marine macroscopic green alga *Udotea flabellum*, when PEPCK was inhibited by 3-mercaptopicolinic acid, there was a dramatic decrease in the rate of photosynthesis. This inhibition of photosynthesis correlated with a reduction of the early products of photosynthesis, namely malate and aspartate. Reiskind and Bowes (1991) argued that PEPCK was acting in an assimilatory capacity to form OAA and malate and that the pathway in *U. flabellum* was a primitive form of C_4 photosynthesis. However it seems unlikely that PEPCK could act as a carboxylase in land plants due to the low affinity of the enzyme for CO_2.

4.3 Fruits

In the climacteric fruits, PEPCK activity can only be detected during ripening, and in tomato is present thoughout the parenchyma of the pericarp. In the non-climacteric fruits, grape and cherry, PEPCK activity was present throughout development (R. P. Walker, unpublished results). As many fruits are known to accumulate high concentrations of organic acids, which subsequently fall at the end of the ripening process, it is likely that PEPCK along with malic enzyme is involved in this metabolism (Knee and Finger, 1992; Kanellis and Roubelakis-Angelakis, 1993). Early in grape berry development, the parenchyma cells of the pericarp all possess crystalline inclusions (probably organic acids), which progressively disappear as the berry develops (Fillion et al., 1999; Famiani et al., 2000), whilst PEPCK and NADP-malic enzyme activity increase. As the activity of sucrose phosphate synthase (an enzyme required for the synthesis of sucrose), also increases during the ripening of both grape (Hawker, 1969) and tomato (Dali et al., 1992), it is likely that carbon is being transferred from organic acids via guconeogenesis, to yield the hexose phosphates required for sucrose synthesis (Leegood and Walker 1999).

4.4 Developing seeds and phloem

4.4.1 Developing seeds

In a recent study, Walker et al. (1999) were able to detect PEPCK activity and protein in a range of developing seeds including grape, iris, pea and tomato. In grape, the amount of PEPCK was shown to be developmentally regulated, with maximum activity coinciding with the maximum activity of amino acid metabolising enzymes and the accumulation of storage proteins. Immunohistochemical localisation showed that in grape, PEPCK was predominantly located in tissues which are involved in the transfer of assimilates within the developing seed, e.g. the phloem and chalaza adjoining the developing storage tissue and palisade layer (Fig. 2). The palisade layer of the grape seed coat is rich in plasmodesmata and may act to distribute imported assimilates symplastically from the vascular supply to the developing sink tissues. Walker et al. (1999) proposed that assimilates diffuse through the palisade cells via plasmodesmata and then enter the nucellus via the transfer cells (Fig. 3), thus coordinating delivery to the demand of the developing endosperm. The enrichment of PEPCK and other enzymes of sugar and amino acid metabolism, as well as amino acids in the pallisade layer would suggest that there is also a considerable amount of metabolism taking place in this layer of cells.

The amount of PEPCK activity and protein in 20 day old grape seeds was greatly induced following incubation with asparagine (100 fold) and to a lesser extent with ammonia (7 fold) and glutamine (3 fold) (Fig. 4). In contrast there was no change in the abundance of PEP carboxylase, NADP malic enzyme, glutamine synthetase or cytosolic aspartate aminotransferase. The large induction of PEPCK by asparagine is consistent with increases in asparaginase activity detected in soybean cotyledons (Tonin and Sodek, 1990) and gene

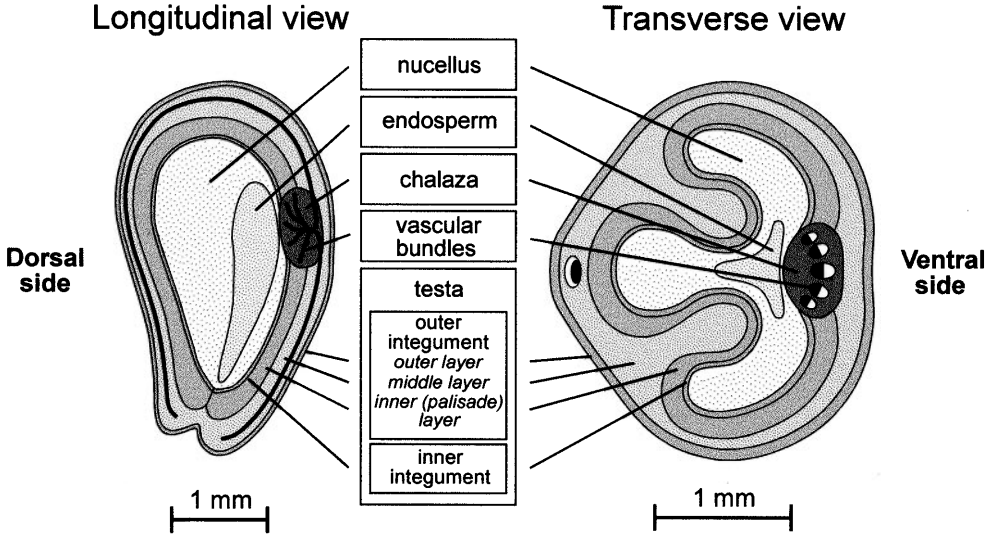

Fig. 2. Diagrammatic structure of a grape seed 28 days after flowering (Walker et al., 1999)

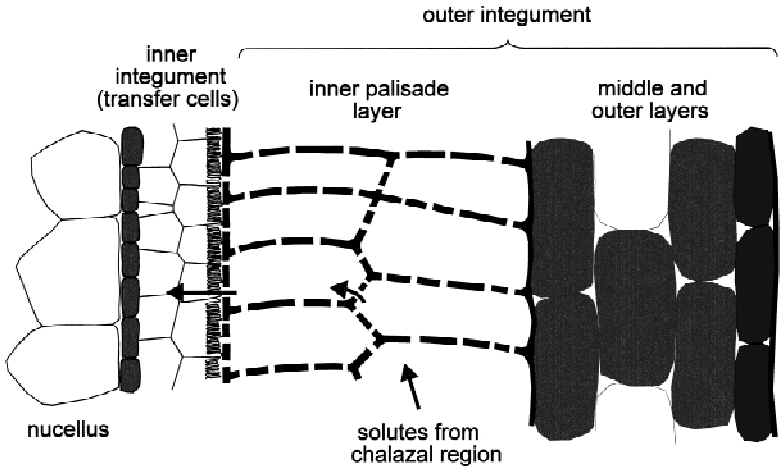

Fig. 3. Diagrammatic structure of the seed coat of grape showing the palisade layer (Walker et al., 1999)

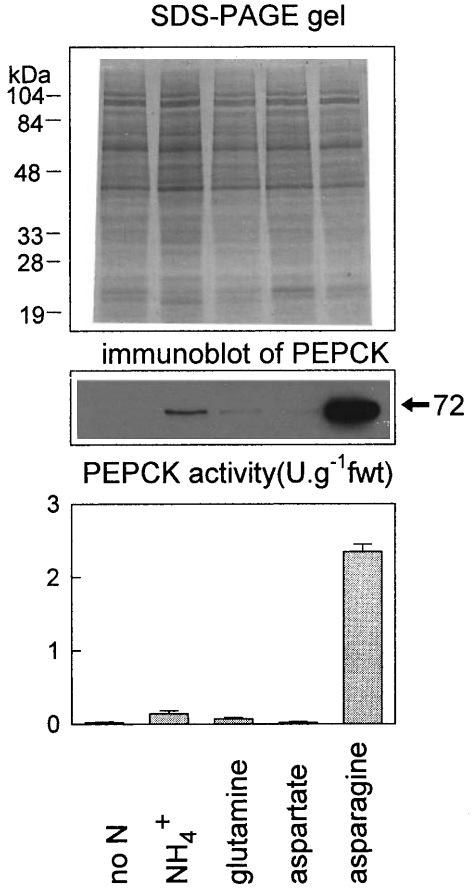

Fig. 4. The induction of PEPCK by nitrogenous compounds. Grape seeds were incubated with the different compounds as indicated for four days and the amount of PEPCK activity determined. The amount of PEPCK protein was determined, following Western blots of extracts subjected to SDS-PAGE (Walker et al., 1999)

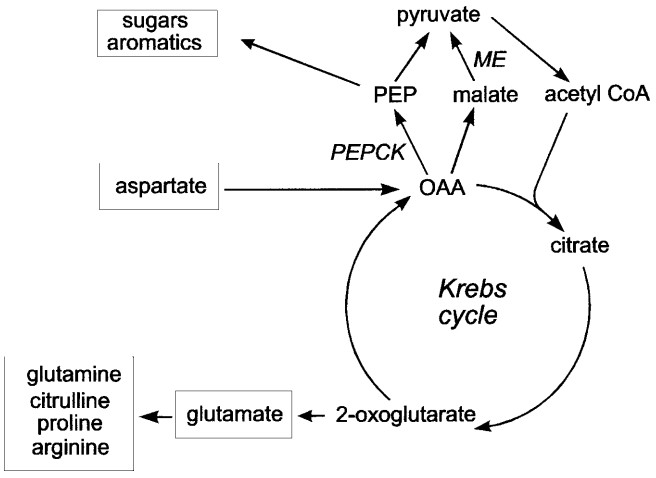

Fig. 5. A proposed involvement of PEPCK in the metabolism of aspartate and oxaloacetate (*OAA*), in either a gluconeogenic role to synthesise carbohydrates or in an anaplerotic role for the formation of 2-oxoglutarate leading to the synthesis of glutamate and other amino acids. Note that PEP is also a precursor of the aromatic amino acids, phenylalanine, tyrosine and tryptophan

expression in tobacco (Grant and Bevan, 1994). Asparagine is present in the phloem of grapevine and the inducibility of PEPCK would allow the developing grape seed to be responsive to changing environmental or nutritional conditions (Glad et al., 1992).

The key question is what is the relationship between asparagine metabolism and PEPCK? The amide group of asparagine is metabolised to ammonia, which must be reassimilated through the glutamate synthase cycle, which requires the net synthesis of 2-oxoglutarate (Ireland and Lea, 1999; Lea and Ireland, 1999). The amino group of aspartate could be used for synthesis of lysine, threonine, methionine or isoleucine directly (Azevedo et al., 1997) or could be transaminated to 2-oxoglutarate to yield glutamate and OAA. There is therefore a requirement for 2-oxoglutarate, which can be synthesised from citrate in the tricarboxylate cycle provided there is a supply of pyruvate or acetyl CoA. It is therefore possible that PEPCK has an anaplerotic role in supplying PEP and thus pyruvate, following the action of PEP phosphatase or pyruvate kinase (Fig. 5). Experiments in which [U-^{14}C-aspartate] fed to asparagine-induced grape seeeds, exhibited labelling of sugars, alanine and glutamate, are consistent with such a role in gluconeogenesis and anaplerosis (Walker et al., 1999).

It is also possible that PEPCK may play a role in maintaining the pH of the cytoplasm following the assimilation of the ammonia liberated from asparagine (Raven, 1988). Plant cells are able to regulate pH in two ways, the first through the use of proton pumps at the plasmamembrane and the second through a biochemical pH-stat (Davies, 1986; Sakano, 1998), in which formation and dissimilation of malate is a key feature. If malic acid is synthesised in one location and exported as malate, decarboxylation of malate in the source produces a OH^- ion. PEP carboxylase is thought to be the enzyme responsible

for malate synthesis and NADP-malic enzyme for malate decarboxylation We have recently suggested that PEPCK might also function as a decarboxylase in this mechanism.

4.4.2 Phloem

PEPCK has frequently been shown to be associated with the phloem. In grape berries and seeds the enzyme protein is present at all stages of development (Walker et al., 1999). PEPCK is also present in the phloem of cucumber, *Coleus blumei*, and *Clusia minor* (Borland et al., 1998), which, like grape, load assimilates into the phloem symplastically. In contrast, PEPCK was not detected in the phloem of tomatoes, barley, maize, *Panicum maximum*, or peas, which are known to be apoplastic loaders (Leegood and Walker, 1999b).

In cucumber, the situation is complex as there are three types of phloem (Esau, 1965). The vascular bundles are bicolatteral, with inner and outer phloem elements. In the minor veins, the inner abaxial phloem elements have symplastic connections, while the outer adaxial phloem elements have apoplastic connections. There is also a third type of phloem, a network of extra-fascicular phloem elements in the leaves, petioles and stems, which lie outside the vascular bundles. Immunolocalisation of PEPCK in cucumber leaves and stems has shown that it is associated with the adaxial phloem of minor veins, in the internal phloem of intermediate veins and throughout the extra-fascicular phloem elements (Leegood et al., 1999). Amino acids, such as glutamate and aspartate have also been immunolocalised in the adaxial and extra-fascicular phloem of leaves and petioles (Leegood et al., 1999). In a related species melon, the mesophyll sap is enriched in aspartate, whereas the phloem sap is enriched in glutamine (Mitchell et al., 1992). Such a difference suggests that there is extensive amino acid metabolism within the cells associated with the phloem, prior to loading into the sieve elements. In addition, cucurbits synthesise a number of compounds that are specifically involved in transport, such as arginine and citrulline, which are derived from glutamate and glutamine. We propose that these compounds are synthesised in or around the vascualture, in cells adjacent to the site of export from the leaf as shown in Fig. 5.

In summary, PEPCK is situated at an important crossroads in plant metabolism, lying between organic and amino acids, lipids and sugars. The enzyme is highly regulated by metabolites and by phosphorylation (only a few enzymes of primary metabolism have been shown to be regulated in this way) and has a far more widespread occurrence then previously realised, as discussed in this review. It occurs in many plant tissues, including economically important ones such as seeds, both at their formation and during germination. In various tissues, PEPCK is present at specific stages of development and in specific cell types. The synthesis of the PEPCK protein can be induced in response to metabolic signals, such as amino acids, a property it shares in common with PEP carboxylase (Suzuki et al., 1994).

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