REVIEW PAPER

Functional evolution of C₄ pyruvate, orthophosphate dikinase

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Abstract

Pyruvate, orthophosphate dikinase (PPDK) plays a controlling role in the PEP-regeneration phase of the C₄ photosynthetic pathway. Earlier studies have fully documented its biochemical properties and its post-translational regulation by the PPDK regulatory protein (PDRP). However, the question of its evolution into the C₄ pathway has, until recently, received little attention. One assumption concerning this evolution is that changes in catalytic and regulatory properties of PPDK were necessary for the enzyme to fulfil its role in the C₄ pathway. In this study, the functional evolution of PPDK from its ancient origins in the Archaea to its ascension as a photosynthetic enzyme in modern C₄ angiosperms is reviewed. This analysis is accompanied by a comparative investigation into key catalytic and regulatory properties of a C₃ PPDK isoform from Arabidopsis and the C₄ PPDK isoform from Zea mays. From these analyses, it is proposed that PPDK first became functionally seated in C₃ plants as an ancillary glycolytic enzyme and that its transition into a C₄ pathway enzyme involved only minor changes in enzyme properties per se.

Key words: C₃ plants, C₄ evolution, C₄ photosynthesis, C₄ plants, pyruvate, orthophosphate dikinase.

Introduction and review

Evolutionary origin and phylogenetic distribution of PPDK

Pyruvate, phosphate dikinase (PPDK, EC 2.7.9.1) was discovered independently in C₄ grasses (Hatch and Slack, 1968) and in the parasitic amoeba, Entamoeba histolytica (Reeves, 1968). The enzyme from all organisms catalyses the freely reversible reaction:

\[ \text{Pyruvate} + \text{ATP} + \text{Pi} \leftrightarrow \text{PEP} + \text{AMP} + \text{PPi} \]

Most of what is known concerning the structure function properties of the enzyme has been derived from studies of PPDK from the bacterium Clostridium symbiosum (Herzberg et al., 1996; Lim et al., 2007). The detail provided from this model PPDK has revealed structural features unique to the enzyme mechanism that allow its gene to be reliably identified in genome databases. These show that PPDK has a phylogenetic distribution restricted to select groups of the Archaea, bacteria, protozoa, lower fungi, and green plants (Liapounova et al., 2006; Slamovits and Keeling, 2006) (Table 1). A PPDK phylogenetic tree analysis assembled from these groups indicates the enzyme originated in the Archaea, but its evolution into prokaryotic and eukaryote taxons is inconsistent with its predicted vertical transmission (Liapounova et al., 2006; Slamovits and Keeling, 2006). For example, PPDKs from eukaryotic protists do not cluster uniformly into a clade, but are isolated into various bacterial subgroups. Because of this irregular transmission, it is proposed that the gene for PPDK has been horizontally transferred among distantly related (unicellular) species. The gene is notably absent in cyanobacteria indicating that plants did not inherit the PPDK gene from the chloroplast endosymbiont ancestor.

PPDK function in non-plant organisms

In most non-plant organisms studied to date (the Archaea, eubacteria, and protists), PPDK is either a primary or secondary enzyme of glycolysis. Its natural selection into these typically metabolically challenged organisms (i.e. anaerobic habitats) was probably promoted by the enzyme’s freely reversible catalytic mechanism. This feature allows
the enzyme to fulfil alternate roles in anaerobic metabolism such as gluconeogenesis [operating in the phosphoenolpyruvate (PEP) forming direction] or for glycolytic ATP synthesis [operating in the pyruvate (Pyr) forming direction]. Bacteria that use PPDK for ATP synthesis are typically anaerobic and lack pyruvate kinase (PK) (Reeves et al., 1968; Pocalyko et al., 1990). Likewise, protozoa that rely on PPDK for ATP synthesis dwell in anaerobic or low oxygen environments and lack mitochondria (Bringaud et al., 1998; Varela-Gomez et al., 2004; Feng et al., 2008).

The utility for using PPDK to generate glycolytically derived ATP is that the enzyme, as operating in the Pyr direction, can harvest the high bond energy inherent in pyrophosphate (Huang et al., 2008). In doing so, the yield of glycolytically derived ATP is boosted from two ATPs per glucose oxidized as in conventional PK-dependent glycolysis, to five ATPs per glucose oxidized (Fig. 1). In certain bacterial species, PPDK is used exclusively in the PEP-forming direction for gluconeogenesis (Benzimann and Eizen, 1971; Østeras et al., 1997; Eisaki et al., 1999). Other bacteria typically initiate gluconeogenesis with PEP synthetase (PEPS). The preference for PPDK in this step may be due to the ability to drive the reaction by hydrolysis of by-product PPi. Only a single study on the role of PPDK in an Archaea species is available (Tjaden et al., 1971). In doing so, the yield of glycolytically derived ATP is boosted from two to five ATPs per glucose oxidized (Fig. 1). In certain bacterial species, PPDK is used exclusively in the PEP-forming direction for gluconeogenesis (Benzimann and Eizen, 1971; Østeras et al., 1997; Eisaki et al., 1999). Other bacteria typically initiate gluconeogenesis with PEP synthetase (PEPS). The preference for PPDK in this step may be due to the ability to drive the reaction by hydrolysis of by-product PPi. Only a single study on the role of PPDK in an Archaea species is available (Tjaden et al., 2006). This investigation linked PPDK expression to alternate roles in gluconeogenesis and glycolytic conversion of PEP to Pyr. Interestingly, the competing PK and PEPS activities also present in this organism are proposed to be co-integrated with PPDK in a carbon-source responsive regulatory scheme.

**PPDK function in C₃ plants**

PPDK is ubiquitous in cells and tissues of C₃ plants, albeit as a very low abundance enzyme (Chastain and Chollet, 2003). However, in specific C₃ plant tissues, C₃ PPDK has been documented to be an abundantly expressed protein. Examples include developing cereal seeds (Kang et al., 2005; Chastain et al., 2006; Mechin et al., 2007; Hennen-Bierwagen et al., 2009), senescing leaves (Tjaden et al., 2010), sperm cells of maturing Arabidopsis pollen (Hruz et al., 2008), and water-saturated roots of Oryza sativa (rice) (Moons et al., 1998). In all plants, PPDK is located in both cytoplasmic and plastid compartments (Chastain and Chollet, 2003). In eudicots, PPDK is encoded by a single-gene locus (Huang et al., 2008), but contains two promoters in the 5'-region to produce cytoplasmic or plastid targeted transcripts (Parsley and Hibberd, 2006). In monocot species, the gene is encoded at two loci (Sheen, 1991; Huang et al., 2008), one of which expresses an isoform exclusive to the cytoplasm and the other loci configured as a two-promoter gene to generate plastid and cytoplasmic targeted polypeptides as in eudicots. The mature cytoplasmic and chloroplast targeted PPDK polypeptides from this gene are identical. In plants with PPDK genes at two loci, the encoded polypeptides are highly homologous (>90%). With the exception of this report, there is little detailed information concerning the biochemical properties of C₃ PPDK isoforms.

Ascribing a role to PPDK in those C₃ cells and tissues where it is present in very low amounts is problematic. The low level of activity prevents tracking the turnover of PPDK substrates and products using conventional 14C-labelling techniques. Furthermore, competing Pyr/PEP converting activities of PK and PEP carboxykinase coexist alongside PPDK, obscuring the metabolic effect of PPDK on the overall pool of these metabolites. Arabidopsis PPDK knockout lines have been isolated but these do not appear to differ markedly from the wild type when grown under optimal conditions (personal communication, K Parsley and JM Hibberd, Department of Plant Science, Cambridge University) and thus do not offer an insight into the low

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**Table 1.** Taxonomic distribution of InterPro Protein sequence entries for PPDK and PDRP/DUF 299 protein

<table>
<thead>
<tr>
<th>Taxonomic group</th>
<th>Database entries for PPDK</th>
<th>Database entries for PDRP and DUF 299 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archaea</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Eubacteria</td>
<td>832</td>
<td>1360</td>
</tr>
<tr>
<td>Eukarya</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plants</td>
<td>65</td>
<td>31</td>
</tr>
<tr>
<td>Diatoms</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Protists</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

*Both gram-positive and gram-negative species are represented in tally.*

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**Fig. 1.** Glycolytic ATP production for PPDK/PPi-dependent glycolysis versus conventional PK-based glycolysis. As illustrated, the yield of glycolytically derived ATP is boosted by the synergistic activities of PPDK and adenylate kinase (AK) versus conventional pyruvate kinase (PK) based glycolysis.
abundance PPDK functions. Progress in establishing a function for C3 PPDK have been made from studies focusing on regions of the plant where the enzyme is abundantly expressed. These point to a principal role for C3 PPDK as an ancillary glycolytic enzyme. An example is PPDKs role in developing rice and Zea mays (maize) seeds, where the enzyme is massively expressed in the cytoplasm of the endosperm (Kang et al., 2005; Chastain et al., 2006; Mechlin et al., 2007; Prioul et al., 2008; Hennen-Bierwagen et al., 2009). Its appearance in this part of the developing seed coincides with the onset of starch, protein, fatty acid, and amino acid biosynthesis. Each of these biosynthetic activities is dependent on glycolytically supplied carbon. PPDK, via its freely reversible catalysis, is proposed to balance the flow of this carbon into each respective biosynthetic activity depending on the maturation stage of the seed. In addition, PPDK may supply ATP to hypoxic regions of the developing endosperm (Chastain et al., 2006). In senescing leaves of Arabidopsis, cytosolic PPDK has been shown to be extensively up-regulated to facilitate remobilization of nitrogen into glutamine (Taylor et al., 2010). It is proposed that Pyr, originating from amino acid deamination, is converted by PPDK into PEP for subsequent glutamine biosynthesis. Notably, the proposed metabolic schemes for each of these functions require the enzyme to be competent in both forward and reverse catalysis. However, assuming a cytoplasmic pH of 6.8 and constitutively high levels of cytosolic pyrophosphate (PPI) (Stitt, 1990; ap Rees et al., 1991), the direction of catalysis is likely to be restricted to the cytoplasm (Chastain et al., 2008). This isoform differs from the C4-like plastid isoform by lacking a Pi-dependent, PPI-forming PPDK phosphotransferase activity. In monocots, only a single PDRP gene (i.e. plastid localized PDRP) has been observed in draft and published genomes. Bacterial genomic databases show that homologues of PDRP, referred to as domain of unknown function (DUF) 299 genes, are present in all PPDK-containing bacteria (Burnell, 2010) (Table 1). However, PDRP homologues are also present in bacterial species that lack PPDK (Burnell, 2010). In these cases, the DUF 299 proteins most probably function in the phospho-regulation of PEPS. A recent study demonstrated how the PDRP homologue gene in E. coli, which lacks PPDK, regulates the on/off activity of its PEPS via reversible phosphorylation of the PEPS active-site Thr (Burnell, 2010). In the annotated genomes of PPDK containing Archaea and protozoa, PDRP homologues are absent suggesting that PDRP first evolved in bacteria for the purpose of regulating the terminal steps of glycolysis via on/off regulation of PPDK and/or PEPS (Burnell, 2010).

**PPDK function in C4 plants**

In the C4 pathway, PPDK catalyses the regeneration of the primary CO2 acceptor PEP in the stroma of leaf-mesophyll cell chloroplasts. It is hugely abundant in C4 leaves, comprising up to 10% of the soluble protein fraction (Edwards et al., 1985; Chastain, 2011). It is maximally active as a homotetramer of ~95 kDa subunits and is inactive in the dimeric and monomeric forms. It requires Mg2+ for oligomerization and NH4 + as a cofactor for optimal catalysis. The C4 enzyme is highly homologous to C3 plant PPDK (Agarie et al., 1997). Residues and regions involved in catalysis are strictly conserved among C4 plant, C3 plant, and non-plant PPDKs (Fisslthaler et al., 1995). An important driver for its evolution as a C4 enzyme was the modification of the PPDK gene promoter to confer leaf mesophyll cell specific expression (Sheen, 1991).

**Regulation of PPDK in plants and bacteria by the PPDK regulatory protein and DUF 299 proteins**

In the C4 pathway, PPDK activity is strictly regulated in an up/down manner by the level of incident light. The PPDK regulatory protein (PDRP), a unique bifunctional enzyme, catalyses this light-dependent regulation by reversible phosphorylation of an active-site Thr residue (Thr456 in maize) (Fig. 2) (Burnell and Hatch, 1985; Roeske and Chollet, 1987; Burnell and Chastain, 2006; for a recent review, see Chastain, 2011). PDRP plays a similar role in the light-regulation of non-photosynthetic PPDK in chloroplasts of C3 plants (Chastain et al., 2002, 2008; Chastain and Chollet, 2003). Arabidopsis and other eudicots (e.g. Cleome species) encode a second PDRP gene, ArRP2, which is localized in the cytoplasm (Chastain et al., 2008). This isoform differs from the C4-like plastid isoform by lacking a Pi-dependent, PPI-forming PPDK phosphotransferase activity. In monocots, only a single PDRP gene (i.e. plastid localized PDRP) has been observed in draft and published genomes. Bacterial genomic databases show that homologues of PDRP, referred to as domain of unknown function (DUF) 299 genes, are present in all PPDK-containing bacteria (Burnell, 2010) (Table 1). However, PDRP homologues are also present in bacterial species that lack PPDK (Burnell, 2010). In these cases, the DUF 299 proteins most probably function in the phospho-regulation of PEPS. A recent study demonstrated how the PDRP homologue gene in E. coli, which lacks PPDK, regulates the on/off activity of its PEPS via reversible phosphorylation of the PEPS active-site Thr (Burnell, 2010). In the annotated genomes of PPDK containing Archaea and protozoa, PDRP homologues are absent suggesting that PDRP first evolved in bacteria for the purpose of regulating the terminal steps of glycolysis via on/off regulation of PPDK and/or PEPS (Burnell, 2010).

![Fig. 2. Reversible phosphorylation of PPDK by PDRP.](http://jxb.oxfordjournals.org/article_access/20130422/03085/03085.html)
Summary

Based on the functional evolutionary history of the enzyme, it is hypothesized that plants inherited a PPDK from a lower organism ancestor that was largely adapted for a role in glycolysis. The functional continuity of PPDK in plants with its glycolysis related role is evident in C₃ angiosperms where PPDK is principally seated as an ancillary glycolytic enzyme. As PPDK evolved into the C₄ pathway, it assumed a radically new function that possibly challenged the enzyme with new catalytic and regulatory requirements. In the present study, an attempt was made to uncover any changes the enzyme may have undergone as it evolved from its C₃-cytoplasmic role into its photosynthetic role in C₄ chloroplasts. Our approach consisted of contrasting key catalytic and regulatory properties of the C₃ PPDK isoform from Arabidopsis with the C₄ PPDK isoform from maize primarily in the context of the cytoplasmic compartment. In so doing, it was possible to demonstrate only minor differences between the PPDK from these representative C₃ and C₄ plants. It is therefore concluded that C₃ PPDK was inherently pre-adapted for its role in C₄ photosynthesis and this probably facilitated its repeated selection as the PEP-regenerating enzyme for the C₄ cycle.

Materials and methods

Expression and purification of recombinant enzyme

All plasmids used for generating recombinant enzymes have been described previously (Burnell and Chastain, 2006; Chastain et al., 2008). Maize C₄ PPDK, Arabidopsis PPDK, and Arabidopsis PDRPs were expressed from a pET 28a plasmid (EMD4Biosciences). Recombinant maize PDRP was expressed from the E. coli protein expression vector pROExC (Invitrogen). Host E. coli BL21 DE3 cells were transformed with the respective plasmid and used for large-scale culture as described before (Chastain et al., 2008). Subsequent extraction and affinity purification of recombinant enzyme was accomplished using a previously described procedure with the following modifications. Cell pellets (harvested from one-litre cultures) were lysed using 20 ml of the 1× BugBuster™+1× Lysonase™ enzyme mix (EMD4Biosciences) in buffer A (50 mM KPi, pH 8.0, 25 mM KCl, 2.5 mM MgSO₄, 5 mM 2-mercaptoethanol, 1× protease inhibitor cocktail VII™, RPI Corporation) and clarified of insoluble matter by high-speed centrifugation (20 min, 74,000 g). The clarified lysate was combined with 1 g of Prepease™ high-yield Ni²⁺-silaica beads (Affymetrix) for batch-binding of 6 His-tagged recombinant proteins at 4 °C for 4 h. The batch-bound slurry was subsequently decanted into a 1.5 cm diameter Bio-Rad Econo column for washing the beads with 20 ml of buffer A + 5 mM imidazole. Recombinant 6 His-bound protein was eluted from the beads with buffer A + 300 mM imidazole (pH 8.0). The protein eluate was brought to 70% ammonium sulphate saturation and placed on wet ice overnight to maximize protein precipitation. The precipitate was brought to a final 20 mM 2-mercaptoethanol, dispensed into 1 ml aliquots for subsequent centrifugation (20 min, 32,000 g). The well-drained protein pellets were sealed under N₂ and stored at –80 °C until use. For recombinant PDRP, no further purification was used. Coomassie-stained gels of recombinant PDRP indicated an approximate 60% purity for each preparation. Recombinant PPDK was further purified using an FPLC-based anion-exchange chromatography procedure (Chastain et al., 1996). PPDK purified in this manner was >95% as indicated by Coomassie-stained gels.

PPDK enzyme assays

PPDK activity was assayed using a previously described spectrophotometric-based procedure and adapted for use with a 96-well plate reader format (Jenkins and Hatch, 1985; Ashton et al., 1990). For assays in the Pyr forming direction, the assay buffer consisted of 25 mM HEPES (pH 7.0 or pH 8.0), 6 mM MgSO₄, 25 mM NH₄Cl, 5 mM DTT, 1 mM PEP, 1 mM PPI, 0.5 mM AMP, 0.25 mM NADH, and 2 U ml⁻¹ lactic acid dehydrogenase (LDH). Assays were initiated by the addition of desalted recombinant PPDK to a final concentration of 25 ng ml⁻¹ at 30 °C. Substrate Kₘ for Pyr and PEP were calculated from double-reciprocal plots of the respective PEP- or Pyr-forming PPDK assays.

PDRP assays

PDRP phosphorylation activity was assayed using a previously established procedure (Chastain et al., 2005) but modified for use at pH 7.0. Assays were initiated by the addition of partially purified PDRP to a final concentration of 0.26 mg ml⁻¹ into a reaction mixture consisting of 0.35 mg ml⁻¹ of affinity-purified recombinant Arabidopsis or maize PPDK, each as a fully activated, non-phospho form, in 50 mM HEPES, pH 7.0, 10 mM MgCl₂, 5mM DTT, 1 mg ml⁻¹ BSA, 1 mM ADP, 1 mM PEP, 0.2 mM ATP, 20 mM NH₄Cl. The reaction mixture was incubated at 30 °C. At specified time intervals, 10 µl aliquots of the reaction were removed for assay of PPDK activity in the PEP to Pyr direction (pH 7.0) as described above. When other nucleotide phosphates were used in the assay in place of ADP, these were present at 1 mM. For the immuno-based PDRP assays, aliquots of the assay were combined with an equal volume of SDS-PAGE sample buffer and subjected to Western blot analysis for phospho-PPDK as previously described (Chastain et al., 2000, 2008). PDRP dephosphorylation activity was assayed using an immuno-based procedure as previously described (Chastain et al., 2008).

Results

Influence of pH on C₃ versus C₄ PPDK specific activities

Nearly all previously published analyses of in vitro plant PPDK properties have been carried out at pH >8.0 for purposes of simulating the behaviour of the C₄ enzyme in the stroma of illuminated chloroplasts. The choice was made to contrast the biochemical properties of C₃ and C₄ PPDKs primarily at a pH of 7.0, the approximate pH of the cytoplasmic compartment. This allowed us to gauge the differences in the properties of the enzyme not with respect to C₄ photosynthesis, but to the enzyme’s competency for glycolytic function. The rationale for this approach was to reveal changes the enzyme may have acquired since it evolved from its ancestral C₃ function. Using recombinant enzyme forms for each species, specific activities for both catalytic directions were obtained (Fig. 3). In agreement with previous studies (Jenkins and Hatch, 1985), these show how the Pyr-forming reaction is strongly favoured at pH 7.0, with catalysis in the PEP-forming direction only 13% for C₃ and C₄ PPDKs primarily at a pH of 7.0. The approximate pH of the cytoplasmic compartment. This allowed us to gauge the differences in the properties of the enzyme not with respect to C₄ photosynthesis, but to the enzyme’s competency for glycolytic function. The rationale for this approach was to reveal changes the enzyme may have acquired since it evolved from its ancestral C₃ function. Using recombinant enzyme forms for each species, specific activities for both catalytic directions were obtained (Fig. 3). In agreement with previous studies (Jenkins and Hatch, 1985), these show how the Pyr-forming reaction is strongly favoured at pH 7.0, with catalysis in the PEP-forming direction only 13% for C₃ PPDK and 8% for C₄ PPDK of the Pyr-forming direction. Interestingly, specific activities of the C₃ and C₄ PPDK in the Pyr-forming reaction were highly similar. Our earlier comparison of these enzymes at pH 8.3 in the PEP-forming direction also showed a nearly equivalent specific activity (4.2 versus 4.4 µmol min⁻¹ mg⁻¹ PPDK for C₃ and C₄ isoforms, respectively) (Chastain et al., 2008).
C₃ and C₄ PPDK substrate affinities for Pyr and PEP

The sole function of PPDK in the C₄-cycle is to catalyse regeneration of the primary CO₂ acceptor PEP from Pyr. Alternatively, it is proposed that C₃ PPDK synthesizes both Pyr from PEP and PEP from Pyr (Moons et al., 1998; Kang et al., 2005; Chastain et al., 2006; Mechin et al., 2007; Hennen-Bierwagen et al., 2009, Taylor et al., 2010). It was therefore surmised that C₃ and C₄ PPDK would have different affinities related to the respective roles of the enzymes. This was examined by comparing the Kₘ for Pyr and PEP at pH 7.0 (Table 2). In agreement with this conjecture, Arabidopsis C₃ PPDK was found to have a 10-fold lower Kₘ for Pyr (17 μM) compared with the maize C₄ enzyme (178 μM). By contrast, the maize enzyme was found to have a significantly higher affinity for PEP (Kₘ=194 μM) than the C₃ enzyme (Kₘ=294 μM).

Effect of PPi on Pyr to PEP catalysis at pH 7.0

PPDK is proposed to catalyse PEP formation in the cytoplasm of C₃ plants (Moons et al., 1998; Kang et al., 2005; Huang et al., 2008; Taylor et al., 2010). At pH 7.0, this reaction should be highly antagonized by the high cytosolic levels of PPi present in plant cells (Dennis and Blakeley, 2000). To examine the potential effect of intracellular PPi on this reaction, initial activities of C₃- and C₄-PPDK were determined for the Pyr to PEP reaction in the presence of 0.1 mM and 1 mM PPi, at pH 7.0. In addition, two levels of substrate Pyr were used (0.1 mM and 1.0 mM) with the lower concentration representing a physiological concentration of cytosolic Pyr (Roeske and Chollet, 1989). When the reaction is assayed in the presence of 1 mM PPi, not only did we fail to observe a predicted inhibition but, on the contrary, a marginal increase was observed compared with the no PPi controls (Fig. 4). At lower levels of Pyr (0.1 mM), the reaction rates declined to 65% and 45% of the rates of saturating Pyr levels for C₃ and C₄ PPDK, respectively. The greater Pyr-dependent decline for the maize C₄ enzyme is consistent with this enzymes higher Kₘ for Pyr (Table 2). Notably, Arabidopsis C₃ PPDK exhibited higher specific activities for the Pyr to PEP reaction in the presence of 0.1 mM and 1 mM PPi (pH 7.0) than maize C₄ PPDK.

Regulation of C₃- and C₄-PPDK by their respective PDRP isoforms at pH 7.0

The Arabidopsis cytosolic PDRP isoform (AtrRP2) is functionally different from its C₄-like chloroplast counterpart (AtrRP1) in that it lacks Pi-dependent PPDK-Thr-P protein phosphotransferase activity. As a monofunctional PDRP, it is capable of regulatory phosphorylation (inactivation) of cytoplasmic PPDK but is unable to reactivate the enzyme by reversible dephosphorylation of the regulatory PThr residue. A plausible reason that may account for the absence of this function is that its properties were originally characterized at a pH more typical of illuminated chloroplast stroma (e.g. pH 8.3) versus at the lower pH of the cytoplasmic compartment (e.g. pH 6.8). This was not the case when AtrRP2 was assayed at a cytosolic-equivalent pH of 6.8, as no dephosphorylation of substrate phospho-PPDK could be detected (Fig. 5).

The phosphoryl-donor nucleotide specificity of AtrRP2 was also examined at pH 6.8 (Fig. 6). This shows that this

Table 2. PPDK substrate affinity constants for Pyr and PEP determined at pH 7.0

<table>
<thead>
<tr>
<th>Km Pyr</th>
<th>Km PEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₃ PPDK</td>
<td>C₄ PPDK</td>
</tr>
<tr>
<td>17 μM</td>
<td>178 μM</td>
</tr>
</tbody>
</table>

Fig. 3. Specific activities of Arabidopsis C₃ and maize C₄ PPDK at pH 7.0 in the PEP- and Pyr-forming directions. Shown are the means ±SE (n=5).

Fig. 4. Influence of exogenous PPi on initial rates of C₃ and C₄ PPDK activity in the PEP-forming direction at pH 7.0. PPDK was assayed as before in the presence of 1 mM PPi at two concentrations of substrate Pyr (Low Pyr=0.1 mM, High Pyr=1.0 mM). Control assays excluded PPi and included Pyr at 1 mM). Values shown are the means ±SE (n=5).
Cytosolic isoform shares similar substrate specificities with maize C₄ PDRP: ADP as the high specificity substrate and GDP as a lower affinity substrate. Furthermore, in this spectrophotometric-based PPDK-phosphorylation assay, no PDRP phosphorylating activity could be found when UDP, an abundant nucleotide in the cytosol of sucrose synthesizing tissues (Dennis and Blakeley, 2000), was used as a substrate for this reaction. A more sensitive immuno-based assay was used to compare nucleotide specificity at pH 7.0 for AtRP2, maize C₄ PDRP and AtRP1, the C₄-like chloroplast isofrom of Arabidopsis (Chastain et al., 2008) (Fig. 7). This assay indicated a similar trend in nucleotide specificity for all three PDRPs, with ADP showing the highest specificity followed by a lesser affinity for GDP. A faint signal for phosphorylated PPDK is evident for the GTP substrate lane but this is probably due to traces of GDP known to contaminate commercial preparations of GTP. Interestingly, a low amount of phosphorylated PPDK was detected when UTP and UDP were used as substrates for AtRP2, but not for AtRP1 and maize PDRP. However, at this low level of PPDK phosphorylation, it is unlikely that these nucleotides are true in vivo substrates for AtRP2. The AtRP2 affinity for its most specific nucleotide substrate, ADP (Kₘ=26 μM, pH 6.8) was also measured. The high affinity for ADP exhibited by this PDRP isoform is similar to maize PDRP measured at pH 8.0 (~50 μM) (Burnell and Hatch, 1985; Roeske and Chollet, 1987).

**Fig. 5.** Immuno-based phospho-PPDK dephosphorylation assays conducted at pH 8.3 and pH 6.8. Shown are representative denaturing Western blots of 20 μl terminated assay reaction aliquots probed with anti-PPDK-ThrP as described in the Materials and methods. Assays were initiated with the addition of 2.5 mM KPi and AtRP2 or AtRP1 (pH 8.3 only) and quenched at 30 min with SDS-PAGE sample buffer. Control lanes correspond to a 15 μl aliquot of substrate phospho-PPDK used in the dephosphorylation reaction that was generated by the first-stage AtRP1 or AtRP2 catalysed ADP-dependent phosphorylation of PPDK as described in the Materials and methods. Arrowheads indicate the position of the bands corresponding to the ~94 kDa Arabidopsis PPDK monomer as estimated by molecular mass standards on the same blot.

**Fig. 6.** Nucleotide specificity of maize PDRP and Arabidopsis AtRP2 for in vitro phosphorylation/inactivation of PPDK. Shown are the results of a spectrophotometric-based PDRP catalysed PPDK-inactivation assay and plotted as a percentage of PPDK inactivation (versus controls) after 15 min incubation with PDRP. Maize C₄- and Arabidopsis C₃-PPDK were used as substrates for the respective AtRP2 and C₄ PDRP catalysed inactivation assays. Values shown are the mean of three independent determinations with associated SEs ≤5%.

**Fig. 7.** Immuno-based PPDK phosphorylation assays of PDRP with alternate nucleotide phosphoryl-donor substrates. The upper panels are representative Western blots of terminated 15 μl reaction aliquots probed with anti-PPDK-ThrP with the lower panels representing the same blots stripped and reprobed with anti-PPDK polyclonal antibodies as described in the Materials and methods. Assays were initiated with the addition of the PDRP as designated (Mz PDRP, maize C₄ PDRP; Arabidopsis C₄-like PDRP, AtRP1; Arabidopsis cytosolic PDRP, AtRP2) and terminated after 20 min with SDS-PAGE sample buffer. All nucleotide phosphates utilized in the assays as noted above each lane were used at a concentration of 1 mM. Control assays were the same as for plus ADP-reactions but without the addition of PDRP. Arrowheads indicate position of bands corresponding to the ~94 kDa Arabidopsis PPDK monomer as estimated by molecular mass standards on the same blot.
Discussion

How PPDK originated in microorganisms to catalyse specific glycolytic reactions has been reviewed here. This same function is evident in C3 plants where PPDK is principally an ancillary enzyme of the glycolytic pathway. It was therefore hypothesized that, prior to the recent evolution of the C4 pathway, plants possessed a PPDK that was functionally adapted for glycolytic metabolism in the cytoplasmic compartment. It was assumed that functional evolution of a glycolytic PPDK into a C4 pathway role would translate into modifications of its catalytic and regulatory properties. However, despite the radically different metabolic roles that each respective isoform fulfils, our analyses of C3- and C4-PPDK isoforms revealed very few differences in these properties. The most significant difference was the 10-fold higher affinity for Pyr exhibited by the C3 isoform. This observation is highly relevant with regard to how C3-PPDK is proposed to carry out its cytoplasmic-

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Concluding remarks

Our investigation of the in vitro properties of recombinantly produced Arabidopsis C3- and maize C4-PPDK revealed few function-specific differences. It is therefore concluded that the evolutionary transition of C3 PPDK into the C4 pathway involved only minor changes in enzyme properties per se. This may account for the preferential selection of PPDK into the C4 pathway as opposed to other PEP-generating enzymes (e.g. PEP carboxykinase). The most significant adaptation for the enzyme to be utilized in C4 photosynthesis had already occurred well before the emergence of the pathway in modern angiosperms. The moment PPDK became a resident enzyme of the chloroplast stroma, its inherent pH sensitivity would have allowed the enzyme to respond to light induced alkalization of this compartment in a manner highly favourable for PEP-synthesis. Further facilitating the immediate functional C4-transition of PPDK is the high amount of stromal pyrophosphatase and adenylate kinase found in chloroplasts of all plants. Finally, light responsive regulation of chloroplast PPDK by PDRP was also long established in C3 plant chloroplasts (Chastain et al., 2002). The remaining enigma for understanding the C4 transition of PPDK is defining its role
in C_3 chloroplasts, where it is in very low abundance. Whatever this function, the co-localization of PPDK along with its companion regulatory protein into chloroplasts had been established by the time the unicellular green algal ancestors of plants emerged (Burnell and Chastain, 2006).

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