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REVIEW

Plant pyruvate kinase

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Abstract

Pyruvate kinase is an important enzyme of glycolytic pathway that also functions in providing carbon skeleton for fatty acid biosynthesis. It has been purified to near homogeneity from *Ricinus communis*, *Selenastrum minutum*, *Cynodon dactylon*, *Brassica campestris* and *B. napus*, and characterised. Partially purified preparations are reported from several other sources. A phosphoenolpyruvate (PEP) phosphatase accompanies pyruvate kinase. In plants, two isozymes of pyruvate kinase are reported, namely cytosolic and plastidic. Isoforms of cytosolic pyruvate kinase have also been reported from spinach. In most cases pyruvate kinase is a tetrameric protein and the molecular mass lies between 200 to 250 kDa. The pH optimum is in the range of 6.2 to 7.5. It requires both Mg^{2+} and K^+ for maximum activity. ATP, citrate, and oxalate inhibit pyruvate kinase in most cases. A sequential compulsory ordered mechanism of binding of substrates to the enzyme has been proposed.

Additional key words: isozymes, kinetics, mechanism, regulation.

Introduction

Pyruvate kinase (PK) (ATP: pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) is an important regulatory enzyme of glycolytic pathway. In earlier literature, this enzyme has also been referred to as pyruvic phosphoferase, pyruvate phosphokinase and phosphoenolpyruvate transphosphorylase (Bücher and Pfleiderer 1955, Boyer 1962). The enzyme is widely distributed in different life forms such as microbes (bacteria, yeast, molds), tissues of animals and plants. The enzyme has been isolated and characterized from various microbial, animal and plant sources. However, available information on PK from plants is not sufficient yet.

The reaction catalyzed by PK is the transfer of phosphoryl group from phosphoenolpyruvate (PEP) to ADP to form pyruvate and ATP in presence of Mg^{2+} and K^+. The reaction is practically irreversible because of the large energy drop in forward direction (ΔG -61.9 kJ mol^{-1}). Unlike rabbit muscle PK, there are no reports of fluorokinase, hydroxylamine kinase and glycolate kinase activity of the plant enzyme.

$$PEP + ADP^{3-} \xrightarrow[PK]{Mg^{2+}, K^+} pyruvate + ATP^{4-}$$

In plants, a phosphatase activity was detected during pyruvate kinase activity assay (McCollum *et al.* 1958, Evans 1963, Duggleby and Dennis 1973a, Tomlinson and Turner 1973). This phosphatase hydrolyzed PEP to pyruvate and Pi and was called phosphoenolpyruvate-phosphatase (PEP-phosphatase) (McCollum *et al.* 1960). Subsequently, this enzyme was accepted by Enzyme Commission in 1992 (phosphoenolpyruvate phospho-

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Abbreviations: ADP-β-S - adenosine 5'-O-(2-thiodiphosphate); BSA - bovine serum albumin; DTE - dithioerythreitol; DTT - dithiothreitol; EDTA - ethylenediaminetetracetic acid; GS/GOGAT - glutamine synthetase/glutamine-2-oxoglutarate transaminase; ORF - open reading frame; PEP - phosphoenolpyruvate; PK_c - cytosolic pyruvate kinase; PK_p - plastidic pyruvate kinase; TCA cycle - tricarboxylic acid cycle.

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hydrolase, EC 3.1.3.60) and has been recently reviewed (Ambasht and Kayastha 1999). This enzyme is unique to plants (McCollum *et al.* 1958, Duff *et al.* 1989a,b, Malhotra and Kayastha 1990). A similar interference has also been observed by PEP carboxylase in both C_3 and C_4 plants (Peterson and Evans 1978, Zervoudakis *et al.* 1997). Pyruvate kinase has been reviewed earlier (Boyer 1962, Kayne 1973). The present review on pyruvate kinase focuses on the plants.

Activity assay

PK activity can be assayed by either monitoring time dependent loss of PEP at 230 nm (Pon and Bondar 1967) or formation of products, pyruvate and ATP. The pyruvate formed can be assayed colorimetrically by its reaction with 2,4-dinitrophenyl hydrazine followed by addition of alkali (Kachmar and Boyer 1953). PK activity is usually assayed by coupling the reaction catalysed by it with that of lactate dehydrogenase (LDH; Bücher and Pfleiderer 1955). The latter is kept high so that the rate of coupled reaction is governed by the PK catalysed reaction. The rate of disappearance of NADH is monitored spectrophotometrically at 340 nm in this assay procedure.

In plants, reaction of PEP with ADP in presence of Mg^{2+} and K^+ represents a sum of PK and PEP-phosphatase activities. The latter is assayed separately by omitting ADP from the assay mixture and this is subtracted from the total value (with ADP) to obtain the PK activity (Plaxton 1988, Ambasht *et al.* 1996).

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

The ATP formed in PK catalysed reaction can be assayed in yet another coupled reaction with hexokinase and glucose-6-phosphate dehydrogenase (Ireland *et al.* 1980). This activity assay procedure finds no interference of PEP-phosphatase and PEP carboxylase, since ATP is assayed. In this assay procedure, however excess of two coupling enzymes are required.

Purification

Attempts were made to isolate PK from plants since early 1950s. Only partially purified preparations of PK were obtained (contaminated with PEP-phosphatase) from lower and higher plants (Tomlinson and Turner 1973, Duggleby and Dennis 1973b, Vaccaro and Zeldin 1974, Besford and Maw 1975, Nakayama *et al.* 1976, Peterson and Evans 1978, Ireland *et al.* 1979, Baysdorfer and Bassham 1984, Gupta and Singh 1989). Earlier attempts to separate the two activities were unsuccessful (Spenser 1952, Duggleby and Dennis 1973b). Experiments were carried out to explore conditions for minimum extraction of PEP-phosphatase. Tris-molybdate buffer was used in some cases because the presence of molybdate inhibited PEP-phosphatase to a greater extent than PK (Spenser 1952, Evans 1963, Wildes and Pitman 1975). Ratio of PK:PEP-phosphatase was examined in a number of plant sources (Duggleby and Dennis 1973a). Ambasht *et al.* (1996) studied contents of PK and PEP-phosphatase during germination in mung beans. PK activity attained a maximum at 24 h of germination. The PK:PEP-phosphatase ratio is maximum at 12 h of germination and therefore 12 h germinated mung beans were used for isolation of PK to minimize interference of PEP-phosphatase. Subsequently, PK free of PEP-phosphatase (Lin *et al.* 1989a,b, Ambasht *et al.* 1996, 1997) and PEP-phosphatase free of PK were isolated and characterized (Duff *et al.* 1989a, Malhotra and Kayastha 1990).

Different additives like ethylene diaminetetracetic acid (EDTA), bovine serum albumin (BSA), dithiotreitol (DTT), dithioerythreitol (DTE) and glycerol were used to stabilize PK during course of isolation (Plaxton 1988). Finally, with use of modern protein purification technologies and fast flow ion exchange and size exclusion resins, PK was isolated to near homogeneity from castor bean (*Ricinus communis*) (Plaxton 1988, Plaxton *et al.* 1990, Hu and Plaxton 1996), green alga *Selenastrum minutum* (Knowles *et al.* 1989), *Cynodon dactylon* (Zervoudakis *et al.* 1997), *Brassica campestris* (Singh *et al.* 1998, 2000) and *Brassica napus* (Smith *et al.* 2000).

Isozymes

In late seventies, two isozymes of PK namely cytosolic and plastidic were reported from developing castor bean endosperm (DeLuca and Dennis 1978, Ireland *et al.* 1979). Subsequently, further reports on cytosolic (Baysdorfer and Bassham 1984, Plaxton 1988, Gupta and Singh 1989, Lin *et al.* 1989a, Podestá and Plaxton 1991,

Sangwan *et al.* 1992, Ambasht *et al.* 1996, Hu and Plaxton 1996, Singh *et al.* 2000, Smith *et al.* 2000) and plastidic (Lin *et al.* 1989a, Knowles *et al.* 1989, Plaxton 1989, Plaxton *et al.* 1990, Sangwan *et al.* 1992, Singh *et al.* 1998) isozymes of PK have come and are called PK_c and PK_p respectively. PK_1 and PK_2 were also used to denote the two isozymes (Ireland *et al.* 1979, Lin *et al.* 1989a). PK_1 represented cytosolic isozyme in castor bean endosperm (Ireland *et al.* 1979) but plastidic isozyme in *S. minutum* (Lin *et al.* 1989a). PK_I and PK_{II} in spinach represented two isoforms of the cytosolic PK (Baysdorfer and Bassham 1984).

PK were adsorbed when chromatographed on anion exchanger column. In some cases, the enzyme was eluted as a single peak (Duggleby and Dennis 1973a, Vaccaro and Zeldin 1974, Nakayama *et al.* 1976, Gupta and Singh 1989, Ambasht *et al.* 1996, Zervoudakis *et al.* 1997). Two peaks of PK activity were also reported (DeLuca and Dennis 1978, Ireland *et al.* 1979, Lin *et al.* 1989a, Plaxton 1989), the first peak represented PK_c and the latter PK_p. The PK_p had a higher negative charge than the corresponding PK_c (DeLuca and Dennis 1978). In contrast, PK_p from *B. campestris* was eluted before PK_c (Singh *et al.* 1998, 2000).

Immunological characterization of the two isozymes has been extensively studied (Lin *et al.* 1989a, Plaxton 1989). PK_2 from *S. minutum* only cross-reacted with PK_c antibodies (Lin *et al.* 1989a). Similarly PK_p from developing endosperm and leaf from castor oil plant did not cross-react with PK_c antibodies (Plaxton 1989). These results suggest that PK_p and PK_c are distinct proteins (Lin *et al.* 1989a, Plaxton 1989). This has been further confirmed by immunoremoval experiments (Plaxton *et al.* 1990).

A variety of cDNA clones have been isolated for PK_c and PK_p (Blakeley *et al.* 1990, 1991, 1995, Ma *et al.* 1993). The two clones of PK_p are $PK_p\alpha$; and $PK_p\beta$ (Blakeley *et al.* 1991) and PK_pA and PK_pG (Blakeley *et al.* 1995). Southern analysis of castor genomic DNA reveals the presence of only one gene for PK_p (Blakeley *et al.* 1991), whereas similar studies on potato indicated the existence of at least six genes for PK_c (Cole *et al.* 1992). The expression of PK_p and PK_c in various tissues of tobacco has been studied, which suggests that tissue specific and developmental expression of these isozymes in tobacco may be controlled by independent transcriptional and post-transcriptional mechanisms (McHugh *et al.* 1995). Further studies have suggested that the plant PK gene and animal or fungal PK genes have descended from divergent isozymes which existed in the proginator of present day eukaryotes and prokaryotes (Hattori *et al.* 1995).

The amino acid sequence of PK from potato and castor endosperm has been derived from nucleotide sequence of cDNA (Blakeley *et al.* 1990). The potato clone is 2000 bp long, with an open reading frame (ORF) of 1530 bp corresponding to 510 amino acids. The castor endosperm clone is slightly longer in comparison to potato (2134 bp), however, it has an ORF of 1164 bp corresponding to 388 amino acids. From the alignment of amino acid sequence, it has been found that 163 amino acid residues are conserved with respect to PK from potato (Blakeley *et al.* 1990).

Stability

In most cases, the finally obtained enzyme was stored at sub-zero temperature and has been found to be very stable (Peterson and Evans 1978, Plaxton 1988, 1989, Lin *et al.* 1989a, Plaxton *et al.* 1990, Hu and Plaxton 1996, Ambasht *et al.* 1996), however spinach leaf PK isoforms were unstable at cold (Baysdorfer and Bassham 1984). PK_c is heat stable, whereas PK_p is heat labile (Ireland *et al.* 1980, Lin *et al.* 1989a). No loss of PK_c activity was observed during heat treatment step (60 °C for 5 min) in the procedure of purification of PK from *R. communis* (Plaxton 1988). PK_p is so unstable, that it can be detected only in presence of ethylene glycol and DTT during organelle breakage (Ireland *et al.* 1979). It can be stabilized only in the presence of ADP, Mg^{2+}, glycerol and DTT, whereas PK_c is stable even in the absence of these additives (Ireland *et al.* 1980). Thus, the stability of PK depends upon storage conditions (temperature and use of additives).

Molecular properties

The molecular mass of PK_c and PK_p lie in the range of 200 to 250 kDa. In *S. minutum*, however PK_c has very high molecular mass *i.e.* 590 kDa (Lin *et al.* 1989a).

PK_c from endosperm of germinating seeds of castor bean and leaves of castor oil plant are reported to be heterotetramer composed of two types of subunits of molecular masses 56 and 57 kDa (Plaxton 1989, Hu and Plaxton 1996). Both polypeptides are present in crude extract prepared under denaturing conditions indicating that 56 kDa subunit has not arisen as a result of proteolysis of 57 kDa subunit during extraction and purification (Plaxton 1989). PK_c from other tissues of

R. communis, however are reported to be homotetramer composed of 57 kDa subunit (Plaxton 1989, Podestá and Plaxton 1994). PK_c from germinating seeds of *B. napus* appears to be composed of a single type of subunit of 56 kDa whereas, the enzyme from cultured embryo contains both 57 and 56 kDa subunits in equal ratio (Sangwan *et al.* 1992). PK_c from green alga *S. minutum* appears to be homodecameric enzyme (Lin *et al.* 1989a).

PK_p in the developing endosperm of the castor oil seeds has a native molecular mass of 305 kDa and appears to be a heterohexamer of four 57.5 and two 44 kDa subunits (Plaxton *et al.* 1990). These subunits are formed by proteolytic cleavage of 63.5 and 54 kDa precursor polypeptides (Plaxton 1991). Earlier, native molecular mass of PK_p from a partially purified preparation was reported to be 290 kDa (Plaxton 1989). PK_p from cultured embryo of *B. napus* consists of 64 and 58 kDa subunits in 1:2 ratio (Sangwan *et al.* 1992). PK_p from green alga *S. minutum* was earlier thought to be homotetrameric enzyme (Lin *et al.* 1989a), however, subsequently, it was reported to be a monomer with a molecular mass 235 kDa and subunit of 210 kDa (Knowles *et al.* 1989).

The amino acid composition of 56 and 57 kDa subunits from *R. communis* has been determined. The 56 kDa subunit has a higher Asp, Glu and Gly content and lower Cys, Tyr, Phe, His and Lys compared to 57 kDa subunit. Both 57 and 56 kDa subunits are hydrolyzed to 51 and 50 kDa polypeptides, respectively in the presence of trypsin, *i.e.* a reduction of 6 kDa in the apparent molecular mass in each subunit takes place. These results suggest that both subunit types have similar site exposed on the enzyme surface, which is accessible to hydrolysis in presence of trypsin (Plaxton 1989).

Steady state kinetic properties

A broad pH optimum has been observed for both PK (6.2 to 7.5) (Tomlinson and Turner 1973, Nakayama *et al.* 1976, Ireland *et al.* 1979, 1980, Podestá and Plaxton 1991, Hu and Plaxton 1996, Zervoudakis *et al.* 1997, Singh *et al.* 2000, Smith *et al.* 2000) and PK_p (6.0 to 7.8) (Lin *et al.* 1989b, Singh *et al.* 1998). Sharp pH optimum has also been reported in both isozymes by Ireland *et al.* 1980, Ambasht *et al.* 1997. PK_p from *R. communis* has slightly higher pH optimum than corresponding PK_c (Ireland *et al.* 1979, 1980), however in *S. minutum* PK_p has a lower pH optimum than corresponding PK_c (Lin *et al.* 1989b) (Table 1).

Normally experiments related to enzyme kinetics are carried out at optimum pH. In some cases, experiments have also been carried out at a pH of highest specificity constants (V_{max}/K_m) for PEP and ADP (Podestá and Plaxton 1991, Hu and Plaxton 1996).

In most cases Michaelis-Menten kinetics has been observed (Ireland *et al.* 1980, Gupta and Singh 1989, Lin *et al.* 1989b, Hu and Plaxton 1996, Zervoudakis *et al.* 1997, Singh *et al.* 1998, 2000, Smith *et al.* 2000). Lineweaver-Burk plot has been extensively used to determine K_m and V_{max} of PK, however, there are no reports of use of Cornish-Bowden plot. Ambasht *et al.* (1996) used secondary double reciprocal plots in addition Lineweaver-Burk plot to determine K_m and V_{max}. In Lineweaver-Burk plot (v^{-1} versus $[ADP]^{-1}$), straight lines cut at a common point above abscissa and left of ordinate (Duggleby and Dennis 1973b, Ireland *et al.* 1980, Gupta and Singh 1989, Podestá and Plaxton 1992, Ambasht *et al.* 1996, Singh *et al.* 1998). Ambasht *et al.* (1996) observed other kinetic patterns also at pH 6.5 and 7.5. At pH 6.5 in v^{-1} versus ADP^{-1} and v^{-1} versus PEP^{-1} plots intersected at a common point on the abscissa suggesting that the K_m values of PEP and ADP are independent of the second reactant. At pH 7.5 in v^{-1} versus ADP^{-1} plot two kinds of patterns were recorded on the basis of PEP concentration. At low [PEP] the plots intersected at a common point on the abscissa similar to the results obtained at pH 6.5. At high [PEP], a set of parallel lines was obtained suggesting that there is an increase in apparent K_m of ADP with increase in [PEP]. In the v^{-1} versus $[PEP]^{-1}$ plot at several-fixed [ADP] the plots are not linear at any ADP concentration. At all ADP concentrations, the plots consist of two linear portions of different slopes, the direction of slope change (concave upward) suggests that K_m of PEP decreases at higher PEP concentrations. A positive cooperativity has been observed in the binding of PEP to the PK.

It is noteworthy that in most cases, the K_m of PEP is somewhat smaller than that of ADP (Tomlinson and Turner 1973, Ireland *et al.* 1980, Baysdorfer and Bassham 1984, Ambasht *et al.* 1996, Zervoudakis *et al.* 1997). Apparent K_m for PEP and ADP were about 2-fold and 4-fold lower, respectively for PK_c as compared to PK_p (Lin *et al.* 1989b). Both isozymes have same K_m for PEP but PK_p has 10-fold higher K_m for ADP than PK_c (Ireland *et al.* 1980). Hyperbolic saturation kinetics is observed when the [ADP] was kept 1 mM in PK_c from endosperm of castor oil seeds, however, a mixed type of inhibition is observed at ADP concentration higher than 1 mM (Podestá and Plaxton 1991). Glycerol increased the $s_{0.5}$ (ADP) 2- to 3-fold and changed the pattern of nucleotide binding from hyperbolic to sigmoidal (Podestá and Plaxton 1991).

Table 1. Kinetic and regulatory properties of PK from different plant sources.

Source	Tissue / Organ	Isozyme	pH	K_m [mM]		pH	Effectors		Reference
				PEP	ADP	optimum	positive	negative	
P. sativum	seed	PK	7.0	0.025	0.017	7.0	-	ATP, citrate	Tomlinson and Turner 1973
D. carrota	root	PK	7.3	0.019	0.055	7.3	-	ATP, citrate	Tomlinson and Turner 1973
Gossypium sp	seed	PK	7.5	ND	ND	6.7	-		Duggleby and Dennis 1973a
Gossypium sp.	seed	PK	7.5				AMP	ATP, citrate	Duggleby and Dennis 1973b
S.oleracea	leaf	PK_I	7.1	0.038	0.101	5.5 - 7.5	Asp, AMP	oxalate, citrate, ATP, Glu, Trp	Baysdorfer and Bassham 1984
		PK_{II}	7.1	0.045	0.114	5.5 - 7.5	Asp, AMP	oxalate, ATP, Glu,Trp	Baysdorfer and Bassham 1984
S. minutum	cells	PK_c	7.0	0.090	0.050	7.0	Glc-1P, Asp, DHAP, Glc-6P	Fru-1,6P, ATP, Gly, Glu, citrate, Pi, oxalate	Lin *et al.* 1989b
		PK_p	6.5	0.180	0.200	6.5	DHAP	Glc-6P, citrate, Asp, Pi, Fru-1, 6P, malate, isocitrate, oxalate, ATP	Lin *et al.* 1989b
C. arietinum	pod wall	PK_c	6.8	0.100	0.170	6.8	Gln, Ala, AMP, UDP	citrate, Fru-1,6P, oxaloacetate, Glu, ATP, oxalate	Gupta and Singh 1989
R. communis	developing endosperm	PK_c	7.9	0.050	0.030	6.8-7.2	-	ATP, AMP, citrate, fumarate, Phe, Fru-1,6P	Ireland *et al.* 1980
		PK_p	7.9	0.050	0.300	8.0	-	fumerate, Phe, citrate, Fru-1,6P, ATP	Ireland *et al.* 1980
	germinating endosperm	PK_c	6.5	0.152	0.078	6.5	-	oxalate, ATP, AMP, citrate, isocitrate, Fru-1,6P	Podestá and Plaxton 1991
		PK_c	7.2	0.052	0.071	6.5	-	ATP, citrate, 3-PGA, AMP, malate, 2-oxogluterate, Fru-1,6P, isocitrate	Podestá and Plaxton 1991
	leaves	PK_c	6.5	0.089	0.087	6.5	Asp	ATP, citrate, Glu, 2-oxogluterate, malate, Fru-1,6P	Hu and Plaxton 1996
		PK_c	7.5	0.033	0.037	6.5	-	ATP, citrate, Glu, 2-oxogluterate, malate, Fru-1,6P	Hu and Plaxton 1996
V. radiata	germinating beans	PK_c	6.5	0.120	0.240	7.5			Ambasht *et al.* 1996
		PK_c	7.5	0.090	0.170	7.5			Ambasht *et al.* 1996
		PK_c	8.5	0.050	0.160	7.5			Ambasht *et al.* 1996
		PK_c	7.5				Ala, Cys, Gln, Met, GMP	oxalate, ATP, citrate, Glu	Ambasht *et al.* 1997
C. dactylon	grass	PK_c	6.2	0.064	0.235	6.2			Zervoudakis *et al.* 1997
B. campestris	developing seeds	PK_c	7.8	0.130	0.140	6.8 - 7.8	AMP, Pi, 3-PGA, Asp	citrate, ATP, oxalate isocitrate	Singh *et al.* 1998
		PK_c	6.8	0.100	0.110	6.8	Glc-6P, Fru-1,6P, Pi	Glu, Gln, citrate, isocitrate, oxalate, 2-PGA	Singh *et al.* 2000
B. napus	cells	PK_c	6.8	0.120	0.078	6.8	Asp	Glu, oxalate, rutin, quercetin	Smith *et al.* 2000

Role of metal ions

In earlier studies, plants were grown in the presence and absence of K^+ and PK activity was assayed (Miller and Evans 1957, McCollum *et al.* 1958, 1960, Evans 1963, Sugiyama *et al.* 1968, Memon *et al.* 1985) and activation of PK by K^+ was recorded (McCollum *et al.* 1960). In grafted watermelon plants, PK activity has been used a bioindicator of cations (Pulgor *et al.* 1996). In *Cucumis sativus* PK activity was maximum at 0.15 $g(K^+)$ dm^{-3} and with increase in K^+ concentration, PK activity decreased (Ruiz *et al.* 1999). PK_p from *S. minutum* had lower affinity for K^+ than PK_c (Lin *et al.* 1989b).

PK requires both Mg^{2+} and K^+ for maximum activity (Vaccaro and Zeldin 1974, Ireland *et al.* 1980, Gupta and Singh 1989, Lin *et al.* 1989b, Podestá and Plaxton 1991, Hu and Plaxton 1996, Singh *et al.* 1998). K^+ is not absolutely required in *V. radiata*, however, it acts as an PK activator (Ambasht *et al.* 1996).

PK_c and PK_p from *S. minutum* had similar affinities for Mg^{2+} (Lin *et al.* 1989b). In PK_c from endosperm of germinating *R. communis* seed, the effect of $[Mg^{2+}]$ was studied at two different pH. Mg^{2+} binding showed a positive cooperativity at pH 6.5 and negative cooperativity at pH 7.2 (Podestá and Plaxton 1991). In PK_c from *R. communis* leaves, Mg^{2+} binding showed only positive cooperativity at pH 6.5 and 7.5 (Hu and Plaxton 1996). Mn^{2+} is found to be partially effective in place of Mg^{2+} (Lin *et al.* 1989b, Ambasht *et al.* 1996). The rates of reaction catalyzed by PK_c from *R. communis* seed in presence of Mn^{2+} and Mg^{2+} were identical when assayed at pH 7.2 (Podestá and Plaxton 1991). The K_m of Mn^{2+} was found to be lower than that of Mg^{2+} (Ireland *et al.* 1980, Ambasht *et al.* 1996). Calcium, nickel and cobalt ions restored only low PK activity (Gupta and Singh 1989, Ambasht *et al.* 1996). Calcium ions are reported to inhibit PK from *Pisum sativum* and *Daucus carrota* (Tomlinson and Turner 1973) and *Euglena gracilis* (Vaccaro and Zeldin 1974). Ca^{2+} inhibited the PK_c from castor oil seed competitively with respect to Mg^{2+} (K_i 2.5 mM) (Podestá and Plaxton 1991). Inhibition by Ca^{2+} in *E. gracilis* could not be reversed by addition of excess Mg^{2+} (Vaccaro and Zeldin 1974).

K^+ ions enhanced the activity in presence of Mg^{2+} or Mn^{2+} but not in the presence of any other divalent cation (Ambasht *et al.* 1996). Ammonium ions could replace K^+ with lower rates (Lin *et al.* 1989b, Podestá and Plaxton 1991). These ions are, however, inhibitory to PK_c (Ambasht *et al.* 1996).

Specificity

In PK_c from *S. minutum*, good activity was observed in presence of UDP besides ADP, GDP and IDP (Lin *et al.* 1989b, Podestá and Plaxton 1991). However, the purine nucleoside diphosphates were not good substitutes of ADP with PK_p (Lin *et al.* 1989b). A similar observation has been reported in isozymes of *R. communis* and *P. sativum* leaves (Ireland *et al.* 1979) and in PK_c of *Cicer arietinum* (Gupta and Singh 1989). PK_p from *B. campestris* could utilize CDP, GDP or UDP as an alternative nucleotide to ADP (Singh *et al.* 1998). Analogues like adenosine 5′-*O*-(2-thiodiphosphate) (ADP-β-S) for ADP and phenyl phosphate for PEP were used to study the kinetic mechanism (Podestá and Plaxton 1992).

PK regulation

Effect of different metabolites on PK from different sources has been studied at respective K_m values for PEP and ADP because K_m is close to the physiological concentration and the effectors are expected to exert a stronger effect under above conditions (Table 1).

Fructose-1,6-bisphosphate does not significantly affect the enzymes from *Gossypium hirsutum* (Duggleby and Dennis 1973b), *R. communis* (Nakayama *et al.* 1976), *Spinacia oleracea* (Baysdorfer and Bassham 1984), and *Vigna radiata* (Ambasht *et al.* 1997). It activates *E. gracilis* enzyme when PEP is limiting, however, has no effect when ADP is limiting (Vaccaro and Zeldin 1974). It acts as a feed-forward activator however, in the presence of inhibitors of PK, it acts as a reactant in the direction of photosynthetic gluconeogenesis (Vaccaro and Zeldin 1974).

AMP has no effect on PK_p from *R. communis* and *S. minutum* (Ireland *et al.* 1980, Lin *et al.* 1989b) and PK_c from *R. communis* and *V. radiata* (Hu and Plaxton 1996, Ambasht *et al.* 1997) suggesting that energy charge has no role in regulation of *in vivo* activities of PK isozymes. It inhibits the PK_c from *R. communis* seed competitively with respect to PEP and non-competitively with respect to ADP (Podestá and Plaxton 1991).

Oxalate, ATP and citrate inhibit PK in most of the cases. The close structural resemblance of oxalate with enolate form of pyruvate may be one of the reasons for inhibition of PK (Lodato and Reed 1987). The inhibition

by ATP is expected, as it is one of the products. ATP inhibits PK_c from mung beans competitively with respect to ADP with $K_{i(compet.)}$ (3.8 mM) which is more than an order of the magnitude higher than the K_m of ADP (0.17 mM) suggesting that enzyme has a higher affinity for ADP than for ATP. ATP inhibits the enzyme non-competitively with respect to PEP, *i.e.*, binding of PEP is unaffected by the presence of ATP (Ambasht *et al.* 1996). In contrast to above, PK_p and PK_c from *B. campestris* are inhibited by ATP competitively with respect to PEP and non-competitively with respect to ADP (Singh *et al.* 1998, 2000). Citrate inhibits mung bean PK_c non-competitively with respect to both PEP and ADP, suggesting the existence of a separate regulatory site (Ambasht *et al.* 1997). The inhibition of PK activity by citrate provides a mechanism for a respiratory control on this enzyme, *i.e.*, PK activity will be suppressed when TCA cycle is inhibited. Additive inhibition in PK from castor bean endosperm and leaf has been reported in the presence of citrate, oxalate and glutamate (Podestá and Plaxton 1991, Hu and Plaxton 1996). Inhibition brought about by ATP and citrate when present together showed synergistic effect (Ambasht *et al.* 1997, Singh *et al.* 1998). This inhibition could not be due to partitioning of Mg^{2+} between ADP, ATP and citrate as very high concentration of the divalent cation was used (Ambasht *et al.* 1997).

Glutamate acts as a mixed type of inhibitor of PK_c with respect to PEP and competitive with respect to ADP (Baysdorfer and Bassham 1984). It inhibited only PK_c and had no effect on PK_p (Lin *et al.* 1989b). As glutamate is the primary product of NH_4^+ assimilation by GS/GOGAT, glutamate inhibition of PK_c is expected to exert an important feed back control (Lin *et al.* 1989b, Hu and Plaxton 1996).

PK_c from endosperm of *R. communis* seed showed activation in the presence of polyethylene glycol. Glycerol, ethylene glycol and BSA also enhanced PK activity (Podestá and Plaxton 1993). PK_p and PK_c from *S. minutum* are inhibited in the presence of inorganic phosphate and are activated in the presence of dihydroxyacetone phosphate. The latter facilitates the binding of PEP to enzyme and reverses inhibition of PK_c by Pi or glutamate (Lin *et al.* 1989b).

Metabolites like 2-oxoglutarate, malate, glucose 6-phosphate, fructose 6-phosphate, 3-phosphoglycerate, 2-phosphoglycerate, glycerol 3-phosphate and phospho-glycolate inhibited the PK_c from castor oil seed at pH 7.2 and had no effect at pH 6.5 suggesting a pH based regulatory mechanism (Podestá and Plaxton 1991, 1994). PK_c activity is known to be regulated by change in activity due to subunit association-dissociation (Kowallik *et al.* 1990, Ruyters *et al.* 1991). Covalent modification by phosphorylation-dephosphorylation is not operative for regulation of PK_c (Podestá and Plaxton 1991).

Mechanism

On the basis of pK_a values, photooxidation in the presence of dyes (methylene blue and rose bengal) and chemical modification studies in the presence of iodoacetate, N-ethyl maleimide, *p*-chloromercuribenzoate and diethyl pyrocarbonate on mung bean PK, it has been suggested that SH and imidazole groups are vital for the activity of the enzyme (Ambasht *et al.* 1997).

Initial velocity studies have suggested a sequential compulsory ordered binding of substrates to enzyme, PEP being a leading substrate and ADP, the lagging substrate (Duggleby and Dennis 1973b, Ireland *et al.* 1980, Gupta and Singh 1989, Singh *et al.* 1998). PK_c and PK_p from *R. communis* show a sequential binding with PEP, the leading substrate, there is difference in sequence of release of products. PK_c releases pyruvate first, whereas ATP is released first by PK_p (Ireland *et al.* 1980). In case of PK_p from *B. campestris* pyruvate is released first (Singh *et al.* 1998). On the basis of fluorescence data, substrate interaction kinetics, pattern of inhibition by products and substrate analogues the above mechanism has been proved (Podestá and Plaxton 1992). Kinetic studies at different pH and effects of ATP suggest the formation of a ternary complex (E.ADP.PEP) by a combination of random and compulsory ordered pathways depending on the experimental conditions. The transfer of phosphate group probably takes place directly from one substrate to the other within the ternary complex. The nature of inhibition by ATP suggests that the enzyme has distinct sites for binding of PEP and nucleotide and the binding of PEP is unaffected by ATP. This is also suggestive of formation of a ternary complex E.ATP.PEP (abortive) and E.ADP.PEP (productive) (Ambasht *et al.* 1996).

Physiological role of PK

PK is primarily involved in controlling the glycolytic pathway (Turner and Turner 1980, Plaxton 1996). It has been shown that 60 % of the glycolytic carbon is processed by PK in developing seeds of soybean (Adams and Rinnie 1981, Adams *et al.* 1982).

The absence of PK should have a major impact on

metabolism. Transgenic tobacco plants were developed with absence of PK_c (-PK_c) in the leaves. The plant growth was not affected, however roots were not examined. These had normal oxygen consumption (McHugh *et al.* 1992). These results suggest that plants have pathways that bypass reaction catalyzed by PK_c (Sung *et al.* 1987, Duff *et al.* 1989a, 1989b, Theodorou *et al.* 1991). A significant decrease in root growth was reported in homozygous progeny of -PK_c throughout development under moderate and low irradiances (Knowles *et al.* 1998). In these plants, the export of fixed carbon dioxide was 40 % lower than that of +PK_c leaves. In addition, the respiratory carbon dioxide was 40 % higher. This provides rationale for reduced root growth (Grodzinski *et al.* 1999).

PK_p from leaves of *R. communis* appears to be involved in providing carbon skeleton for fatty acid biosynthesis (Ireland *et al.* 1979). Since PK_p is inactive at pH below 7.0 and is fully active at pH 8.0, it is expected that PK_p would be functional in light when stromal pH increases to 8.0 (Werden *et al.* 1975). Further, it has also been demonstrated that fatty acid biosynthesis takes place in light and not in dark (Yamada and Nakamura 1975) suggests that PK_p is involved in fatty acid biosynthesis (Ireland *et al.* 1979).

PK_c can also cater for cellular requirements of ATP during early stages of seed germination, when mitochondria are incapable of conducting oxidative phosphorylation (Perl 1980). An increase in glycolytic flux has been observed during initial stages of seed germination leading to formation of pyruvate and ATP. This is due to *de novo* synthesis of PK_c (Sangwan *et al.* 1992). PK_c is also involved in providing carbon skeleton for ammonia assimilation as it has been demonstrated that PK is activated during periods of enhanced ammonium assimilation (Larsen *et al.* 1981).

PK_p from *S. minutum* has pH optimum 6.5 (Lin *et al.* 1989b). In *S. minutum*, PK has been shown to regulate the partitioning of carbon skeleton between starch, respiration and ammonium assimilation (Turpin *et al.* 1990). The model proposed by Lin *et al.* (1989b) on partitioning of carbon from starch to respiration and nitrogen assimilation suggest that assimilation via GS/GOGAT system, causes a significant fall in levels of glutamate and ATP causing increase in concentration of ADP and glutamine. PK is thus relieved from adenylate control.

Concluding remarks

In glycolytic pathway, PK is one of the three key enzymes (hexokinase, phosphofructokinase and PK). Despite the progress in the analysis of the organization and regulation of plant PK, many issues remain unresolved. This review clearly reveals that PK has been isolated from a few sources and has been mostly studied from plant seed rich in fatty acids. There is a need for further purification and characterization of PK from non-fatty sources. The amino acid composition has been worked out only from one source. The active site studies are lacking and are required to be done. Substrate specificity studies with PEP analogues are also lacking in plant PK.

Developments in molecular genetics have led to exciting strategies. Primary structures of PK_c and PK_p have been deduced from the respective cDNA sequences. This valuable information has provided new insights into molecular mechanism of catalysis and regulation of PK. Transformation of plants with sense or anti-sense cDNA constructs provides new information on the function and control of this enzyme *in vivo*.

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