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Kinetic and regulatory properties of cytosolic pyruvate kinase from germinating castor oil seeds

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The kinetic and regulatory properties of cytosolic pyruvate kinase (PK_c) isolated from endosperm of germinating castor oil seeds (*Ricinus communis* L.) have been studied. Optimal efficiency in substrate utilization (in terms of $V_{max.}/K_m$ for phosphoenolpyruvate or ADP) occurred between pH 6.7 and 7.4. Enzyme activity was absolutely dependent on the presence of a bivalent and a univalent metal cation, with Mg^{2+} and K^+ fulfilling this requirement. Mg^{2+} binding showed positive and negative co-operativity at pH 6.5 ($h = 1.6$) and pH 7.2 ($h = 0.69$) respectively. Hyperbolic saturation kinetics were observed with phosphoenolpyruvate (PEP) and K^+, whereas ADP acted as a mixed-type inhibitor over 1 mM. Glycerol (10%, v/v) increased the $s_{0.5}$(ADP) 2.3-fold and altered the pattern of nucleotide binding from hyperbolic ($h = 1.0$) to sigmoidal ($h = 1.79$) without modifying PEP saturation kinetics. No activators were identified. ATP, AMP, isocitrate, 2-oxoglutarate, malate, 2-phosphoglycerate, 2,3-bisphosphoglycerate, 3-phosphoglycerate, glycerol 3-phosphate and phosphoglycolate were the most effective inhibitors. These metabolites yielded additive inhibition when tested in pairs. ATP and 3-phosphoglycerate were mixed-type inhibitors with respect to PEP, whereas competitive inhibition was observed for other inhibitors. Inhibition by malate, 2-oxoglutarate, phosphorylated triose sugars or phosphoglycolate was far more pronounced at pH 7.2 than at pH 6.5. Although ^{32}P-labelling studies revealed that extensive phosphorylation *in vivo* of soluble endosperm proteins occurred between days 3 and 5 of seed germination, no alteration in the ^{32}P-labelling pattern of 5-day-germinated endosperm was observed after 30 min of anaerobiosis. Moreover, no evidence was obtained that PK_c was a phosphoprotein in aerobic or anoxic endosperms. It is proposed that endosperm PK_c activity of germinating castor seeds is enhanced after anaerobiosis through concerted decreases in ATP levels, cytosolic pH and concentrations of several key inhibitors.

INTRODUCTION

Pyruvate kinase (PK; ATP:pyruvate phosphotransferase, EC 2.7.1.40) catalyses the irreversible final step of aerobic glycolysis, as follows (PEP, phosphoenolpyruvate):

$$PEP + ADP + H^+ \xrightarrow{Mg^{2+},\,K^+} \text{pyruvate} + ATP$$

It is considered to be a key regulatory enzyme of glycolysis, and many efforts have been devoted to elucidate its molecular and catalytic properties. Both allosteric mechanisms and reversible phosphorylation can be utilized to co-ordinate the activity of animal PKs with the energy or carbohydrate demands of the cell [1–3]. Although a growing body of evidence indicates that PK may be the primary control site of plant glycolytic flux to pyruvate [4,5], far less is known about the kinetic and regulatory characteristics of plant cytosolic PK (PK_c) or plastid PK isoenzymes [6–10]. Only recently have the molecular and immunological properties of several highly purified or homogeneous plant PKs been examined in detail [11–15], and some of their cDNAs cloned and sequenced [16].

The aerobic carbon metabolism of 5-day-germinated castor-bean endosperm is dominated by substantial transformation of reserve lipids into sucrose [17]. This process involves the cytosolic gluconeogenic conversion of PEP, derived from fatty acids, into hexose phosphates. The maximal rate of gluconeogenic flux in germinating castor-oil-seed endosperm (COS) is about 10 times that of glycolysis [17], and occurs in the presence of relatively large quantities of PK_c [11]. In their classic 1971 study, Kobr & Beevers [17] demonstrated that PK probably plays a fundamental role in regulating a metabolic reorganization from gluconeogenesis to glycolysis that occurs when germinating COS undergoes anaerobiosis. Plaxton [11] purified germinating-COS PK_c to electrophoretic homogeneity, and in a subsequent study [12] demonstrated that the enzyme probably exists *in vivo* as a heterotetramer composed of two types of similar but non-identical subunits having molecular masses of about 57 and 56 kDa. In the present study, we report various kinetic and regulatory properties for purified COS PK_c. The results are used to formulate a model for the regulation of this enzyme in relation to the control of the glycolytic pathway in germinating COS.

EXPERIMENTAL

Chemicals and plant material

Mes, bis-tris-propane, Hepes, PEP and dithiothreitol were from Research Organics Inc. (Cleveland, OH, U.S.A.). NADH was from Boehringer Mannheim (Montreal, Que., Canada). All other fine biochemicals, coupling enzymes and *Staphylococcus aureus* cells were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade obtained from BDH Chemicals (Toronto, Ont., Canada). All solutions were prepared with Milli-Q-processed water.

Seeds of castor bean (*Ricinus communis* L., cv. Hale) were obtained from Bothwell Enterprises (Plainview, TX, U.S.A.). Seeds were soaked overnight with running water at 20 °C, made sterile by washing with 5% (v/v) sodium hypochlorite solution, and rinsed with distilled water before planting. Seeds were germinated in the dark at 30 °C in vermiculite and 70% relative humidity. After 5 days, germinated seeds were dissected and the endosperms were frozen in liquid N_2 and stored at −80 °C until used. PK_c purification was performed as described in [11], except

Abbreviations used: PK, pyruvate kinase; PK_c, cytosolic PK; PEP, phosphoenolpyruvate; COS, castor-oil-seed endosperm.

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that the potentially deleterious heat step was omitted. The final preparation's specific activity of 180 μmol of pyruvate produced/min per mg of protein compares favourably with the value of 200 μmol of pyruvate produced/min per mg of protein previously obtained for homogeneous germinating-COS PK_c [11]. No plastidic PK was present in the purified enzyme, as no immunological cross-reactivity was observed when a Western blot of the final preparation (0.25 μg) was probed with rabbit polyclonal antibodies directed against developing-COS leucoplast PK [15].

Enzyme assay

The PK reaction was routinely coupled with the lactate dehydrogenase reaction and assayed at 30 °C by monitoring NADH utilization at 340 nm with a Varian DMS 200 spectrophotometer. Standard assay conditions for PK were: 2 mM-PEP, 1 mM-ADP, 20 mM-KCl, 10 mM-$MgCl_2$, 0.2 mg of BSA/ml, 2 mM-dithiothreitol, 0.15 mM-NADH and 2 units of rabbit muscle lactate dehydrogenase in a final volume of 1 ml. Assays were buffered by 50 mM-imidazole/HCl (pH 6.5) or by 25 mM-Mes/bis-tris-propane/HCl for all other pH values. For examination of the pyruvate analogue glycolate as a potential metabolite effector of COS PK_c, NADH and lactate dehydrogenase were replaced by 2 mM-glucose, 0.15 mM-NAD^+, 2 units of yeast hexokinase and 2 units of *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase. Coupling enzymes were desalted before use. Assays were initiated by addition of PK_c. One unit of enzyme activity is defined as the amount of PK resulting in the production of 1 μmol of pyruvate/min at 30 °C. For coupling enzymes, units of activity are as defined by the supplier.

Kinetic studies

Apparent K_m or $s_{0.5}$ and h (Hill coefficient) values for all substrates and cofactors were calculated from the Hill equation, fitted to a non-linear least-squares regression computer kinetics program (S. Brooks, unpublished work). For determination of kinetic constants for univalent and bivalent cation cofactors, samples (300 μl) of the final PK_c preparation were desalted on a column (0.7 cm × 7.5 cm) of Sephadex G-25 that had been pre-equilibrated with 25 mM-imidazole/HCl (pH 6.5) containing 0.4 mg of BSA/ml, 0.5 mM-EDTA, 20% (v/v) glycerol and 2 mM-dithiothreitol. When screening for enzyme effectors, a stock solution of each metabolite was adjusted to the corresponding assay pH being used. I_{50} values (inhibitor concentration producing 50% inhibition of enzyme activity) were determined by the method of Job *et al.* [18]. Competitive inhibition constants (K_i values) were determined from Dixon plots, whereas uncompetitive inhibition constants (K_i' values) were determined from Cornish-Bowden [19] plots. Patterns of inhibition were evaluated by Lineweaver–Burk plots.

Concentrations of free and Mg-complexed metabolites or substrates were calculated by using the relationship:

$$C = \frac{(K_d + M + S) - \sqrt{(K_d + M + S)^2 - 4MS}}{2}$$

where C is the concentration of the complex, M and S are the total concentrations of Mg^{2+} and metabolites (or substrates) respectively, and K_d is the dissociation constant of the complex. Values were computed by using a program written in Turbo Pascal, which also automatically corrects for pH. Apparent dissociation constants were obtained from [20]. Free Mg^{2+} concentration was in excess of 5 mM under all conditions. Enzyme activity was independent of free Mg^{2+} concentrations in the range of 3–10 mM. Metabolite or substrate concentrations stated in the text refer to their total concentration in the assay medium.

All kinetic parameters are the means of duplicate determinations performed on two separate preparations of the purified enzyme, and are reproducible to within 10%.

Other calculations

Estimated cytosolic concentrations of several metabolites in 5-day-germinated COS were calculated from data of Kobr & Beevers [17], by using the assumptions of Kubota & Ashihara [21].

Phosphorylation studies *in vivo*

Endosperms of intact 3-day-germinated castor seeds (14 g total fresh wt.) were injected with 2 mCi of [^{32}P]P_i (ICN Radiochemicals, Irvine, CA, U.S.A.; 1000 mCi/mmol). The seeds were allowed to germinate for a further 2 days, after which the endosperms were dissected free of seed coats, hypocotyls and roots. Half of the endosperms were immediately homogenized with a Polytron, together with 2 vol. of 50 mM-potassium phosphate (pH 7.6) containing 50 mM-NaF, 2 mM-EDTA, 1 mM-EGTA, 2 mM-dithiothreitol, 2.5 mM-$MgCl_2$, 2.5% (w/v) insoluble polyvinylpolypyrrolidone, 20% (v/v) glycerol, 0.1 mM-PEP, 1 mM-phenylmethanesulphonyl fluoride, 5 μg of chymostatin/ml and 5 μg of leupeptin/ml [11]. To impose anoxia, the remaining endosperms were placed in flasks containing distilled water at 30 °C, and N_2 gas was continuously bubbled through the water for 30 min. After homogenization, both samples were centrifuged at 4 °C in an Eppendorf microcentrifuge for 5 min at 16000 *g*, and the fat-free supernatants were removed and stored on ice. Centrifugation of the homogenate prepared from anoxic endosperms was done in sealed tubes under N_2.

The supernatants were subjected to immunoprecipitation in accordance with [22] as follows: to 1 ml of crude supernatant, 50 μl of rabbit pre-immune serum was added. After 1 h incubation on ice, a washed pellet of *S. aureus* cells [obtained by centrifuging 500 μl of 10% (w/v) cell suspension in extraction buffer] was resuspended with the above mixture and incubated for 30 min on ice. After centrifuging for 5 min as above, the supernatant was removed. Rabbit anti-(COS PK_c) immune serum (20 μl) [12] was added to 50 μl of supernatant. The mixture was incubated for 1 h on ice, followed by addition of 50 μl of a 10% (w/v) *S. aureus* cell suspension (washed as before) and a further 30 min incubation, and then centrifugation as above. The immunoprecipitate was washed three times with extraction buffer, and the final pellet was resuspended in SDS/PAGE sample buffer [15]. Crude supernatants and solubilized immunoprecipitates were subjected to SDS/PAGE in a Bio-Rad mini-gel apparatus as described in [15] (10%-acrylamide separating gel). The gels were analysed by autoradiography, or the lanes of interest were cut into 2 mm segments. Each gel segment was placed into a scintillation vial and digested with 0.2 ml of 30% (v/v) H_2O_2/0.9 mM-$CuSO_4$. Aquasol II was added to each vial, and ^{32}P radioactivity was determined in a scintillation counter.

Protein determination

Protein concentration was determined by the dye-binding method of Bradford [23], with BSA as standard.

RESULTS AND DISCUSSION

Kinetic properties

Effect of pH and temperature. In the presence of saturating concentrations of substrates and cofactors, PK_c showed a broad pH optimum centred at about pH 6.5 (Fig. 1*a*). However, the apparent K_m values for both substrates were lower and approxi-

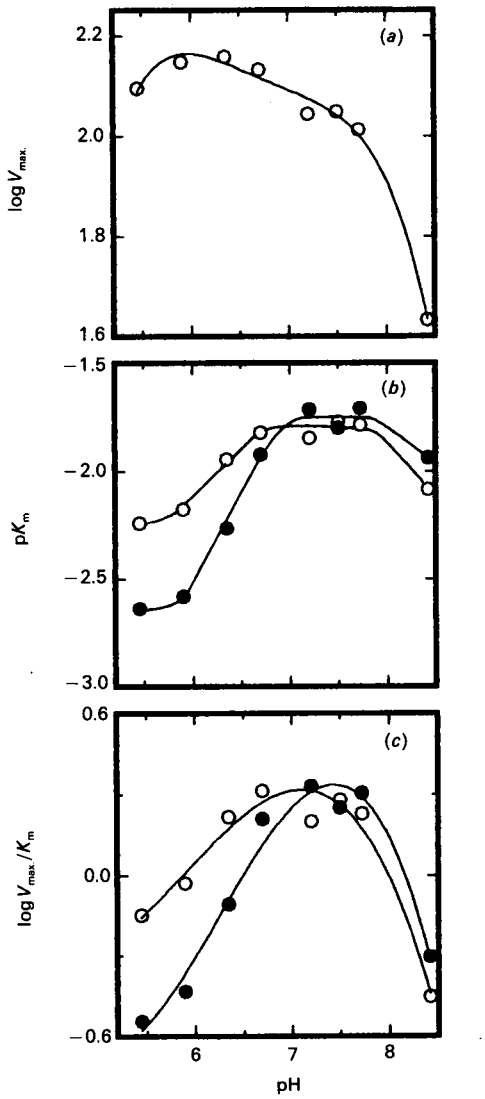

Fig. 1. Dependence of various PK_c kinetic parameters on assay pH

(*a*) $V_{max.}$, (*b*) K_m and (*c*) $V_{max.}/K_m$. Data for (*b*) and (*c*) were obtained by varying the concentrations of ADP (○) or PEP (●); invariant co-substrate concentrations were 2 mM-PEP or 1 mM-ADP. The units for $V_{max.}$ and K_m were ΔA_{340}/min and mM respectively. Enzyme assays at each pH value were buffered by a mixture of 25 mM-Mes and 25 mM-bis-tris-propane.

mately constant, in the range pH 6.8–7.5 (Fig. 1*b*). Consequently, the highest specificity constants ($V_{max.}/K_m$) for ADP and PEP occurred at approx. pH 7.2 (Fig. 1*c*). The slope of the ascending part of the curves in Fig. 1(*b*) (about 0.8) suggests that the change in the protonation state of one species, either in the free enzyme itself or one of the substrates, is responsible for the corresponding change in affinity. The pK_a of such a group is approx. pH 6.8. Since the curves for ADP and PEP both show a bend at about pH 6.8 (Fig. 1*b*), a group of the enzyme is probably responsible.

Similar to PK from cucumber, but not that from winter rape [24], an Arrhenius plot for the COS enzyme was linear over the temperature range 2–37 °C (results not shown). The activation energy (E_a) for the enzyme was calculated from the Arrhenius equation to be 52.4 kJ/mol.

Substrate saturation kinetics. Table 1 summarizes the apparent K_m or $s_{0.5}$ and Hill coefficient (h) values obtained for substrates

Table 1. Kinetic constants for substrates and cofactors of PK_c

Fixed substrate/cofactor concentrations were: PEP, 2 mM; ADP, 1 mM; K^+, 20 mM; and Mg^{2+}, 10 mM. Numbers in parentheses are values for the Hill coefficient (* determined with ADP less than 1 mM). Hill coefficients and affinity constants were determined by using a Hill plot. Abbreviation: n.d., not determined.

Parameter	pH 6.5	pH 7.2
K_m(PEP) (mM)	0.152 (1.0)	0.052 (0.96)
K_m(ADP) (mM)	0.078 (1.0)	0.071 (0.99)*
K_m(K^+) (mM)	0.720 (0.98)	n.d.
$s_{0.5}$(Mg^{2+}) (mM)	0.450 (1.6)	0.090 (0.69)

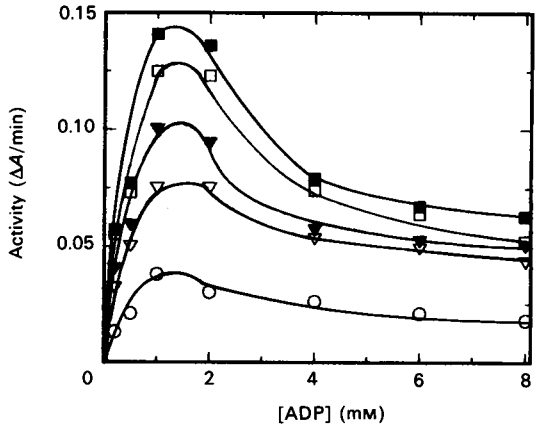

Fig. 2. Effect of ADP concentration on reaction velocity for PK_c

PK_c activity was tested at pH 7.2, 15 mM-$MgCl_2$ and 0.025 mM- (○), 0.100 mM- (▽), 0.200 mM- (▼), 0.400 mM- (□) and 0.800 mM- (■) PEP.

and metal cofactors of PK_c at pH 6.5 and 7.2. In contrast with many animal regulatory PKs [1–3], but similar to most other plant PKs [6–10], germinating-COS PK_c exhibited hyperbolic PEP saturation kinetics. ADP binding shifted from normal Michaelis–Menten kinetics at concentrations higher than 1 mM, owing to substrate inhibition occurring above this value (Fig. 2). Substrate inhibition by ADP has also been reported for several non-plant PKs [1,3,25].

ADP inhibition was determined to be of the mixed type (results not shown). From Dixon and Cornish-Bowden plots, a K_i of 6.7 mM and a K_i' of 2.8 mM were calculated (results not shown). Plotting the slopes or ordinate intercepts of Lineweaver–Burk plots (obtained with PEP as variable substrate at different inhibitory ADP levels) against ADP concentration produced straight lines (results not shown), indicating that the inhibitory bound ADP was catalytically ineffective. Downward-curved lines would be expected if the reaction could still proceed. Metal complexing seems not to be the cause of this inhibitory effect, since at 15 mM total $MgCl_2$ the minimum free Mg^{2+} level is greater than 7 mM (at 8 mM-ADP). The mixed-type inhibition suggests that interaction of ADP with COS PK_c may involve: (i) the formation of inactive complexes resulting from binding of ADP to the PEP site (although with much less affinity), or (ii) binding of a second molecule of ADP to a second site that would inhibit catalysis. The physiological significance of substrate inhibition by ADP is, however, unclear, since the K_i and K_i' values are in excess of estimated cytosolic levels of ADP in this tissue (1.2–1.5 mM; see [17]).

Glycerol (10%, v/v), a compound that can promote aggregation of proteins [26], increased the $s_{0.5}$(ADP) 2.3-fold and induced an allosteric behaviour of PK_c, as suggested by a h value of 1.79 for nucleotide binding. By contrast, 10% glycerol had no effect on either $V_{max.}$ or PEP saturation kinetics.

Similar to the enzyme isolated from other sources [1,3,6–9], COS PK_c could utilize alternative nucleoside diphosphates as substrates. UDP, GDP and IDP yielded 100, 51 and 60%, respectively, of the $V_{max.}$ observed with ADP. The efficiency in the use of alternative nucleotides, according to the relative $V_{max.}/K_m$ ratios (ADP = 100) was as follows: UDP = 36; GDP = 18; IDP = 8.

Cation requirements. In common with other plant and non-plant PKs [1,3,6–10], COS PK_c showed an absolute dependence for both a univalent and a bivalent cation. Increasing the pH from 6.5 to 7.2 elicited: (i) a 5-fold decrease in the $s_{0.5}(Mg^{2+})$, and (ii) negative, instead of positive, co-operativity in the pattern of Mg^{2+} binding (Table 1). These data suggest that major structural modifications of PK_c could be occurring within this pH range. Mn^{2+} (10 mM) yielded 45% of the $V_{max.}$ achieved with saturating Mg^{2+} at pH 6.5. At pH 7.2, however, identical $V_{max.}$, $s_{0.5}$ and h values were obtained with both bivalent cations. Ca^{2+} behaved as a competitive inhibitor with respect to Mg^{2+} (K_i = 2.5 mM) and was, like Co^{2+} and Zn^{2+}, catalytically ineffective.

Hyperbolic saturation kinetics were observed with K^+ at pH 6.5 (Table 1). NH_4^+ could replace K^+ as the univalent cation for PK_c. However, the $V_{max.}$ value in the presence of saturating NH_4^+ was only 41% of that obtained with K^+.

Evidence that germinating-COS PK_c is not phosphorylated *in vivo*

Various animal PKs have been shown to be regulated *in vivo* by alterations in phosphorylation state in response to physiological signals such as anoxia or starvation [2,3,27]. Homogeneous COS PK_c has been phosphorylated *in vitro* by several plant Ca^{2+}-dependent protein kinases (including a germinating-COS protein kinase) (W. C. Plaxton, A. C. Harmon, D. D. Randall & L. J. Klimczak, unpublished work). We therefore investigated whether, as has been reported for red muscle and hepatopancreas PKs of the anoxia-tolerant marine gastropod mollusc *Busycotypus canaliculatum* [2,3], phosphorylation–dephosphorylation could be involved in modulating the activity *in vivo* of germinating-COS PK_c after the aerobic-to-anoxic transition.

The amount of radioactivity incorporated into PK_c immunoprecipitated from crude extracts of [^{32}P]P_i-injected aerobic or anoxic 5-day-germinated COS was determined. Dephosphorylation of COS phosphoproteins after tissue extraction was unlikely, owing to the presence of 50 mM-NaF (a phosphatase inhibitor) in the homogenization buffer. An autoradiogram of a SDS gel of the respective crude extracts showed appreciable ^{32}P labelling of many protein bands (Fig. 3). However, no radioactivity was found to co-migrate with PK_c after SDS/PAGE of immunoprecipitated samples prepared from the aerobic or anoxic endosperm tissue. More than 80% of the initial PK activity was removed immunologically from the soluble fractions, compared with controls in which extraction buffer was substituted for the anti-PK_c immune serum. Moreover, PK_c obtained from aerobic or anoxic endosperms of 5-day-germinated COS did not significantly differ in $V_{max.}$ or apparent K_m(PEP) values, nor in the pattern of PEP binding (results not shown). These results suggest that the enhancement *in vivo* of germinating-COS PK_c activity that accompanies anoxia stress [17] does not involve reversible (de)phosphorylation, and are consistent with the absence from potato tuber and COS PK_cs of the regulatory phosphorylation site found on mammalian and yeast PKs [16]. Our findings, however, do not completely exclude the possibility of a plant PK being a phosphoprotein in other tissues, or under other physiological conditions.

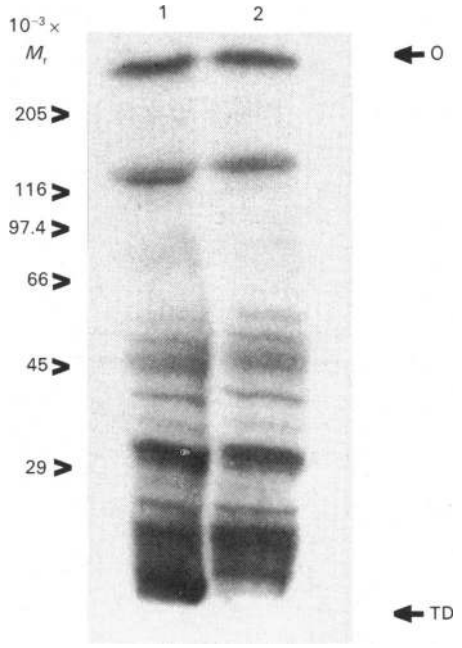

Fig. 3. Phosphorylation pattern *in vivo* of soluble proteins from 5-day-germinated castor bean endosperm

Crude supernatants were prepared from [^{32}P]P_i-injected endosperm tissue and analysed by SDS/PAGE (10%-acrylamide separating gel) and autoradiography as described in the Experimental section. The autoradiogram was exposed for 3 h at −80 °C. Lane 1 contains 60 μg of protein from extracts of aerobic tissue, whereas lane 2 contains 60 μg of protein from extracts of endosperms subjected to 30 min of anaerobiosis. O, origin; TD, tracker dye front.

Regulatory properties

Metabolite effects. A wide variety of compounds were tested as possible effectors of PK_c at pH 6.5 and 7.2 with K_m concentrations of PEP and ADP. The following compounds had no effect (±10% control velocity) on enzyme activity: 15 mM-P_i; 10 mM-sucrose; PP_i, acetaldehyde, acetate, NH_4Cl, fumarate, succinate, arginine, aspartate, histidine, phenylalanine, alanine, glycine, serine, glutamate, glutamine, lysine, methionine, proline and cystine (all 5 mM); glucose 1-phosphate, ribose 5-phosphate, 6-phosphogluconate, glucose, glycolate, leucine, isoleucine, threonine, valine, tryptophan and tyrosine (all 2 mM); phytin, acetyl-CoA, UDP-glucose and ADP-glucose (all 0.5 mM); 100 μM-fructose 2,6-bisphosphate; 50 μM-trifluoperazine. No activators were found. Also, no effect was found for 0.5 mM-dihydroxyacetone phosphate, an important activator of a green-algal PK_c [8]. Similarly, the following substances had no effect (±10% control velocity) on enzyme activity after a 30 min preincubation at 30 °C: dithiothreitol (50 mM); oxidized or reduced β-mercaptoethanol, oxidized or reduced glutathione, and dehydroascorbate (all 10 mM); and a mixture containing 100 μg of spinach leaf thioredoxin$_{fm}$/ml and 20 mM-dithiothreitol.

Table 2 lists those compounds that were found to inhibit the activity of the purified enzyme. As has been demonstrated for PK from several other sources [7,8,28], oxalate was found to inhibit COS PK_c. Oxalate inhibition of PK is believed to arise from a close structural similarity between oxalate and the enolate form

Table 2. Effect of various metabolites on PK_c activity

Standard assay conditions were used, except that the concentrations of PEP and ADP were sub-saturating (0.05 mM-PEP, 0.15 mM-ADP). Enzyme activity in the presence of effectors is expressed relative to the control set at 100. Abbreviation: n.d., not determined.

Addition	Concn. (mM)	Relative activity pH 6.5	pH 7.2
Oxalate	0.5	61	n.d.
ATP	2	85	82
	5	32	47
AMP	2	66	65
	5	29	51
Citrate	2	82	81
	5	67	76
Isocitrate	2	80	78
	5	55	47
2-Oxoglutarate	5	100	64
Erythrose 4-phosphate	2	68	94
Sedoheptulose 1,7-bisphosphate	2	70	n.d.
Malate	5	100	62
Glucose 6-phosphate	5	100	72
Fructose 6-phosphate	5	100	73
Fructose 1,6-bisphosphate	2	90	81
	5	61	67
2,3-Bisphosphoglycerate	2	83	56
3-Phosphoglycerate	2	100	71
	5	n.d.	50
2-Phosphoglycerate	2	100	85
	5	n.d.	56
Glycerol 3-phosphate	2	100	68
	5	n.d.	49
Phosphoglycolate	5	100	47

of pyruvate [28]. In agreement with our findings (Table 2), Nakayama *et al.* [29] reported that 3-phosphoglycerate, malate and isocitrate had no effect on partially purified germinating-COS PK_c at pH 6.5. By contrast, 2-oxoglutarate was described as an activator [29], instead of an inhibitor (the present work), of this enzyme. The reason for this discrepancy is not apparent. Citrate, described as a potent inhibitor of PK from spinach leaves [7] and a green alga [8], caused minor inhibition (Table 2). ATP, AMP, isocitrate, several phosphorylated triose sugars and phosphoglycolate were the most effective inhibitors of COS PK_c when tested at pH 7.2 (Table 2). Essentially the same results as described in Table 2 for pH 7.2 were obtained when assays were conducted in the presence of 10% (v/v) glycerol or at pH 7.8 (results not shown). Additive inhibition was observed for the following metabolites when tested in pairs: ATP, AMP, 2-phosphoglycerate, 3-phosphoglycerate, citrate, isocitrate and fructose 1,6-bisphosphate (Table 3).

Remarkably, 2-oxoglutarate, malate, glucose 6-phosphate, fructose 6-phosphate, 3-phosphoglycerate, 2-phosphoglycerate, glycerol 3-phosphate and phosphoglycolate, all PK_c inhibitors at pH 7.2, showed no effect at pH 6.5 (Table 2). This finding raises the possibility of an efficient regulatory mechanism based on pH (see below). As arises from Table 4, more significant inhibitory effects were exerted at the level of PEP binding. However, when assay pH is lowered from 7.2 to 6.5 the increase in apparent K_m(PEP) is proportionally higher than that for ADP (Fig. 1, Table 1). Thus, more significant inhibition by these metabolites should be expected as assay pH decreases. As this was not the case, other mechanisms that could include structural reorganization could be involved.

Table 3. Interacting effects of various metabolites on the activity of PK_c at pH 7.2

Conditions are identical with those described for Table 2, except that the concentration of Mg^{2+} was 15 mM. Enzyme activity is expressed relative to control set at 100. Metabolites were tested at individual concentrations of either 2 or 5 mM. Values in parentheses indicate the expected value (obtained from the individual inhibition values in Table 2) if additive inhibition is observed.

Additions	Relative activity at: 2 mM	5 mM
3-Phosphoglycerate		
+2-Phosphoglycerate	60 (60)	38 (28)
+ATP	64 (58)	24 (33)
+AMP	42 (46)	36 (36)
+Fructose-1,6-bisphosphate	68 (57)	35 (34)
+Citrate	52 (57)	40 (38)
+Isocitrate	53 (55)	27 (33)
2-Phosphoglycerate		
+ATP	60 (70)	27 (20)
+AMP	42 (55)	34 (28)
+Fructose-1,6-bisphosphate	50 (69)	41 (37)
+Citrate	65 (69)	39 (42)
+Isocitrate	62 (66)	31 (26)
ATP		
+Fructose-1,6-bisphosphate	65 (66)	36 (31)
+Citrate	65 (66)	33 (36)
+Isocitrate	58 (64)	29 (22)
AMP		
+Fructose-1,6-bisphosphate	53 (53)	30 (34)
+Citrate	52 (53)	46 (39)
+Isocitrate	53 (51)	45 (24)
Isocitrate		
+Citrate	53 (63)	45 (36)
+Fructose-1,6-bisphosphate	47 (63)	40 (32)
Citrate		
+Fructose-1,6-bisphosphate	53 (65)	48 (51)
2-Phosphoglycerate		
+3-Phosphoglycerate	42 (40)	
+glycerol 3-phosphate		
3-Phosphoglycerate		
+2-Phosphoglycerate	26 (32)	
+glycerol 3-phosphate		
+fructose-1,6-bisphosphate		

A mixed-type inhibition by 3-phosphoglycerate with respect to PEP at pH 7.2 (Table 4) suggests that it may interact at an allosteric inhibitor site. AMP inhibited germinating-COS PK_c (Tables 2 and 4), as was observed for developing-COS PK_c [6]. This differs with reports on other plant PKs indicating that AMP had no effect [6,8] or was an activator [7,9]. AMP inhibition was non-competitive with respect to ADP, but competitive with respect to PEP (Table 4). ATP inhibition of PK is widespread, as is expected for a product of the enzyme's reaction. This compound also inhibited COS PK_c (Tables 2 and 4), and its effect, like that of AMP and ADP, appeared not to be due to Mg^{2+} chelation, probably reflecting the high capacity of PK to form dead-end complexes [24]. ATP inhibition was of the mixed type with respect to both ADP and PEP (Table 4).

Proposal for the regulation of PK_c in relation to glycolytic control in germinating castor beans

(I) Inhibition of PK_c in aerobic germinating COS. The most striking metabolic event that occurs in germinating COS is the massive conversion of storage triacylglycerols (mainly triricinolein) into sucrose, which is absorbed by the cotyledons of the

Table 4. Inhibition pattern and enzyme–inhibitor dissociation constants for some PK_c effectors at pH 7.2

K_i and K_i' were determined as described in the Experimental section at pH 7.2. Saturating concentrations of the invariant substrate were used throughout (2 mM-PEP or 1 mM-ADP). Abbreviations: C, competitive inhibition; NC, non-competitive inhibition; M, mixed-type inhibition.

	Variable substrate					
	ADP			PEP		
Effector	Inhibition type	K_i (mM)	K_i' (mM)	Inhibition type	K_i (mM)	K_i' (mM)
3-Phosphoglycerate	C	8.2	–	M	6.2	4.2
2-Phosphoglycerate	C	5.5	–	C	4.0	–
Citrate	C	7.1	–	C	4.8	–
Isocitrate	C	6.0	–	C	4.0	–
AMP	NC	7.2	–	C	3.2	–
ADP	–	–	–	M	6.7	2.8
ATP	M	9.6	3.8	M	10	5.0

growing embryo. This process involves the gluconeogenic conversion of PEP (derived from the glyoxylate cycle, malate dehydrogenase and PEP carboxykinase) to hexose phosphates in the cytosol, where appreciable amounts of PK_c co-exist. A very low glycolytic flux (relative to the gluconeogenic one) is observed, and the vast majority of the carbon mobilized from fatty acids ends up as sucrose [17,30]. Kobr & Beevers [17] identified PK as a major control point in glycolysis during gluconeogenesis in aerobic 5-day-germinated COS. PK_c purified from this tissue, however, appears to show substrate saturation kinetics characteristic of a fully 'active' enzyme, e.g. non-sigmoidal kinetics, low apparent K_m values for PEP and ADP (Table 1), and a relatively high $V_{max.}$ of about 200 units/mg of protein [11]. Moreover, although we tested some 55 different metabolites as potential effectors of the purified enzyme, none were potent inhibitors (Tables 2–4). We cannot yet rule out the possibilities that: (i) there is an unidentified effector that significantly regulates COS PK_c, and/or (ii) in aerobic germinating COS, this enzyme is sequestered away from the cytosolic pools of PEP and/or ADP via some form of micro-compartmentation. The patterns of metabolite inhibition that we did observe at pH 7.2, however, are consistent with several of these effectors combining to inhibit COS PK_c significantly *in vivo* (Table 3). Furthermore, although the K_i and K_i' values of COS PK_c for ATP appear to be relatively high (Table 4), the physiological relevance of this inhibitor should not necessarily be disregarded. From data of Kobr & Beevers [17], a cytosolic ATP concentration of 6 mM, falling to 2 mM under anoxia, can be calculated. Thus ATP could contribute along with the various gluconeogenic and glyoxylate-cycle intermediates that were found to inhibit COS PK_c (i.e. 3- and 2-phosphoglycerate, glycerol 3-phosphate, fructose 1,6-bisphosphate, fructose 6-phosphate, glucose 6-phosphate, isocitrate and malate; see Tables 2–4) to the almost complete inhibition of this enzyme in aerobic germinating COS.

(II) Enhancement of PK_c activity in anoxic germinating COS. The onset of anoxia in 5-day-germinated COS is accompanied by a significant decrease in the ratio *in vivo* of [PEP]/[pyruvate] [17]. This clear positive cross-over between PEP and pyruvate occurs within 10 min of anaerobiosis and indicates a key role for PK_c in co-ordinating the metabolic switch from gluconeogenesis to glycolysis that anoxia elicits in this tissue [17]. Within 15 min of anoxia, the level of ATP falls to one-third of the aerobic value and remains stationary thereafter [17]. Our findings indicate that this lowered ATP concentration could partially account for the anoxia-induced enhancement of COS PK_c activity. A 4-fold decrease in PEP levels follows 20 min of anaerobiosis [17]; as PEP is kept in equilibrium with 2- and 3-phosphoglycerate by enolase and phosphoglyceromutase, the concentrations of these two inhibitors (Table 2) should also show a concomitant 4-fold decrease over this initial anoxic period. Moreover, the response of PK_c to these and several other inhibitors was pH-dependent, being far more pronounced at pH 7.2 than at pH 6.5 (Table 2). Significant accumulation of the anaerobic glycolytic end-product lactate occurs over the first 20 min of anoxia in 5-day-germinated COS [17]. A consequent decrease in cytosolic pH could alleviate the pH-dependent inhibitions of PK_c by 2- and 3-phosphoglycerate, glycerol 3-phosphate and several tricarboxylic acid- and glyoxylate-cycle intermediates (i.e. isocitrate, 2-oxoglutarate and malate).

Conclusions

These studies provide further insights into some of the biochemical characteristics of a plant PK_c. Based on a previous report on carbohydrate metabolism in germinating COS [17], an important role for PK_c in regulating cytosolic glycolytic carbon flow could be envisaged. However, no metabolites have been identified that drastically affect the activity *in vitro* of the purified enzyme, nor has it been possible to detect PK_c phosphorylation *in vivo*. Nonetheless, our data indicate that this enzyme is not insensitive to its metabolite environment. In this regard, special attention should be directed to ATP, since its intracellular concentration could reach unusually high levels in germinating COS. Thus the activity *in vivo* of germinating-COS PK_c could be modulated by concerted changes in the concentrations of several metabolites, cytosolic pH, as well as the overall energetic status of the cell, resulting in an almost entirely inhibited enzyme under aerobiosis and the release of this inhibition following the initiation of anoxia stress.

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