Purification and characterization of cytosolic pyruvate kinase from banana fruit

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Cytosolic pyruvate kinase (PK_c) from ripened banana (*Musa cavendishii* L.) fruits has been purified 543-fold to electrophoretic homogeneity and a final specific activity of 59.7 μ mol of pyruvate produced/min per mg of protein. SDS/PAGE and gel-filtration FPLC of the final preparation indicated that this enzyme exists as a 240 kDa homotetramer composed of subunits of 57 kDa. Although the enzyme displayed a pH optimum of 6.9, optimal efficiency in substrate utilization [in terms of $V_{\max}/K_m$ for phosphoenolpyruvate (PEP) or ADP] was equivalent at pH 6.9 and 7.5. PK_c activity was absolutely dependent upon the presence of a bivalent and a univalent cation, with Mg²⁺ and K⁺ respectively fulfilling this requirement. Hyperbolic saturation kinetics were observed for the binding of PEP, ADP, Mg²⁺ and K⁺ (K_m values of 0.098, 0.12, 0.27 and 0.91 mM respectively).

Although the enzyme utilized UDP, IDP, GDP and CDP as alternative nucleotides, ADP was the preferred substrate. L-Glutamate and MgATP were the most effective inhibitors, whereas L-aspartate functioned as an activator by reversing the inhibition of PK_c by L-glutamate. The allosteric features of banana PK_c are compared with those of banana PEP carboxylase [Law and Plaxton (1995) *Biochem. J.* **307**, 807–816]. A model is presented which highlights the roles of cytosolic pH, MgATP, L-glutamate and L-aspartate in the co-ordinate control of the PEP branchpoint in ripening bananas.

Key words: carbohydrate metabolism, plant glycolysis, phosphoenolpyruvate carboxylase, ripening.

INTRODUCTION

Fruit ripening involves a series of complex biochemical events that have evolved to aid seed dispersal. Numerous studies indicate that this process is not akin to senescence, as cellular integrity and intracellular compartmentation are maintained throughout [1,2]. A wide variety of co-ordinated changes occur during banana (*Musa cavendishii* L.) fruit ripening, including *de novo* protein synthesis, major alterations in carbohydrate composition, cell wall disassembly, synthesis of volatile compounds, changes in phenolic constituents, and chlorophyll degradation in the peel [3–5]. Of particular significance in ripening bananas is the massive conversion of starch, which comprises approx. 20% of the fresh weight of unripe fruit, into soluble sugars. Associated with this transformation is a marked rise in respiration, termed the respiratory climacteric, as indicated by an increase in CO₂ evolution, which falls gradually to an elevated post-climacteric level [5]. Increased CO₂ production during the respiratory climacteric arises from an increased flux of starch-derived hexose phosphates through the glycolytic pathway to the mitochondria. Mitochondria of climacteric fruit, which remain tightly coupled throughout ripening, function to generate the ATP required for the conversion of starch into sucrose [6]. Due to the highly predictable pattern of carbohydrate metabolism during ripening, the banana fruit represents an ideal model system in which to investigate the regulation of glycolytic and gluconeogenic carbon flux in vascular plants.

Concomitant with the onset of the respiratory climacteric in banana fruit is a rapid decline in the tissue's content of phosphoenolpyruvate (PEP), with a corresponding elevation in the pyruvate content [7,8]. This positive crossover indicates that

activation of the PEP-metabolizing enzymes pyruvate kinase (PK; EC 2.7.1.40) and/or PEP carboxylase (PEPC; EC 4.1.1.31) is the initial response of glycolysis at the climacteric. This agrees with many reports indicating that the primary control of plant glycolytic flux from hexose phosphate to pyruvate is exerted at the level of PEP utilization [9]. PEPC and PK may exist as compartment- and/or tissue-specific isoforms, and can be differentially expressed during development or in response to environmental stress [9].

PK catalyses the irreversible transfer of P_i from PEP to ADP, yielding pyruvate and ATP. In most eukaryotes PK exists as a cytosolic homotetramer composed of subunits of 55–60 kDa [10]. Many animal and yeast PKs are regulated by allosteric effectors (with fructose 1,6-bisphosphate acting as a potent activator), as well as by reversible protein kinase-mediated phosphorylation [10,11]. In vascular plants and green algae, PK exists as cytosolic (PK_c) and plastidic (PK_p) isoenzymes that differ markedly with respect to their physical, immunological and kinetic characteristics [9,12]. In addition, plant PK_c appears to occur as tissue-specific isoenzymes that demonstrate substantial differences in their kinetic and regulatory properties [13–17], differences that reflect the distinctive metabolic requirements of the respective tissues. Kinetic studies of purified plant PK_c indicated that the enzyme exists naturally in an activated state (similar to the non-plant enzyme activated by fructose 1,6-bisphosphate) [9]. Feedback regulation of PK_c by various inhibitors serves to modulate its activity *in vivo* in accordance with the cell's momentary demands for tricarboxylic acid cycle and respiratory end-products, namely ATP and/or carbon skeletons that serve as biosynthetic precursors. Regulatory properties of PK_c that may be of particular significance include inhibition by:

Abbreviations used: DTT, dithiothreitol; PEG, poly(ethylene) glycol; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PK, pyruvate kinase; PK_c and PK_p, cytosolic and plastidic isoenzymes respectively of PK.

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(i) ATP, (ii) various tricarboxylic acid cycle intermediates, and/or (iii) glutamate, which may be reversed by the activator aspartate [9,12,15–17]

PEP can also be metabolized by PEPC in a Mg^{2+} -dependent β -carboxylation, yielding oxaloacetate and P_i . A ubiquitous cytosolic enzyme in vascular plants and green algae, PEPC is particularly abundant in C_4 and CAM (crassulacean acid metabolism) leaves, where it catalyses the initial fixation of atmospheric CO_2 [18]. Proposed roles for non-photosynthetic and C_3 -leaf PEPCs are diverse, and include: (i) regulation of cellular pH and cation balance; (ii) production of respiratory substrate for N_2 -fixing bacteroids; and (iii) anaplerotic replenishment of tricarboxylic acid cycle intermediates [17]. PEPC has been purified from several non-photosynthetic tissues, and shown to be controlled by a variety of allosteric effectors (particularly inhibition by malate and activation by hexose phosphates) and by reversible protein kinase-mediated phosphorylation [18–22].

Despite the critical importance of PK_c and PEPC in controlling respiration and PEP partitioning in plants, it is surprising that the two enzymes have thus far been subjected to concurrent analysis in only two systems: rapeseed (*Brassica napus*) suspension cell cultures [12,20] and germinating castor (*Ricinus communis*) seed cotyledons [16]. We previously reported the purification and characterization of banana fruit PEPC [21,22]. In the present study, we describe the purification to homogeneity of banana fruit PK_c and document the physical, immunological and kinetic properties of the purified enzyme. A model is presented for the co-ordinate regulation of banana PEPC and PK_c by allosteric effectors. In particular, the combined results of the present and earlier [22] studies indicate roles for cytosolic pH, MgATP, glutamate and aspartate in the control of the PEP branchpoint in climacteric fruit.

EXPERIMENTAL

Chemicals and plant material

ADP, ADP-agarose, EDTA, EGTA, rabbit muscle lactate dehydrogenase, NADH, PEP and phospholipids were purchased from Sigma Chemical Co. Butyl-Sepharose, PD-10, Mono-Q and Superose 6 columns and molecular mass standards were from Amersham Pharmacia Biotech. Acrylamide, bisacrylamide, dithiothreitol (DTT) and PMSF were from ICN Pharmaceuticals. PVDF membranes (0.45 μ m) were obtained from Millipore, DE-52 cellulose was from Whatman, DEAE-650-Fractogel was from Merck, and alkaline phosphatase-conjugated goat anti-(rabbit IgG) IgG was from Promega. All other chemicals were of analytical grade obtained from BDH Chemicals. Ripe banana (*M. cavendishii* L. cv. Cavendish) fruit were purchased from a local retailer and used the same day.

Enzyme and protein assays

The PK_c reaction was coupled to the lactate dehydrogenase reaction and assayed at 25 °C by monitoring the oxidation of NADH at 340 nm using a SpectraMax 250 microplate reader (Molecular Devices). Standard PK_c assay conditions were 50 mM Hepes/KOH (pH 6.9), 25 mM KCl, 10 mM $MgCl_2$, 2 mM PEP, 1 mM ADP, 1 mM DTT, 5% (w/v) poly(ethylene) glycol 8000 (PEG-8000), 0.15 mM NADH and 2 units/ml desalted lactate dehydrogenase, in a final volume of 250 μ l. PEG-8000 was added routinely to the reaction mixture because this organic solute significantly enhances the activity of homogeneous castor-seed PK_c by stabilizing the native enzyme in dilute solutions [23]. All assays were: (i) initiated by the addition of ADP; (ii) corrected for contaminating PEP phosphatase by omitting ADP from the

reaction mixture; and (iii) linear with respect to time and concentration of enzyme assayed. One unit of PK_c activity is defined as the amount of enzyme resulting in the utilization of 1 μ mol of PEP/min at 25 °C.

Apparent K_m values were calculated from the Michaelis–Menten equation fitted to a non-linear least-squares regression computer kinetics program [24]. IC_{50} values were determined using the aforementioned computer kinetics program. Competitive inhibition constants (K_i values) were obtained using Dixon plots [25], whereas uncompetitive inhibition constants (K'_i values) were determined using Cornish-Bowden plots [26]. Kinetic parameters are means of three or more independent determinations, and are reproducible to within $\pm 10\%$ of the mean.

Protein concentration was determined by the dye-binding method of Bradford [27] as modified by Bollag et al. [28], or by the bicinchoninic acid method of Hill and Straka [29]. Bovine γ -globulin was used as the protein standard.

Buffers used in the purification of banana PK_c

Buffer A contained 50 mM Tris base (pH unadjusted), 20 mM KCl, 10 mM thiourea, 3.5 mM $MgCl_2$, 2 mM DTT, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol, 2% (w/v) PEG-8000, 1.5% (w/v) insoluble polyvinylpyrrolidone and 0.1% (v/v) Triton X-100. Buffer B contained 50 mM imidazole/HCl (pH 7.1), 3.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA and 10% (v/v) glycerol. Buffer C contained 50 mM imidazole/HCl (pH 6.5), 3.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol and 20%-satd $(NH_4)_2SO_4$. Buffer D contained 50 mM imidazole/HCl (pH 7.1), 3.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol and 20%-satd $(NH_4)_2SO_4$. Buffer E contained 50 mM imidazole/HCl (pH 6.5), 3.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol and 20% (v/v) ethylene glycol. Buffer F contained 50 mM imidazole/HCl (pH 7.1), 50 mM KCl, 3.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA and 20% (v/v) glycerol. Buffer G contained 50 mM Tris/HCl (pH 7.1), 3.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA and 20% (v/v) glycerol. Buffer H contained 50 mM Tris/HCl (pH 7.4), 50 mM KCl, 3.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA and 20% (v/v) glycerol.

Purification of banana fruit PK_c

All procedures were carried out at 0–4 °C, unless otherwise noted.

Preparation of clarified homogenate

Peeled banana fruit (900 g) was diced and homogenized in 1.4 litres of buffer A using a Waring blender followed by a Polytron. The homogenate was centrifuged at 14000 g for 20 min and filtered through two layers of Miracloth.

Batchwise DE-52 fractionation

The supernatant was stirred gently for 30 min in the presence of 200 g (wet wt) of DE-52 cellulose which had been pre-equilibrated in buffer B and collected with suction on a Büchner funnel. The matrix was isolated by filtration through Miracloth on a Büchner funnel. The filtrate was treated with DE-52 a further two times as described above. The combined DE-52 resin was washed with 1.5 litres of buffer B by filtration as above. Absorbed proteins were eluted by stirring the washed matrix for 15 min with 300 ml of buffer C, followed by filtration as above. This process was repeated twice, and the combined filtrates were adjusted to pH 7.1 with 1 M KOH.

Butyl-Sepharose hydrophobic-interaction FPLC

Butyl-Sepharose (50 ml resin volume) that had been pre-equilibrated in buffer C was added to the DE-52 eluate and mixed for 30 min. The suspension was transferred to a 2 litre graduated cylinder and the resin was allowed to settle for 60 min. The settled resin was packed into a column (2.2 cm $\times$ 13 cm), connected to a FPLC system and washed with buffer D at 4 ml/min until the A_{280} decreased to baseline. The column was developed at 2 ml/min with a linear gradient (200 ml) of 0–100 % buffer E (100–0 % buffer D). PK_c activity was eluted as a single peak of activity at approx. 95 % buffer E. Fractions of 6 ml were collected, and those containing more than 20 % of peak PK_c activity were pooled and concentrated 5-fold by ultrafiltration over a YM-30 membrane. The concentrate was diluted 5-fold in buffer F and reconcentrated. This process was repeated twice, and the concentrate was clarified by centrifugation at 14000 *g* for 10 min.

Fractogel EMD-650 (S) anion-exchange chromatography

The clarified extract was loaded at 2 ml/min on to a column (1.6 cm $\times$ 4 cm) of Fractogel EMD-650 (S) that had been connected to an FPLC system and pre-equilibrated in buffer F. The column was washed with buffer F until the A_{280} decreased to baseline, and then developed at 2 ml/min with a linear gradient (60 ml) of 50–250 mM KCl in buffer F while collecting 1.5 ml fractions. PK_c was eluted as a single peak of activity at approx. 125 mM KCl. Fractions containing greater than 20 % of peak PK_c activity were pooled, concentrated to 2.5 ml by ultrafiltration over a YM-30 membrane, and desalted using a PD-10 column that had been pre-equilibrated in buffer G.

ADP–agarose affinity chromatography

This step was performed at room temperature. The desalted sample was loaded on to a column (1.0 cm $\times$ 6.3 cm) of ADP–agarose (Sigma; cat. no. A-4398) at 0.3 ml/min. The column was washed at 0.3 ml/min with 15 ml of buffer G, and PK_c was eluted with 5 ml of buffer G containing 3 mM ADP. Fractions of 0.5 ml were collected, and those containing PK_c activity were pooled.

Mono-Q anion-exchange FPLC

The pooled fractions were loaded at 1 ml/min on to a Mono-Q (HR 5/5) column that was connected to an FPLC system and pre-equilibrated with buffer H. The column was washed at 1 ml/min with 10 ml of buffer H and developed with a linear gradient (20 ml) of 50–250 mM KCl. Fractions (1.0 ml each) containing PK_c activity were pooled, concentrated to 1.1 ml by ultrafiltration over a YM-30 membrane, frozen in liquid N₂ in 25 μ l aliquots, and stored at -80°C . PK_c activity was stable for at least 4 months when stored at -80°C .

Determination of native molecular mass by gel-filtration FPLC

This was performed by FPLC on a Superose 6 HR 10/30 column pre-equilibrated with 50 mM Hepes/KOH (pH 6.9) containing 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, 1 mM EDTA and 10 % (v/v) glycerol. Purified PK_c (100 μ l; 0.45 mg/ml) was chromatographed at 0.2 ml/min. Fractions of 0.25 ml were collected and assayed for PK_c activity and A_{280} . Native molecular mass was calculated from a plot of K_D (partition coefficient) against log(molecular mass) using the following protein standards: thyroglobulin (669 kDa), apoferritin (443 kDa), catalase (232 kDa), aldolase (158 kDa) and BSA (66 kDa). The column's void volume was determined using Dextran Blue.

Electrophoresis and immunoblotting

SDS/PAGE was performed with a Bio-Rad Minigel apparatus using the method of Laemmli [30]. The final acrylamide concentration in the 0.75 mm-thick slab gels was 10 % (w/v) for the separating gel and 4 % (w/v) for the stacking gel. Extraction of bananas under denaturing conditions using 10 % (w/v) trichloroacetic acid was performed as described by Wu and Wang [31]. Before electrophoresis, all samples were incubated for 3 min at 100°C in the presence of 50 mM Tris/HCl (pH 6.8) containing 50 mM DTT, 10 % (v/v) glycerol and 1 % (w/v) SDS. SDS/PAGE was performed at 200 V for 45 min, and the gel was stained with Coomassie Brilliant Blue R-250. The subunit molecular mass was calculated from a plot of relative mobility against log[molecular mass (kDa)], with the following standard proteins: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (22 kDa), lysozyme (14 kDa) and aprotinin (7 kDa).

Immunoblotting was performed using affinity-purified rabbit antibodies against *B. napus* PK_c or castor-seed PK_p, as described previously [12,32]. Antigenic polypeptides were visualized using an alkaline phosphatase-conjugated secondary antibody. Phosphatase staining was for 5–10 min at 30°C . Immunological specificity was confirmed by performing immunoblots in which rabbit preimmune serum was substituted for the anti-(*B. napus* PK_c) IgG.

RESULTS

Purification of PK_c from banana fruit

The use of unbuffered Tris in the homogenization buffer prevented acidification of the extract, and resulted in a clarified homogenate of approx. pH 7.1. The initial batchwise DE-52 fractionation step was necessary to remove high levels of gelatin and pectin present in banana fruit. Unless rapidly removed, these compounds would cause the crude extract to gel within 30 min and therefore preclude conventional methods of protein fractionation.

As shown in Table 1, PK_c was purified 543-fold to a final specific activity of 59.7 units/mg and an overall recovery of 6 %. The protein concentration of the final preparation using the Coomassie Brilliant Blue R-250 dye binding and bicinchoninic acid methods was 0.48 and 0.45 mg/ml respectively. Using the latter method of protein determination, the specific activity of the purified PK_c is thus increased from 59.7 to 63.7 units/mg.

Physical and immunological properties

The purified enzyme was relatively heat stable, retaining 92 % of its activity following incubation at 55°C for 5 min. Denaturation, followed by SDS/PAGE of the final preparation, resolved a single Coomassie Brilliant Blue-staining polypeptide of 57 kDa (Figure 1A, lanes 1 and 2) that cross-reacted strongly with anti-(*B. napus* PK_c) IgG (Figure 1B, lane 2). No cross-reaction was observed when an immunoblot of 50 ng of the final preparation was probed with anti-(castor seed PK_p) IgG. An immunoblot of a clarified banana extract prepared under denaturing conditions also resulted in a single immunoreactive polypeptide of 57 kDa (Figure 1B, lane 1), indicating that proteolysis of the enzyme did not occur during its isolation.

The native molecular mass of the enzyme, as estimated by gel-filtration FPLC of the final preparation, was 240 ± 12 kDa (mean $\pm$ S.E.M., $n = 3$). The molar absorption coefficient of banana PK_c was determined to be $1.17 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm ($A_{280}^{0.1\%} 0.487$). This value was based upon the Coomassie

Table 1 Purification of PK_c from 900 g of ripe banana fruit

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Clarified extract	1750	6563	730	0.11	—	100
Batchwise DE-52	1200	1800	370	0.21	1.9	51
Butyl-Sepharose	30.5	132	162	1.2	10.9	22
DEAE-Fractogel	10	57	119	2.1	19.1	16
ADP-agarose	3.3	2.54	63	24.8	225	9
Mono-Q FPLC	1.5	0.72	43	59.7	543	6

Figure 1 SDS/PAGE and immunoblot analysis of banana fruit PK_c

(A) SDS/PAGE (10% separating gel) analysis of purified banana PK_c. Lane 1 contains 8 µg of various protein standards. Lanes 2 and 3 contain 1 and 3 µg respectively of the final PK_c preparation. Protein staining was performed with Coomassie Brilliant Blue R-250. (B) Immunoblot analysis performed using anti-(*B. napus* PK_c) IgG. Antigenic polypeptides were visualized using alkaline phosphatase-tagged secondary antibody. Lane 1 contains 10 µg of protein from a clarified extract prepared from banana under denaturing conditions [31]. Lane 2 contains 20 ng of purified banana PK_c. TD, tracking dye.

Table 2 Use of metal ion cofactors by banana PK_c

Assays were conducted at pH 6.9 using the standard spectrophotometric assay, except that the concentration of the univalent or bivalent cation was varied.

Cofactor	$V_{\max}$ (units/mg)	K_m (mM)	$V_{\max}/K_m$ (units/mg · mM ⁻¹)
K ⁺	59.7	0.91	66
NH ₄ ⁺	40.0	3.2	13
Mg ²⁺	59.7	0.27	221
Mn ²⁺	42.8	0.35	122

Brilliant Blue G-250 dye-binding protein assay [28] and calculated by assuming a native molecular mass of 240 kDa.

Kinetic properties

The enzyme displayed a broad and symmetrical pH/activity profile, with maximal activity at pH 6.9. Kinetic studies were performed at this pH and at pH 7.5, where activity was 70 % of the maximum. The enzyme's pH/activity profile was unchanged when 25 mM Mes/25 mM Bis-Tris/propane was substituted for Hepes/KOH, or when 10 % (v/v) glycerol was used instead of 5 % (w/v) PEG-8000.

Table 3 Influence of assay pH on kinetic constants for substrates of banana PK_c

The standard spectrophotometric assay was used, except that the concentration of PEP or MgADP was varied.

Parameter	pH 6.9	pH 7.5
$V_{\max}$ (units/mg of protein)	59.7	41.8
K_m^{PEP} (mM)	0.098	0.067
K_m^{MgADP} (mM)	0.121	0.082
$V_{\max}/K_m^{\text{PEP}}$ (units/mg · mM ⁻¹)	610	620
$V_{\max}/K_m^{\text{MgADP}}$ (units/mg · mM ⁻¹)	490	510

Cofactor requirements and substrate saturation kinetics

Tables 2 and 3 summarize the $V_{\max}$ and apparent K_m values obtained for metal cation cofactors and substrates of PK_c at pH 6.9 and 7.5. The enzyme exhibited hyperbolic saturation kinetics for Mg²⁺, K⁺, PEP and ADP. Its activity showed an absolute requirement for both a univalent and a bivalent cation, with K⁺ and Mg²⁺ respectively satisfying this requirement. NH₄⁺ and Mn²⁺ could substitute for K⁺ and Mg²⁺ respectively, albeit with lower catalytic efficiencies (Table 2). Although $V_{\max}$ was approx. 30 % lower at pH 7.5 than at pH 6.9, the K_m^{PEP} and K_m^{ADP} values were also lower at the higher pH. Thus the enzyme's catalytic efficiencies ($V_{\max}/K_m$) with both PEP and ADP were very similar at the two pH values (Table 3). In contrast with castor endosperm PK_c, which demonstrated substrate inhibition by ADP at concentrations greater than 1 mM [15], no inhibition of banana PK_c by ADP (up to 5 mM) was observed.

Banana PK_c could utilize alternative nucleotide diphosphates as substrates (Table 4). However, as judged by the respective specificity constants, ADP is the preferred nucleotide substrate for the enzyme.

Table 4 Use of alternative nucleotide diphosphates by banana PK_c

Assays were conducted at pH 6.9 using the standard spectrophotometric assay, except that the NDP concentration was varied. Abbreviation: n.d., not determined.

Nucleotide	$V_{\max}$ (units/mg)	K_m (mM)	$V_{\max}/K_m$ (units/mg · mM ⁻¹)
MgADP	59.7	0.121	493
MgUDP	53.3	0.22	242
MgIDP	43.9	1.6	27
MgGDP	37.2	1.3	29
MgCDP	39.7	4.5	9
MgTDP	6.6	n.d.	n.d.

Table 5 Effects of various metabolites on the activity of banana PK_c

Assays were conducted at pH 6.9 and pH 7.5 in the presence of subsaturating concentrations (0.1 mM each) of PEP and ADP, and 10 mM MgCl₂. Enzyme activity is expressed relative to the control (set at 100%), and was determined in the presence of 5 mM effector, except for AMP, where the final concentration was 0.5 mM. Abbreviation: n.d., not determined.

Metabolite	Relative activity (%)		IC ₅₀ (mM)	
	pH 6.9	pH 7.5	pH 6.9	pH 7.5
MgAMP	80	90	n.d.	n.d.
MgATP	52	58	4.8	5.5
Magnesium citrate	71	88	8.0	16.0
Magnesium isocitrate	78	85	8.1	14.0
Fructose 1,6-bisphosphate	64	82	9.3	18.0
L-Glutamate	55	30	5.3	2.2
L-Malate	82	97	38.0	39.0
2-Phosphoglycerate	78	100	n.d.	n.d.
3-Phosphoglycerate	72	95	11.0	34.0

Metabolite effects

A wide variety of compounds were tested as possible effectors of PK_c at pH 6.9 and pH 7.5 with subsaturating concentrations of PEP and ADP (0.1 mM each). The following compounds had little or no influence ($\pm 15\%$ control velocity) on PK_c activity: alanine, arginine, aspartate, asparagine, glutamine, phenylalanine, proline, 2-oxoglutarate, succinate, glycerol 3-phosphate, dihydroxyacetone phosphate, P_i, PP_i, UDP-glucose, ADP-glucose, fructose 6-phosphate, glucose 6-phosphate, glucose 1-phosphate, glucuronate 6-phosphate, ribose 5-phosphate, glucose, fructose, sucrose, NAD⁺, γ -aminobutyrate, lactate, acetaldehyde and ethanol (5 mM each), 1 mM acetyl-CoA, 0.5 mM shikimate, 0.4 mM rutin or quercetin, and 5 μ M fructose 2,6-bisphosphate. Under the test conditions employed, activation of PK_c by any of these compounds was not observed at either pH 6.9 or pH 7.5. In contrast with bovine heart PK, which is activated by liposomes containing phosphatidylserine [33], liposomes composed of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol or phosphatidylserine (formed as described in [33]) did not influence the activity of banana PK_c.

Table 5 lists those metabolites that were found to inhibit PK_c. The enzyme displayed pH-dependent modulation of activity such that, with the exception of glutamate, inhibition was more pronounced at pH 6.9 than at pH 7.5. When tested in pairs, the effectors listed in Table 5 produced additive inhibition. Inhibition by glutamate and ATP was characterized further, and the patterns of inhibition and inhibition constants, determined at pH 6.9 and pH 7.5, are presented in Table 6. With PEP or ADP as the vari-

Figure 2 Influence of L-aspartate on the inhibition of banana fruit PK_c by L-glutamate

Assays were conducted at pH 6.9 and pH 7.5 in the presence of subsaturating concentrations of PEP and ADP (0.1 mM each). IC₅₀ (*I*_{0.5}) values for glutamate were determined in the presence of various concentrations of aspartate.

able substrate, inhibition by glutamate increased with an increase in pH, whereas inhibition by ATP was essentially unchanged (Table 6). Mixed-type inhibition by glutamate with respect to PEP or ADP at either pH suggests that it may interact at an allosteric site. ATP inhibition of PK is widespread, as is expected for a product of the enzyme's reaction. At pH 6.9 or pH 7.5, inhibition by ATP was competitive with respect to ADP, but mixed with respect to PEP.

As reported for some plant PK_cs [12,17,34], aspartate reversed the inhibitory effect of glutamate on PK_c, with the effect being more pronounced at pH 6.9 than at pH 7.5 (Figure 2). This appears to be a very specific effect, since aspartate did not influence the response of PK_c to the other inhibitors listed in Table 5, and did not activate the enzyme in the absence of glutamate. Similarly, the following metabolites did not influence inhibition of PK_c by glutamate: glucose, fructose, sucrose, glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate (all 10 mM), and 10 μ M fructose 2,6-bisphosphate.

DISCUSSION

The present study was undertaken with the goals of purifying and characterizing PK_c from banana fruit, and of comparing its kinetic and regulatory properties with those documented previously for banana PEPC. As the maximal extractable activities of banana fruit PEPC and PK_c are equivalent and remain

Table 6 Inhibition pattern and enzyme-inhibitor dissociation constants for two banana PK_c inhibitors at pH 6.9 and pH 7.5

The invariant substrate concentration was 2 mM. *K_i* and *K_i'* values were determined as described in the Experimental section. Abbreviations: C, competitive inhibition; M, mixed-type inhibition.

Inhibitor	Variable substrate...	MgADP			PEP		
		Inhibition type	<i>K_i</i> (mM)	<i>K_i'</i> (mM)	Inhibition type	<i>K_i</i> (mM)	<i>K_i'</i> (mM)
L-Glutamate	pH 6.9	M	19.2	116	M	13.6	40.1
	pH 7.5	M	3.7	52.1	M	4.4	19.4
MgATP	pH 6.9	C	3.9	—	M	9.6	22.7
	pH 7.5	C	4.0	—	M	10.0	22.7

constant throughout ripening [22], the stimulation of PEP utilization at the initiation of the climacteric [7,8] must thus arise from fine control of PK_c and/or PEPC, and not from *de novo* enzyme synthesis.

Purification of PK_c and its physical and immunological properties

The specific activity of approx. 60 units/mg obtained with purified banana PK_c (Table 1) compares favourably with those of homogeneous PK_c preparations from other plant sources [12,13,15,17]. SDS/PAGE of the final preparation confirmed that the enzyme was purified to homogeneity (Figure 1A). Gel-filtration FPLC and SDS/PAGE analyses indicated that, similar to many other PKs, the native banana PK_c exists as a homotetramer of 240 kDa. That it corresponded to a PK_c, and not a PK_p, was established by three observations: (i) the purified enzyme was relatively heat stable, a characteristic of PK_c but not PK_p [12–14,16,17,32]; (ii) the enzyme's pH optimum of 6.9 is characteristic of other plant PK_cs [12,15–17], whereas PK_p has a relatively sharp pH optimum of approx. pH 8 [32]; and (iii) the purified enzyme cross-reacted with anti-(*B. napus* PK_c) IgG (Figure 1B), but not with anti-(castor-seed PK_p) IgG. The immunological distinctiveness of plant PK_c and PK_p isoenzymes has been well documented [9,12,14,17,32].

Kinetic studies

The activity of banana PK_c exhibited an absolute dependence on a univalent and a bivalent cation cofactor, hyperbolic substrate saturation kinetics, similar affinities for PEP and MgADP, and good use of alternative purine and pyrimidine nucleotide diphosphates, particularly UDP (Tables 2–4).

Our results demonstrate that banana PK_c (Tables 5 and 6) is regulated by metabolites involved in amino acid and energy metabolism in a pH-dependent fashion. In particular, this PK_c demonstrated significant inhibition by glutamate and ATP, with the response to glutamate being somewhat diminished at pH 6.9 relative to pH 7.5 (Tables 5 and 6, Figure 2). The possible significance of these effectors *in vivo* is indicated by comparing the IC₅₀ values with their corresponding cytosolic concentrations in ripened banana fruit. Assuming that ATP is localized mainly in the cytosol, and that the cytosol comprises 5% of the cell's volume, then one can estimate from a previous study [35] that the cytosolic ATP concentration of ripening banana fruit is approx. 1 mM. Thus the physiological relevance of the inhibition of banana PK_c by ATP is not obvious, since the enzyme's IC₅₀ value for ATP is approx. 5 mM (Table 5). Nevertheless, inhibition by ATP could potentially be involved in the control of banana PK_c *in vivo*, particularly during situations when the cytosolic ATP concentration becomes elevated relative to that of PK_c's co-substrate, ADP. No information is available on the cytosolic glutamate concentration of ripened banana fruit. However, next to sucrose, glutamate is the most highly concentrated compound in the cytosol of many plant cells [36]. For example, the glutamate concentration of the leaf cytosol ranges from approx. 20 to 90 mM [37]. Glutamate is thus expected to have major significance in the allosteric control of banana PK_c (Tables 5 and 6, Figure 2) and PEPC [22] *in vivo*. Both glutamate and ATP have been implicated in the regulation of PK_c and PEPC in various plant tissues and in green algae [9,12,16–18,20,22,23,34,38,39]. Inhibition of banana PK_c by glutamate was mixed with respect to PEP and ADP, with enzyme-inhibition dissociation constants inversely proportional to assay pH (Table 6). It is intriguing that aspartate functioned as an activator of banana PK_c only in the presence of glutamate, with the effect being more pronounced at

pH 6.9 than at pH 7.5 (Figure 2). These data suggest that the activity of banana PK_c *in vivo* may be a balance between cytosolic effector concentrations (e.g. aspartate/glutamate concentration ratio, and possibly ATP levels) and cytosolic pH. The influence of pH was also apparent on the substrate saturation kinetics and effector sensitivity of banana PEPC, with PEPC activity becoming greater and responses to effectors being decreased as the assay pH was increased [22]. Therefore cytosolic pH may be an important factor in co-ordinating the relative flux of PEP through PK_c and PEPC in ripening bananas, with the balance moving from PEPC to PK_c upon cytosolic acidification and vice versa.

The ability of aspartate to reverse the inhibition by glutamate of plant PK_c has been documented previously for the enzyme from castor seed and spinach leaves [17,34], and from heterotrophic *B. napus* cell cultures [12]. This differs from banana PEPC, which was potently inhibited by both glutamate and aspartate (IC₅₀ values of 0.34 and 0.07 mM respectively at pH 7.0), but activated by hexose phosphates [22]. By contrast, hexose phosphates did not influence banana PK_c activity or its susceptibility to inhibition by glutamate.

Co-ordinate regulation of PK_c and PEPC during banana fruit ripening

The stimulation of respiration that occurs during ethylene-induced banana ripening has been ascribed to an initial activation (or de-inhibition) of PK_c and/or PEPC, leading to the increased production of ATP, which is required to power the conversion of starch into sucrose and associated substrate (futile) cycles [6–8]. Activation of either enzyme would be expected to alleviate PEP inhibition of ATP-dependent phosphofructokinase, and thus allow increased entry of hexose phosphates into glycolysis.

Scheme 1 presents a model summarizing the allosteric features of banana PK_c and PEPC that may be important in co-ordinating carbohydrate metabolism and PEP partitioning in this tissue. Starch-derived hexose phosphates are believed to be the major form of carbon exported from the amyloplast of ripening bananas [6]. Activation of PEPC by glucose 6-phosphate would balance the rate of starch mobilization with the production of dicarboxylic acids via PEP carboxylation. Inhibition of PEPC by L-malate provides a tight feedback control that closely links PEPC activity with the overall rate of L-malate metabolism. Inhibition by ATP of banana PEPC (IC₅₀ 1.3 mM [22]) and PK_c (Tables 5 and 6) agrees with earlier proposals that the respiration rate of ripening bananas depends largely on the tissue's demand for ATP, and that the respiratory climacteric is created by rapid adenylate turnover brought about by the massive conversion of amyloplastic starch into sucrose [6,40]. However, the allosteric features of banana PEPC and PK_c suggest that, in addition to regulating the provision of respiratory substrates for mitochondrial ATP production, both enzymes may also play a pivotal role in co-ordinating carbon and nitrogen metabolism in this tissue.

Significant rates of protein synthesis, as well as differential gene expression, have been associated with the ripening process of bananas [3,41]. Thus PK_c and/or PEPC may have an additional function to replenish carbon skeletons (e.g. oxaloacetate and 2-oxoglutarate) consumed during NH₄⁺ assimilation and/or transamination reactions in ripening bananas. It has similarly been proposed that allosteric modulation of PK_c and PEPC in *B. napus* suspension cells by glutamate and aspartate regulates the anaplerotic replenishment of tricarboxylic acid cycle intermediates during NH₄⁺ assimilation [12]. However, the interactive effects of glutamate and aspartate on *B. napus* cell culture and banana fruit PK_c and PEPC indicates that anaplerotic

Scheme 1 Model for the allosteric regulation of PK_c and PEPC in banana fruit

The co-ordinate control of banana PK_c and PEPC [22] by allosteric effectors provides a mechanism for the control of glycolytic flux and PEP partitioning during respiration and amino acid biosynthesis, as discussed in the text. Abbreviations: Cit, citrate; OAA, oxaloacetate; Mal, malate; 2-OG, 2-oxoglutarate; PFK, phosphofructokinase; Pyr, pyruvate; TCA, tricarboxylic acid; E.T.C., electron transport chain; -P denotes phosphate.

flux will respond not just to nitrogen assimilation, but also to the manner in which carbon is drained from the tricarboxylic acid cycle as a consequence of amino acid biosynthesis. If NH₄⁺ assimilation via glutamine synthetase/glutamine-2-oxoglutarate aminotransferase occurs, the tricarboxylic acid cycle will be drained from citrate (leading to cytosolic 2-oxoglutarate production via cytosolic isoenzymes of aconitase and isocitrate dehydrogenase). The synthesis of aspartate and aspartate-derived amino acids will result in a depletion of oxaloacetate. In banana fruit, the production of citrate by the combined action of PEPC, malate dehydrogenase and NAD⁺-malic enzyme, rather than by PK_c, has been shown to be negligible [6]. Following incorporation of ¹⁴CO₂ into fruit tissue slices, more than 95% of the incorporated ¹⁴C was found in malate and aspartate, and was not rapidly re-metabolized [6]. Therefore, as noted previously [39,42] and outlined in Scheme 1, replenishment of citrate withdrawn from the tricarboxylic acid cycle necessitates equal contributions from PK_c and PEPC, whereas replenishment of oxaloacetate requires only PEPC. The allosteric control of banana PK_c (Tables 5 and 6, Scheme 1) and PEPC [22] by aspartate and glutamate allows for such co-ordinated anaplerotic

replenishment. Feedback inhibition of banana PK_c and PEPC by glutamate provides a rationale for the activation of the two enzymes that occurs *in vivo* during periods of enhanced nitrogen assimilation (when cellular glutamate concentrations are decreased) [39]. In contrast with its effects on PEPC [22], aspartate functions as an activator of banana PK_c by effectively reversing inhibition of the enzyme by glutamate (Figure 2). This provides a mechanism for decreasing flux from PEP to aspartate (via PEPC and aspartate aminotransferase), while promoting PK_c activity when cytosolic L-aspartate concentrations become elevated. Neither banana PK_c nor PEPC [22] were responsive to asparagine or glutamine, two metabolites thought to signal nitrogen status in fruits and seeds [43].

Further work is required to determine the extent to which regulation of the PEP branchpoint occurs *in vivo* throughout the ripening process. In particular, it would be of interest to apply the methodologies of Edwards and co-workers [42] to determine the relative flux of PEP through PK_c compared with PEPC during banana fruit development and ripening. In addition, time-course studies of ripening-induced alterations in banana cytosolic pH, as well as PEPC and PK_c effector levels (particularly ATP, glutamate and aspartate), are required in order to define further the precise roles and relative contributions of PEPC and PK_c to metabolism in ripening fruit.

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