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PHYSICAL AND KINETIC PROPERTIES OF PHOTOSYNTHETIC PHOSPHOENOLPYRUVATE CARBOXYLASE IN DEVELOPING APPLE FRUIT

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Abstract—Phosphoenolpyruvate carboxylase (PEPC) was partially purified from young developing apple fruit, cultivars Golden Delicious and Cox's Orange Pippin. Freeze-drying of tissue reduced the yield of PEPC activity compared to samples stored at 4°. Activities measured by $H^{14}CO_3^-$ incorporation exceeded the spectrophotometric assay for the enzyme with coupled NADH-malate dehydrogenase (MDH) by up to 60%. The enzyme could be stored at −16° with glycerol and bovine serum albumin for several months without loss of activity. Thermal inactivation of PEPC occurred after heating to 75° for 3 min when MDH was still slightly active. Inhibition of PEPC activity by endogenous phenolics could be prevented by grinding in liquid nitrogen in the presence of polyvinylpyrrolidine and dithiothreitol. Apparent K_m (PEP) and V_{max} values compared more favourably with those obtained from a C_3-species (spinach) than from a C_4-species (maize). L-Malate (5 mM) inhibited fruit PEPC by 22%; this was decreased to 12% by addition of glucose-6-phosphate (2 mM). From kinetic and effector experiments PEPC in the apple fruit is concluded to be a non-C_4 photosynthetic enzyme.

INTRODUCTION

Phosphoenolpyruvate carboxylase [orthophosphate: oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31] (PEPC), catalyses the carboxylation of phosphoenolpyruvate (PEP) with HCO_3^- to produce oxaloacetate (OAA) (Equation 1) in the presence of Mg^{2+} and/or Mn^{2+} under physiological conditions [1]:

$$(1)\ \text{PEP} + HCO_3^- \xrightarrow{Mg^{2+}(Mn^{2+})} \text{OAA} + \text{Pi}.$$

PEPC occurs in most plants and most plant tissues [2]. In plants photosynthesizing in a typical C_4/CAM pattern, this key enzyme is responsible for primary photosynthetic carboxylation/CO_2 assimilation into C_4 acids [1]. In CAM plants [2] and certain other plant tissues, e.g. in some fruit, it is involved in dark CO_2 fixation. In plants with predominant C_3-photosynthesis, PEPC is involved elsewhere in the carbon metabolism, e.g. in the regulation of the cellular pH and cation balance [3]. Apart from the CAM-exhibiting pineapple [4], PEPC has been reported in developing fruit of plants with C_3 type photosynthesis, such as citrus [5–7], avocado [8], grape [9–12] and apple [13]. In these heterotrophic tissues, an internal CO_2 concentration exceeding the ambient is built up by mitochondrial respiration of predominantly imported carbon. A possible function of PEPC therefore is to reassimilate respired CO_2 into organic, C_4 acids [14]. Inhibition of this reassimilation *in vitro* starts above 3–5% CO_2 concentration [15]. As with other typical C_4/CAM enzymes, PEPC activity in some fruit was commensurate to that of C_4/CAM leaves [14], suggesting that CO_2 recycling may follow some C_4/CAM patterns.

In the apple fruit, PEPC activity was detected in the cortex. The fruit, harvested at the preclimacteric minimum, was examined for PEPC activity after storage. PEPC activity, on a fresh weight basis, was constant and small relative to aspartate aminotransferase (GOT) and NADP-malic enzyme (m.e.) in the same tissues [13]. PEPC was not originally proposed to account for the dark CO_2 fixation of the apple fruit, although the similarity to CAM patterns was pointed out [16].

For fruit, a dualism of chloroplasts similar to that existing in the C_4 anatomy, has so far only been discovered in the apple [17]. Hypodermal apple chloroplasts are analogous to the C_4 mesophyll type, whereas the chloroplasts surrounding the internal vascular bundle resemble the C_4 bundle sheath type. No attempt has yet been made to categorize apple and other fruit chloroplasts according to the three decarboxylating subtypes i.e. phospho(enol)pyruvate carboxykinase (PEPCK), NAD- and NADP-m.e., as developed with the C_4 Gramineae. Based on the chloroplast dualism in apple fruit, C_4-photosynthesis has been suggested for the internal tissues surrounding the vascular bundle [17].

Kinetic values for multiple forms of leaf PEPCs showed larger K_m (PEP, Mg) and V_{max} values for the C_4 than the C_3 *Atriplex* subspecies [18]. PEPC from non-autotrophic tissues had C_3-PEPC properties; PEPCs of CAM type tissue were reported to exhibit small K_m (PEP, Mg), but large V_{max} values [19]. Some species of the Panicum family ('C_3/C_4 intermediates') were shown to have PEPC with small K_m and V_{max}, commensurate to C_3 photosynthetic types [20].

These results indicate that the K_m values of PEPC in CAM and C_3/C_4 intermediate species and non-

autotrophic tissues are similar in magnitude to characteristic PEPC C_3 K_m values; only tissues photosynthesizing exclusively to the C_4 pattern exhibit very different kinetic properties, i.e. generally several-fold larger both K_m and V_{max} values than non-C_4 species.

In developing fruit, PEPC has only been partially purified and characterized in the grape [11], where the enzyme, as in apple fruit, is involved in accumulation and degradation of the malic acid [14]. In developing grape berry a PEPC was identified in the flesh with apparent kinetic properties (K_m = 0.09 mM PEP; inhibition by L-malate) similar to the C_3- or non-autotrophic photosynthetic type.

This paper presents data on the influence on sample preparation and variations in assay systems on detectable PEPC activity. We characterize some properties of PEPCs in three different tissues of two apple cultivars, Golden Delicious and Cox's Orange Pippin. To categorize these PEPC alloenzymes in developing apple fruit, we have compared the apparent kinetic properties with those achieved from a typical C_4 species, maize, and a C_3 species, spinach. An attempt is made to discuss the intermediate position of the apple fruit photosynthesis between the C_3, non-autotrophic, CAM and the strict C_4 type.

RESULTS

Freeze-drying whole apple organs or particular tissues, diminished extractable PEPC activity compared with fresh or cold-stored material. Freeze-dried seeds and vascular bundle tissue yielded respectively 36% and 61% of the PEPC activity of cold-stored material. A similar result has been reported using tissue of *Kalanchoe* leaves (CAM) [21]. The extraction and partial purification of fruit PEPC from fresh tissue resulted in a final enzyme recovery of 30% of the activity of the centrifuged crude extract. This recovery could be increased to 44% by the addition of HCO_3^- (5 mM final concentration) to both extraction and suspension buffers, confirming previous results [Priestley, C. A., personal communication].

Most of the apple fruit PEPC activity precipitated from crude extracts at 65% saturation with ammonium sulphate. The precipitate was redissolved and passed through a G-25 Sephadex column to remove potential enzyme substrates and nucleotides. Optimum pH for extraction of the enzyme varied within species, tissue and season, but values were usually between pH 7.7 and 8.9 for the apple fruit, in general agreement with values for other tissues of *ca* pH 8 [22].

Using commercial purified PEPC from maize, both the isotope method and the spectrophotometric method gave similar activities. By contrast, with partially purified PEPC from apple tissue, the activity detected using the isotope method exceeded that of the spectrophotometric method by up to 60%. In the isotope method, two ways of removing excess $^{14}CO_2$, i.e. acid or heat treatment, gave similar results, provided samples were treated for several hours. In the spectrophotometric assay, the substrate for PEPC can be added either as PEP or generated from 2-phosphoglyceric acid and enolase [23]. After an initial time of a few minutes with the PEP-generating system, the two methods (addition of PEP and PEP-generating system), provided the same rates. The spectrophotometric assay gave a linear relationship between enzyme and rates, over the range used. The partially purified extracts were found to be substantially free of endogeneous phosphatase activity, thereby ensuring the stability of the PEP.

Apple fruit preparations are severely affected by endogenous phenoloxidases [24]. Crude apple extracts, left at room temperature without additives, to allow 'browning' to proceed, strongly inhibited (by 64%) a PEPC preparation from apple fruit, which had been partially purified in the cold with DTT and PVP as additives. In contrast to PEPC from CAM plants [25], carbonic anhydrase (30 units), added to ensure rapid HCO_3^-/CO_2 equilibrium, did not enhance PEPC activity; pyridoxal-5-phosphate (40 μM) was similarly non-enhancing (Table 1).

Our partially purified apple PEPC preparations could be stored without loss of activity up to 1 year at −16° in the presence of 10% glycerol and 1% BSA. At −16° without glycerol, or at 4° with glycerol, 7% of the initial enzyme activity was lost after 10 weeks. Without glycerol the PEPC extract could be stored at 4° for up to 3 days without detectable decay while at 25° the activity was lost completely after 7 days.

The PEPC from a C_4 plant was reported to be more heat sensitive, i.e. complete loss of activity at 50° [26], than malate dehydrogenase and malic enzyme in the grape berry [12]. Thermal inactivation of PEPC from seeds of mature apple fruit occurred after heating the enzyme at 75° for 3 min or 67° for 10 min, although MDH was still active after this latter treatment when assayed at 20° (Table 2). The effect of temperature on the PEPC reaction was studied by progressively raising the temperature from 20 to 67° in the spectrophotometer over a 10–15 min period. Slopes were obtained from the resulting curve (Fig. 1). Under these conditions maximal activity was observed around 60–64°, compared to 38° for PEPC from 2–3 week old grape berries [12] when no exogenous MDH was added to the assay. Maximum activity and thermal inactivation *in vitro* of PEPC for mature apple fruit occurred at around 20°, higher temperatures relative to previously reported data for the grape berry [12] and *Eleusine* leaves (C_4) [26].

The kinetic data for apple fruit PEPC alloenzymes were evaluated at the beginning of maturation, when PEPC activity was increasing (results not shown). Both apple cultivars, Golden Delicious and Cox's Orange Pippin and two fruit tissues were investigated, i.e. the vascular bundle region and the seeds, these showing the greater activities.

Table 1. Effect of crude extract and effectors on the activity of partially-purified apple PEPC

Additive	PEPC activity* (% of purified apple PEPC)
None	100
Crude apple extract	36
Pyridoxal-5-phosphate (40 μM)	103
Carbonic anhydrase (30 units)	100

*PEPC activity measured spectrophotometrically. 100% = 0.05 nkat/200 μl.

†Apple vascular bundle tissue (2 g) ground with 7.5 ml Tris buffer, pH 7.8, and the centrifuged extract left to stand for 15 min at room temperature. 200 μl added to 200 μl of purified apple PEPC.

Table 2. Effect of pre-incubation time and temperature on PEPC activity, using the coupled NADH-MDH system

Pre-incubation time (min)	Temperature (°)	PEPC activity	MDH activity
0	20	1	3.20
10	65	0.08	1.77
10	67.5	0.04	1.68
3	70	0.02	1.37
3	75	0	0.61
3	80	0	0.06

PEPC activity measured spectrophotometrically with excess PEP and MDH present. PEPC obtained from apple seeds cv. Golden Delicious sampled at the end of September.

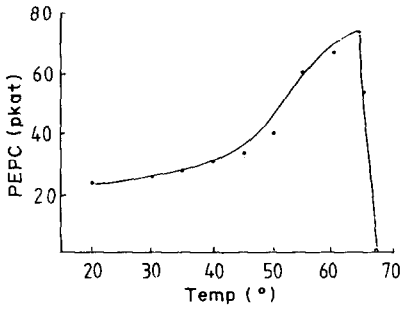

Fig. 1. Effect of temperature on the reaction velocity of phosphoenolpyruvate carboxylase from apple fruit cv. Cox's Orange Pippin vascular bundle.

To categorize the alloenzymes from these tissues they were compared with PEPCs from young spinach and maize leaves, considered respectively, to be representatives of typical C_3- and C_4-photosynthesis species. Our K_m (PEP) value (1.5 mM) for maize confirms previous findings, with values around 1 mM at pH 8 [27] compared with a spinach value of 0.088 mM. Other reported K_m (PEP) values for C_3-PEPC alloenzymes have been only five-fold lower than C_4-PEPCs [18]. For the apple PEPCs we found apparent K_m (PEP) and V_{max} values (Table 3) of the order of those for spinach, compared to much larger values for maize. Within the apple cultivars the K_m (PEP) of both Cox's Orange Pippin vascular bundle and seeds was *ca* two-fold the values of the same tissues of Golden Delicious which may reflect their physiological difference, e.g. larger respiration rates of the Cox's Orange Pippin relative to Golden Delicious [28]. Our Golden Delicious K_m (PEP) value (0.09 mM) for PEPC was identical with that reported for the grape berry enzyme [11].

The K_m (Mg^{2+}) values for the apple fruit PEPCs were commensurate within apple tissues of both varieties, resembling our C_3 spinach and previous maize (C_4) value [22] and smaller than the value reported previously for spinach (0.08 mM) when extrapolated to infinite PEP concentration [29].

The K_m (Mn^{2+}) apple PEPCs were also smaller than previously reported for other tissues and were lower relative to the K_m (Mg^{2+}) values, confirming previous results [29]. The K_m values for the apple tissues suggest one common form of PEPC for both types of tissues and probably for both varieties.

Values of V_{max}, expressed as nkat/g fr. wt, were 50% of V_{max} of spinach (1.05) which was 14% of V_{max} of maize (7.5). Values for vascular bundle were less than those for seed, in both cultivars.

Among the PEPC effectors (Table 4) glucose-6-phosphate (1 or 5 mM) activated PEPC from apple fruit as reported for C_4-plants [18, 30], and could relieve the inhibition by L-malate (5 mM) especially with our C_4-maize enzyme extract. This relief has been previously reported for C_4-plants, in a similar way for CAM^+ [25] and to a smaller extent for C_3-species [18, 31]. Glycine (5 mM) also activated apple fruit PEPC, although this activation was previously found exclusive to maize or other monocotyledonous C_4-plants [32]. Contrary to other results [26, 33] pyruvate (1 mM) but not aspartate (1 or 5 mM), inhibited the C_4-maize enzyme and neither compound inhibited the C_3-spinach or apple fruit PEPC. Since effector properties of PEPC appear to relate to its isoform, our inhibition studies with apple fruit PEPC suggest a non-C_4 alloenzyme, underlining the kinetic results, particularly with PEP.

DISCUSSION

Based on kinetic and effector data, we suggest that there is one common form of PEPC present in the apple fruit tissues and this enzyme is, in accordance with a previous

Table 3. K_m and V_{max} values of PEPCs of apple fruit tissue compared with spinach leaf (C_3-type) and maize (NADP-m.e.-C_4-type photosynthesis)

Tissue	K_m (mM) PEP	Mg^{2+}	Mn^{2+}	V_{max} (nkat/g fr. wt)
Golden Delicious				
Vascular bundle	0.09	0.027	0.0029	0.15
Seeds	0.09	0.029	0.0050	0.38
Cox's Orange Pippin				
Vascular bundle	0.15	0.024	0.0024	0.22
Seeds	0.17	0.025	0.0024	0.43
Spinach leaf	0.088	0.020	0.0032	1.05
Maize leaf	1.5	0.03–0.015 [40]	0.010–0.0067 [40]	7.5

Table 4. Effect of activators and inhibitors on PEPCs of apple fruit seeds cv. Golden Delicious, compared to C_3 (spinach leaf) and C_4 (maize leaf) photosynthetic types

Effector	PEPC activity*		
	Apple	Spinach	Maize
A. Activators			
Glucose-6-phosphate			
1 mM	104	138	138
5 mM	109	146	185
L-Glycine			
5 mM	109	107	138
B. Inhibitors			
L-Malate			
1 mM	100	100	94
5 mM	78	81	76
L-Malate (5 mM) + glucose-6-phosphate			
2 mM	88	93	100
Aspartate			
1 mM or 5 mM	100	100	100
Pyruvate			
1 mM	100	100	92

*PEPC activity of all three PEPCs adjusted to 1 nkat before addition of potential effectors. Results expressed as % of the activity obtained in absence of effectors.

scheme [19], of C_3- or non-autotrophic type, possibly of CAM-type, but differs from the C_4-phytosynthesis enzymes. In this the malate dominated, climacteric apple fruit resembles the tartrate–malate-dominated, non-climacteric grape berry [11], contrary to the suggestion [17] of C_4-type in the internal tissues and C_3-type photosynthesis in the hypodermal tissues of apples. Table 5 shows fruit characteristics, listed according to their photosynthetic category and consequently indicating the intermediate position of fruit photosynthesis. A CAM feature in fruit, including the apple [16], is dark fixation of CO_2.

Some CAM species exhibit some C_3 properties; their PEPC K_m (PEP) values are commensurate to those of C_3-species, they have intermediate carbon isotope ratios and can switch to C_3-type photosynthesis during the day [14].

Some C_3/C_4 intermediate features in various fruit are kinetic properties of PEPC [18], carbon isotope ratios [34] and high CO_2 compensation points [34], all commensurate to values in C_3-species [14].

The idea of some C_4 features in certain fruit is underlined by the presence and activity of metabolites and enzymes, chloroplast morphology and CO_2 concentrating mechanism. In some fruit, C_4 organic acids predominate and enzymes associated with C_4 photosynthesis are present. The presence of chloroplasts normally associated with bundle sheath in C_4 plants in internal apple tissues points towards a similar type of photosynthesis, although it has been stated [14] that C_4 photosynthesis only functions when all parameters of the C_4 syndrome are present and properly co-ordinated.

A familiar function of the C_4/CAM mechanism is to provide substantial concentrations of CO_2 by means of carboxylation and consequent metabolic storage and/or transfer. Some fruit produce larger CO_2 concentrations than found in bundle sheath cells of C_4 leaves by means of metabolic transfer and/or storage followed by decarboxylation of these carbon sources, which may be derived from assimilate import from the leaves or, to a smaller extent, from fruit photosynthesis, and are respired in the mitochondrial TCA cycle after glycolytic transformation. With both carbon sources, the primary carboxylation is by RubP-Carboxylase, while PEPC refixes respired CO_2 in a secondary carboxylation. This might indicate reversed

Table 5. C_4-, CAM-, C_3/C_4-intermediate and non-autotrophic properties of fruit (with special reference to the apple fruit)

Photosynthetic feature	C_4	CAM	C_3, C_3/C_4 intermediate, non-autotrophic
1. Anatomy			
	C_4 chloroplast dualism (apple)	Stomatal frequency	Chlorophyll $a:b$ ratio (tomato)
2. Physiology			
	High internal CO_2 concentration up to 2% (apple, pear, tomato)		High CO_2 compensation point (tomato, grape)
	Small loss of assimilates in photosynthesizing tissue (apple)		Photorespiration (grape)
	—	Slow photosynthesis rate (apple, grape)	—
	—	Dark $^{14}CO_2$ fixation (apple, grape)	—
3. Biochemistry			
	—	Primary CO_2 fixation by RubP Carboxylase	
	—	$\delta^{13}C$ (grape)	—
	C_4 metabolites (malate in apple, grape)	—	
	C_4 enzymes (PEPC, PEPCK, MDH, m.e.) (apple, grape, tomato, kiwi, aubergine)		—
	No diurnal pH fluctuations (apple, grape)	Vacuole stores malate; malate is intermediate not end-product	No diurnal pH fluctuations (apple, grape, tomato)

sequence of primary and secondary carboxylating enzymes as defined in C_4/CAM photosynthesis.

To clarify the situation of fruit photosynthesis, further investigations are necessary. Determination of PEPC:RubPC ratio in fruit and leaf, the presence or absence of pyruvate-Pi-dikinase and PEPCK in fruit, photorespiration, compensation point, diurnal (acid) rhythm, chloroplast types and subtypes and the carbon isotope ratios of fruit sugars, will enable better categorization and interpretation of present results. At the moment we can only conclude that some fruit, including the apple, fix and metabolize CO_2 in a different manner, i.e. intermediate between the photosynthesis types, from their C_3 leaves. These fruit exhibit properties of all photosynthetic types, although in the case of C_4/CAM with a reverse sequence of carboxylation. However, they probably do not possess strict C_4 photosynthesis in any fruit tissue.

EXPERIMENTAL

Materials. Apple cvs. Golden Delicious and Cox's Orange Pippin were grown in 1982 and 1983 on 5-year-old EMLA 106 rootstock and harvested after sunrise in the early morning throughout the growing season. Spinach cv. Fabris was grown using NFT [35] with Long Ashton complete nutrient [36] and maize cv. HO81 grown in controlled environment. Biochemicals and purified maize PEPC were obtained commercially.

Preparation and designation of plant material. Hypodermis is the first layer of fruit tissues underlying the epidermis; vascular bundle is the central vascular bundle linking mainly the peduncle with the core.

Preparation of extracts. All extractions were made at 4° in the dark. Plant material (0.5–2.5 g fr. wt) was homogenized in 7.5 ml buffer consisting of 200 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM $NaHCO_3$, 0.25 mM EDTA, 1 mM DTT and 2% (w/v) insoluble PVP, with a pestle and mortar using either sand or liquid N_2 to aid the extraction as necessary. The crude extract was centrifuged at 20 000 *g* for 15 min and the supernantant fluid brought to 65% (v/v) $(NH_4)_2SO$ satn with satd $(NH_4)_2SO_4$ soln, after adjustment with NH_4OH to pH 7.8 After 45 min the mixture was centrifuged at 23 000 *g* for 25 min and the resulting pellet dissolved in 1 ml of suspension buffer consisting of 50 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM $NaHCO_3$, 0.25 mM EDTA and 2 mM DTT. The soln was applied to a column (20 × 15 mm) of Sephadex G-25 (fine) equilibrated with the suspension buffer and protein was eluted with the same buffer. Fractions of 0.5 ml were collected and enzyme activities determined within 3 hr.

Enzyme assays. PEPC activity was measured as $H^{14}CO_3^-$ incorporation into the acid stable fraction, modifying a previous method [37]. The reaction mixture for PEPC contained 0.2 ml of enzyme soln in suspension buffer, 10 μl of 0.5 mM $NaH^{14}CO_3$ at pH 8.4 equivalent to 0.5 μCi (18.5 kBq). The reaction was started by adding substrate, 40 μl of PEP (10 mM final concn) and stopped after 40 min in the dark at 25° by adding 0.025 ml HOAc. The mixture was left overnight to remove unbound $^{14}CO_2$. 1,2,4-Trimethylbenzene based scintillator (3 ml) was added to the acidified soln and well mixed. Radioactivity was measured in a liquid scintillation counter. A quench correction curve was constructed using the ext. standard channels ratio (ESCR-) method with the samples assayed, resulting in a counting efficiency of 85–90%. The samples were counted for 10 min and the counting repeated after 24 hr. For each sample, the same prepns without substrate (PEP) served as blanks. Activity was calculated as the difference between samples with and without the particular substrate, corrected for variations of the standard enzyme prepn and as the mean of at least two 10 min counts.

PEPC activity (carboxyl) was also measured spectrophotometrically at 340 nm and 20° by coupling the reaction to the oxidation of NADH from malate dehydrogenase (MDH). The assay medium contained 50 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 0.25 mM EDTA, 5 mM $NaHCO_3$, 2 mM DTT, 10 units MDH, 0.1 mM NADH and 2 mM PEP in a total of 3 ml. The reaction was started by the addition of PEP.

Evaluation of kinetic values. The above assay was used to obtain the kinetic values. Initial rate measurements were recorded using a double beam spectrophotometer linked to a computer. K_m-values were calculated with on-line computer programmes [Hucklesby, D. P., unpublished] using a linear least squares analysis method and assuming a mixed relative and simple error situation [38].

Malate dehydrogenase (EC 1.1.131) activity was measured spectrophotometrically at 340 nm in the direction of OAA reduction using a standard procedure [39].

Protein was estimated using the Biorad protein assay reagent according to the manufacturer's instructions with ovalbumin as the standard.

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