Plant Physiology and Biochemistry 108 (2016) 323–327

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Short communication

Phosphorylation of phosphoenolpyruvate carboxykinase (PEPCK) and phosphoenolpyruvate carboxylase (PEPC) in the flesh of fruits

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ARTICLE INFO

Article history:
Received 20 June 2016
Received in revised form
3 July 2016
Accepted 19 July 2016
Available online 21 July 2016

Keywords:
Developing seeds
Fruit
Gluconeogenesis
Malate
Phosphoenolpyruvate carboxylase
Phosphoenolpyruvate carboxykinase
Phosphorylation

ABSTRACT

This study determined whether phosphoenolpyruvate carboxykinase (PEPCK) and phosphoenolpyruvate carboxylase (PEPC) are phosphorylated in the flesh of a range of fruits. This was done by incubating fruit flesh with $^{32}P[P]$ (where $^{32}P[P] = {}^{32}PO_4^{3-}$), then PEPCK and PEPC were immunoprecipitated from extracts using specific antisera. The incorporation of $^{32}P[P]$ into these enzymes was then determined by autoradiography of SDS-PAGE gels. Both enzymes were subject to phosphorylation *in vivo* in the flesh of grape, tomato, cherry and plum. PEPCK was also subject to phosphorylation *in vivo* in developing grape seeds. Proteolytic cleavage of PEPCK showed that it was phosphorylated at a site(s) located on its N-terminal extension. Potentially phosphorylation of these enzymes could contribute to the coordinate regulation of their activities in the flesh of fruits and in developing seeds.

1. Introduction

Phosphoenolpyruvate carboxykinase (PEPCK) is only known to be located in the cytosol in flowering plants (Leegood and Walker, 2003; Walker and Chen, 2002). Similarly, phosphoenolpyruvate carboxylase (PEPC) is almost exclusively located in the plant cytosol (O'Leary et al., 2011). PEPCK catalyses the reversible reaction oxaloacetate (OAA) to phosphoenolpyruvate (PEP) (OAA + ATP ↔ PEP + CO_2 + ADP) and PEPC the reaction PEP + HCO_3 → OAA + phosphate. In *in vitro* assay PEPCK can operate in either the decarboxylation or carboxylation direction (Leegood and Walker, 2003; Walker and Chen, 2002). However, *in vivo* the cytosolic ATP/ADP ratio (in the range that has been reported in the cytosol of plant cells) is thought to bias the reaction strongly in favour of decarboxylation (i.e. synthesis of PEP) (Leegood and Walker, 2003; Walker and Chen, 2002; Walker et al., 2002). Hence, if the enzymes are present in the same cell their activities need to be coordinated in order to avoid a cycle of interconversion of PEP and OAA. In the flesh of many fruits PEPCK and PEPC are located in the same cells and at the same time (Famiani et al., 2005, 2012; Walker et al., 2011, 2015). It is not certain how the activities of PEPCK and PEPC are coordinately regulated in these fruits, however, it is possible that phosphorylation could play a role, and this, in combination with metabolite effectors and other factors, effectively switches on or off flux through the enzymes. This mechanism is thought to be used in leaves of Crassulacean acid metabolism (CAM) plants in which PEPCK and PEPC are also located in the cytosol of the same cells, where they function in the provision of CO_2 for photosynthesis (Freschi et al., 2010; Leegood and Walker, 2003; Walker and Chen, 2002).

In this study we show that PEPCK and PEPC are phosphorylated *in vivo* in the flesh of a range of fruits. We then describe how phosphorylation of these enzymes could potentially contribute to the coordinate regulation of their activities in the flesh of fruits.

2. Materials and methods

2.1. Plant material

Fruits of ripening cherry (*Prunus cerasus* L., cv Morello), plum

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http://dx.doi.org/10.1016/j.plaphy.2016.07.021
0981-9428/

(*Prunus domestica* ssp. *intermedia*, cv Victoria), and tomato (*Solanum lycopersicum* L., cv Moneymaker) were harvested from plants growing in a garden in Bolton, UK, in August 1996. Both developing seeds and ripening berries of grape (*Vitis vinifera* L., cv Black Hamburg) were obtained from vines growing in a glasshouse in Bolton, UK. Mature leaves of *Panicum maximum* Jacq. were taken from plants growing in a glasshouse in Sheffield, UK. Cucumber cotyledons (*Cucumis sativus* L., cv Masterpiece) were obtained from seeds germinated and grown in vermiculite, under darkness at 25 °C for 5 d.

2.2. In vivo phosphorylation assay

This was done as described previously (Walker and Leegood, 1996). Briefly, detached leaves of *P. maximum* were fed radiolabelled phosphate (^{32}P[P], where $^{32}P[P] = {}^{32}PO_4^{3-}$) by placing them in a 1 ml cuvette containing 200 μl water and 50 μCi (5 μl) ^{32}P[P] (specific radioactivity 200 mCi nmol^{-1} - Amersham, UK), under illumination (500 μmol quanta m^{-2} s^{-1}) at 25–30 °C. When 90% of the solution had been taken up, a further 200 μl of water was added. Then when 90% of the solution had been taken up a further 750 μl of water was added, and the leaves were incubated overnight under darkness at 25–30 °C. Cucumber seeds, developing grape seeds (dissected from the berry) and slices of fruits were placed in a petri dish containing moist filter paper. Then 5 μl (50 μCi) ^{32}P[P] was applied directly to each of the tissues. In the case of cucumber ^{32}P[P] was applied to the cotyledon. Then tissues were incubated overnight at 25–30 °C under darkness. The following morning (09.00–10.00 h) tissues were extracted by homogenisation in a mortar with 10 vol of buffer. For tissues other than grape, the buffer was 200 mM Bicine-KOH (pH 9.0) containing 5 mM DTT, and for grape tissues 500 mM Bicine-KOH (pH 9.0) containing 5 mM DTT, 200 mM KCl and 5% (w/v) polyethyleneglycol-4000 was used. Then extracts were centrifuged at 20 000 g for 5 min and the supernatant removed. The supernatant was subjected to another two cycles of centrifugation using the same conditions. For immunoprecipitation 200 μl of supernatant was then added to 25 μl of either PEPCK or PEPC antiserum and then incubated on a shaker for 1 h at 25 °C. Afterwards, 10 μl Protein A-Sepharose (Sigma, UK) was added to each sample and shaken for 15 min. Immune complexes were harvested by centrifugation at 20 000 g for 15 min at 25 °C. Pellets were washed by resuspension in 200 μl Tris-HCl (pH 7.4) containing 150 mM NaCl. Immune complexes were harvested by centrifugation at 20,000*g* for 15 min at 25 °C. The pellet was then resuspended in 5 μl SDS-PAGE loading buffer (62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 5% (w/v) SDS, 50 mM ascorbate, 5% (v/v) 2-mercaptoethanol and 0.002% (w/v) bromophenol blue), incubated at 100 °C for 5 min and then centrifuged at 12 000 *g* for 5 min. Then 1–5 μl of this were loaded on gels.

For the preparation of the cleaved form of PEPCK (Fig. 3) the procedure employed was identical to the above except for all tissues 200 mM Tris-HCl (pH 7.4) replaced 200/500 mM Bicine-KOH (pH 9.0), and after homogenisation extracts were incubated at 25 °C for 1 h.

2.3. Preparation of a nitrogen powder

For enzyme activity measurements, a nitrogen powder was used to ensure that the sample was representative of the tissue. Slices of fruit flesh similar to those used for *in vivo* phosphorylation assay were frozen in liquid nitrogen. Then, they were ground in a mortar containing liquid nitrogen and the resulting powder was used either immediately or after storage at −80 °C.

2.4. Enzyme assay

Two hundred milligrams of frozen powder was ground in a mortar containing 800 μL of ice cold 200 mM Bicine-KOH (pH 9.0), 50 mM DTT and clarified by centrifugation at 12,000 *g* for 5 min. Phosphoenolpyruvate carboxykinase (PEPCK) activity in the supernatant was measured, using an enzyme coupled method, in the carboxylation direction as described by Walker et al. (1999) and Famiani et al. (2005). PEPC activity was measured using an enzyme coupled assay as described by Ashton et al. (1990). For both enzymes one unit (U) of activity is that which produces 1 μmol product min^{-1} at 25 °C.

2.5. SDS-PAGE, immunoblotting and autoradiography

SDS-PAGE, immunoblotting and autoradiography were done as described previously (Walker and Leegood, 1996; Walker et al., 1992).

2.6. Source of antibodies

Both antisera were polyclonal and raised in rabbits. PEPCK antiserum was raised against the enzyme purified from cucumber cotyledons (Walker et al., 1995). PEPC antiserum was raised against the enzyme purified from *P. maximum* leaves (R.P. Walker unpublished data).

3. Results and discussion

3.1. PEPCK and PEPC activity in intact flesh of fruits

The maximum activities of PEPCK and PEPC were measured in extracts of the flesh of the fruits studied in order to have an indication of their abundance. PEPCK activity was 0.26 ± 0.05, 0.11 ± 0.03, 0.14 ± 0.03 and 0.05 ± 0.01 U g^{-1} FW in cherry, grape, plum and tomato, respectively. PEPC activity was 0.26 ± 0.05, 0.32 ± 0.05, 0.24 ± 0.04 and 0.22 ± 0.01 U g^{-1} FW in cherry, grape, plum and tomato, respectively. These values were comparable to those of previous studies of these fruits (Bahrami et al., 2001; Famiani et al., 2012; Guillet et al., 2002; Walker et al., 2011, 2015).

3.2. In vivo phosphorylation of PEPCK in the flesh of fruits

PEPCK is subject to phosphorylation *in vivo* in a range of plant tissues, however, whether this occurs in the flesh of fruits is unknown (Chao et al., 2014; Walker and Leegood, 1995, 1996; Walker et al., 1997). To investigate this, ripening fruit flesh was incubated with ^{32}P[P] and then phosphorylation of PEPCK was subsequently assessed by SDS-PAGE and autoradiography of immunoprecipitated PEPCK (Fig. 1). In the flesh of grape, tomato, cherry and plum, ^{32}P[P] was incorporated into PEPCK, and the amount of incorporation per unit PEPCK polypeptide was similar in each fruit (Fig. 1).

3.3. In vivo phosphorylation of PEPC in the flesh of fruits

PEPC has been shown to be phosphorylated in the flesh of banana (Law and Plaxton, 1997), and observations have been made that are consistent with it being subject to phosphorylation in the flesh of grape, orange and tomato (Diakou et al., 2000; Guillet et al., 2002; O'Leary et al., 2011; Perotti et al., 2010; Sweetman et al., 2009). However, whether PEPC is subject to phosphorylation in the flesh of most fruits has not been determined directly. Slices of ripening fruits from a range of species were incubated with ^{32}P[P]