Biochimie 92 (2010) 1362–1370

Contents lists available at ScienceDirect

Biochimie

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

Research paper

Characterization of phosphoenolpyruvate carboxylase from mature maize seeds: Properties of phosphorylated and dephosphorylated forms

Martin Černý [a], Veronika Doubnerová [a], Karel Müller [b], Helena Ryšlavá [a,*]

[a] Department of Biochemistry, Faculty of Science, Charles University, Hlavova 2030, Prague 2 128 40, Czech Republic
[b] Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojova 263, Prague 6 165 02, Czech Republic

ARTICLE INFO

Article history:
Received 5 January 2010
Accepted 18 June 2010
Available online 30 June 2010

Keywords:
Phosphoenolpyruvate carboxylase
Phosphorylation
Seed
Sigmoidal kinetics
Zea mays

ABSTRACT

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) from mature maize seeds (*Zea mays* L.) was purified to homogeneity and a final specific activity of 13.3 μmol min^{-1} mg^{-1}. Purified PEPC was treated with phosphatase from bovine intestinal mucosa or protein kinase A to study its apparent phosphorylation level. Kinetic parameters of the enzyme reaction catalyzed by phosphorylated and dephosphorylated forms under different conditions were compared, as well as an effect of modulators. The enzyme dephosphorylation resulted in the change of hyperbolic kinetics to the sigmoidal one (with respect to PEP), following with the decrease of maximal reaction rate and the increase of sensitivity to L-malate inhibition. The hyperbolic kinetics of native PEPC present in dry maize seeds was not changed after the protein kinase A treatment, while it was converted to the sigmoidal one after dephosphorylation. Level of PEPC phosphorylation was not affected during seed imbibition.

1. Introduction

Phospho*enol*pyruvate (PEP) carboxylase (PEPC; E.C. 4.1.1.31) is a ubiquitous and highly regulated cytosolic enzyme that is widely distributed in organisms from bacteria to higher plants. PEPC catalyzes the irreversible β-carboxylation of PEP to yield oxaloacetate and inorganic phosphate (P_i) with participation of HCO_3^- ion as a cosubstrate and divalent metal ion as a cofactor. The enzyme participates in primary CO_2 fixation by C_4 and CAM plant leaves [1–3]. PEPC plays a significant role in many other processes in plants, particularly in the anaplerotic replenishment of tricarboxylic acid cycle intermediates for biosynthetic purposes and nitrogen assimilation. PEPC in cooperation with malate dehydrogenase (MDH; E.C. 1.1.1.37) and NADP-malic enzyme (NADP-ME; E.C. 1.1.1.40) balances pH in the cell cytoplasm and PEPC-MDH reaction may also serve in the utilization of NAD^+ in the process of malate fermentation [2,4]. Most PEPCs are homotetramers, composed of identical subunits. Crystal structures of PEPC were established for *Escherichia coli* and for maize (*Zea mays* L.) [2]. The size of PEPC polypeptide varies significantly depending on the origin: the approximate molecular masses of 60 kDa, 100 kDa, 110 kDa, 116 kDa and 134 kDa were determined for PEPCs from *archaea*, bacteria, vascular plants, cyanobacteria and protozoa, respectively [2,5].

All known PEPCs show allosteric properties: L-malate and L-aspartate belong to the most important inhibitors and glucose-6-P to activators. In addition to that, sensitivity to various compounds differs depending on the origin of PEPC. Some plant PEPCs are sensitive to the presence of citrate and pyruvate (e.g. C_4 isoform from maize). Glycine is also a potent activator of C_4 PEPC isoform from monocots such as maize. Bacterial PEPC from *E. coli* is inhibited by succinate and is modulated by acetyl-CoA and fatty acids [2,6,7].

The activity of most vascular plant PEPCs is regulated by reversible phosphorylation catalyzed by an endogenous Ca^{2+}-independent PEPC kinase (PEPC-k). Phosphorylated PEPC is usually characterized by an increase in activity [1–3]. In addition, phosphorylation results in a reduced sensitivity of the PEPC to inhibition by L-malate and to an increased sensitivity to activation by glucose-6-P [1–3]. The posttranslational control of plant PEPC by reversible phosphorylation is regulated primarily by PEPC-k expression [3]. This expression is in correlation with light and circadian clock in C_4 and CAM plants, respectively [2]. The regulation of PEPC-k expression in C_3 plants is less understood, light seems to participate in it too [8]. Enhanced phosphorylation of PEPC has been observed in leaves of sorghum plants under conditions of salt stress, in leaves of tobacco plants infected by *Potato virus Y* and in phosphate-starved *Arabidopsis thaliana* plants [9–11].

Abbreviations: Glucose-6-P, D-glucose-6-phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MDH, malate dehydrogenase; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PEPC-k, PEPC protein kinase; PK A, protein kinase A.

* Corresponding author. Tel.: +420 221 951 282; fax: +420 221 951 283.
E-mail address: rysl@natur.cuni.cz (H. Ryšlavá).

0300-9084/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2010.06.019

Small gene family encoding PEPC has been identified in plants. PEPC gene family in *A. thaliana* is composed of a housekeeping gene, which is widely expressed in most plant organs and is probably involved in the anaplerotic function, whereas the other members of the family show tissue-specific expression and participate in more specialized functions. Moreover, one gene belongs to the bacterial type PEPC [12].

C_4/CAM and C_3 PEPC isoforms in leaves have been widely studied [1,2,13], but limited amount of information is available on occurrence and properties of PEPC in seeds. High PEPC activity in seeds of maize has been reported by Ryslava et al. [14]. Since that, PEPC activity has been found in seeds or fruits e. g. castor oil seeds [15–17], barley [18], wheat [19,20], bean [21], alfalfa [22], grape berries [23] or in seeds of germinating sorghum [24]. PEPC mRNAs were isolated from developing soybean and castor seeds [25,26]. Genetic experiments have shown that PEPC is important for the protein level in seeds: an increase in the protein content has been observed (150%) after the insertion of bacterial PEPC in beans [27]. Moreover, L-malate (product of PEPC-MDH cooperation) has been found to support very high rates of fatty acids synthesis in leucoplasts from developing castor oil seeds [16]; this phenomenon is in an agreement with the recent study describing the relation between lipid content and the PEPC activity in developing seeds of spring rapeseed [28]. Seed PEPC is also regulated *via* phosphorylation. In developing castor oil seed PEPC was found in phosphorylated form as the typical homotetramer, marked as Class-1. Moreover, the hetero-octameric Class-2 PEPC complex composed of plant and bacterial type PEPC tetramers was detected there. On the contrary, in mature dry seeds only non-phosphorylated homotetrameric PEPC was present [17,29,30]. Castor seed PEPC is monoubiquitinated at a conserved lysine residue during the early stages of seed germination, resulting in novel Class-1 PEPC heterotetramer composed monoubiquitinated 110 kDa and non-ubiquitinated 107 kDa plant-type PEPC subunits. This phenomenon caused decreased sensitivity to modulation by L-malate and glucose-6-P [31]. In developing barley seeds PEPC was also present in phosphorylated form. In dry seeds PEPC was stored in non-phosphorylated form, however PEPC kinase was found active there [32].

Studies concerning localization, isoforms content and phosphorylation status of PEPC in seeds were performed only for C_3 plants [15–32]. In this work we focused on PEPC from maize seeds (C_4 plant). Photosynthetic PEPC from this plant species is the best investigated plant PEPC [1–3,5]. The aim was to find out i) influence of reversible phosphorylation on kinetic properties of PEPC purified from mature maize seeds, and ii) apparent phosphorylation status of native PEPC of dry maize seeds.

2. Materials and methods

2.1. Chemicals and plant material

Seeds of maize (*Z. mays* var. 2013 Čejč, 2002) were obtained from Cezea, Czech Republic. Alkaline phosphatase from bovine intestinal mucosa; malate dehydrogenase from porcine heart; phosphatase inhibitor cocktail (sodium orthovanadate, sodium molybdate, sodium tartrate, imidazole); phosphoenolpyruvate (sodium salt); protease inhibitor cocktail for plant cell and tissue extracts (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, bestatin hydrochloride, *N*-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, leupeptin hemisulphate, pepstatin A, 1,10-phenanthroline) and other chemicals were from Sigma, USA. The catalytic subunit of porcine heart PK A was from Promega, UK. Hydroxyapatite (Bio-Gel HTP Hydroxyapatite) was from Bio-Rad USA and Superdex 200 10/300 GL column from GE Healthcare, UK.

2.2. Purification of PEPC from mature maize seeds and relative molecular mass estimation

PEPC was purified from 100 g of maize seeds, which were homogenized in 200 ml of 100 mM Tris–HCl extraction buffer (pH 7.5) containing 1 mM dithiothreitol, 1% (v/v) protease inhibitor cocktail for plant cell and tissue extracts, 1% (v/v) phosphatase inhibitor cocktail, 1 mM EDTA, 5% (v/v) glycerol and 5 mM $MgCl_2$ (buffer A). The extract was centrifuged for 30 min at 10 000×*g* and 4 °C. The supernatant was precipitated with ammonium sulphate (30–60% saturation), the precipitate was dissolved in 10 ml of 50 mM Tris–HCl (pH 7.8), containing 5 mM $MgCl_2$, 1 mM EDTA, 1% (v/v) protease inhibitor cocktail for plant cell and tissue extracts, 5% (v/v) glycerol and 0.5 mM dithiothreitol (buffer B) and dialyzed against buffer B at 4 °C overnight. Desalted sample was applied to a DEAE-cellulose column (1.5 × 15 cm) equilibrated with buffer B. PEPC was eluted with a linear gradient (0–300 mM) of NaCl in buffer B and applied to hydroxyapatite column (1.5 × 15 cm) previously equilibrated with 20 mM potassium phosphate (pH 7.0) containing 5 mM $MgCl_2$, 1 mM EDTA, 5% (v/v) glycerol and 0.5 mM dithiothreitol (buffer C). After washing the column with buffer C, the protein was eluted with stepwise increases of the phosphate concentration of the buffer: 50 ml of each 50 mM, 100 mM and 200 mM phosphate buffer pH 7.0. The PEPC was eluted at the phosphate buffer concentration of 100 mM (pH 7.0) and concentrated to 200 µl using an Amicon Ultra-15 Centrifugal Filter Unit (100 kDa cut off) and applied onto Superdex 200 10/300 GL column previously equilibrated with 25 mM Tris–HCl buffer (pH 7.8) containing 5 mM $MgCl_2$ (buffer D). Peak with the PEPC activity was eluted with buffer D at a flow rate of 0.5 ml/min (ÄKTA FPLC system). The fractions containing the PEPC activity were pooled and stored at 4 °C. The specific activity of PEPC remained unchanged for at least 1 week under these conditions. Relative molecular mass was estimated on a calibrated Superdex 200 gel filtration column.

2.3. Native electrophoresis and PEPC activity staining

Native electrophoresis in 6% polyacrylamide gels was performed according to Lee and Lee [33]. After electrophoretic separation, activity of PEPC was detected in gel according to Karn et al. [34].

2.4. Determination of PEPC activity and amount of protein

The PEPC assay mixture contained 250 mM HEPES-KOH (pH 8.1), 2 mM $MgCl_2$, 2 mM PEP, 0.2 mM NADH, 5 mM $NaHCO_3$, 4 units of malate dehydrogenase in total volume of 1 ml. The reaction was followed at 340 nm (spectrophotometer Helios α, Thermo Spectronic). PEPC activity was expressed as the amount of PEPC resulting in the formation of 1 µmol of oxaloacetate per minute at given temperature and pH. Soluble proteins were determined according to Bradford [35] and specific activities were calculated as µmol min^{-1} mg^{-1}.

2.5. Kinetic studies

The dependence of initial reaction rate on varying concentration of substrate PEP was performed at 25 °C or 34 °C and at pH 7.3 or 8.1. The effect of L-malate, glucose-6-P, glycine, L-aspartate, phosphate, sulphate, citrate, pyruvate, acetyl-CoA, CoA was tested; concentrations of modulators and assay conditions are given in each experiment. Experimental data were fitted to the Michaelis–Menten equation (1), Hill equation (2), or the equation for mixed inhibition (3). Kinetic constants were calculated by non-linear regression.