Journal of Plant Physiology 181 (2015) 1–8

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Biochemistry

Expression and properties of the glyoxysomal and cytosolic forms of isocitrate lyase in *Amaranthus caudatus* L.

Alexander T. Eprintsev[a], Dmitry N. Fedorin[a], Alexei V. Salnikov[a], Abir U. Igamberdiev[b,*]

[a] Department of Biochemistry and Cell Physiology, Voronezh State University, Voronezh 394006, Russia
[b] Department of Biology, Memorial University of Newfoundland, St. John's, NL A1B 3X9, Canada

ARTICLE INFO

Article history:
Received 15 January 2015
Received in revised form 27 February 2015
Accepted 27 February 2015
Available online 15 April 2015

Keywords:
Amaranthus caudatus
Isocitrate lyase
Glyoxylate cycle
Glyoxysome
Cytosol

ABSTRACT

Isocitrate lyase (EC 4.1.3.1) catalyzes the reversible conversion of D-isocitrate to succinate and glyoxylate. It is usually associated with the glyoxylate cycle in glyoxysomes, although the non-glyoxysomal form has been reported and its relation to interconversion of organic acids outside the glyoxylate cycle suggested. We investigated the expression of two isocitrate lyase genes and activities of the glyoxysomal (ICL1) and cytosolic (ICL2) forms of isocitrate lyase in amaranth (*Amaranthus caudatus* L.) seedlings. Both forms were separated and purified. The cytosolic form had a low optimum pH (6.5) and was activated by Mn^{2+} ions, while Mg^{2+} was ineffective, and had a lower affinity to D, L-isocitrate (K_m 63 μM) as compared to the glyoxysomal form (optimum pH 7.5, K_m 45 μM), which was activated by Mg^{2+}. The highest ICL1 activity was observed on the 3rd day of germination; then the activity and expression of the corresponding gene decreased, while the activity of ICL2 and gene expression increased to the 7th day of germination and then remained at the same level. It is concluded that the function of ICL1 is related to the glyoxylate cycle while ICL2 functions independently from the glyoxylate cycle and interconverts organic acids in the cytosol.

Introduction

Isocitrate lyase (ICL; D-threo-isocitrate: glyoxylate lyase, EC 4.1.3.1) has been studied in plants mostly in connection with its operation in the glyoxylate cycle and with breakdown of stored lipids during germination of oily seeds, fern spores or pollen grains, and with lipid degradation during aging (reviewed in Igamberdiev and Lea, 2002). In the glyoxylate cycle, located in the glyoxysome-type peroxisomes, ICL operates together with malate synthase, where it converts isocitrate to succinate (directed to gluconeogenesis) and glyoxylate (which is condensed with acetyl-CoA by malate synthase forming malate). The glyoxysomal form of isocitrate lyase has been purified from several plants and its mechanism and regulation by Mg^{2+} ions and metabolites were characterized in detail (Igamberdiev and Zemlyanukhin, 1987). However, numerous data indicate that the activity of this enzyme in plants may not be always accompanied by the presence of malate synthase activity, assuming participation of ICL in the processes not associated with the glyoxylate cycle.

Indications of the role of ICL in synthesis of organic acids and amino acids not related to the glyoxylate cycle appeared in the scientific literature from the 1960s. Possible mitochondrial (non-glyoxysomal) localization of ICL and its participation in the biosynthesis of glycine and serine was demonstrated in pea leaves (Hunt and Fletcher, 1977) and in rose leaf cell culture (Hunt et al., 1978). The role of ICL in glycine and serine biosynthesis is supported by the data obtained in malate synthase-deficient Arabidopsis mutants (Cornah et al., 2004). Zelitch (1988) reported ICL activity in green leaves of tobacco associated mainly with cytosol and partly with mitochondria. He suggested a pathway of glycolate formation from the glyoxylate formed in ICL reaction, which is alternative to the photorespiratory glycolate pathway. The existence of magnesium-independent ICL was claimed for preclimacteric banana fruits (Surendranathan and Nair, 1978). Millerd et al. (1962, 1963) detected ICL in the absence of malate synthase in Oxalis seedlings and indicated a possibility of ICL participation in the biogenesis of oxalic acid. In this plant, ICL may also be involved in the formation of 4-hydroxy-2-oxoglutaric acid (Morton and Wells, 1964). A role of ICL in accumulation of oxalate was also demonstrated in Atriplex leaves (Osmond and Avadhani, 1968). More recently, the role of isocitrate lyase in oxalate biosynthesis was confirmed in Rumex (sorrel) species by using metabolomics methods (Miyagi et al., 2013). Feeding the plants with $^{13}CO_2$

* Corresponding author. Tel.: +1 709 864 4567; fax: +1 709 864 3018.
E-mail address: igamberdiev@mun.ca (A.U. Igamberdiev).

http://dx.doi.org/10.1016/j.jplph.2015.02.014
0176-1617/

revealed that oxalate accumulation in young leaves was affected by citrate translocation from stems and that the inhibitor of isocitrate lyase itaconate suppressed it.

Godavari et al. (1973) reported that ICL is present in green leaves of several plants; its presence in green leaves of a dozen plants, in non-photosynthetic spring shoots of horsetail (*Equisetum arvense* L.), in achlorophyllous stems of heterotrophic plant *Lathraea squamata* L. and in all parts of maize (*Zea mays* L.) seedling (scutellum, aleurone layer, endosperm, leaf, root and mesocotyl) was demonstrated (Igamberdiev et al., 1986). It was suggested that the role of ICL is diverse and confined not only to participation in the glyoxylate cycle. However, for many years it was still unclear whether there exists a form of ICL that differs in properties and localization from the glyoxysomal form until the magnesium-independent ICL was partially purified from corn leaves (Igamberdiev et al., 1986) and its low pH optimum (6.0) and cytosolic localization were shown. Later Eprintsev et al. (2009) further characterized the two forms of ICL from corn and showed that the second form can be activated by Mn^{2+} ions. Similar results were obtained in soybean [*Glycine max* (L.) Merr.] (Eprintsev et al., 2010).

The purpose of the present study was to characterize the expression and activity of both forms of ICL in amaranth (*Amaranthus caudatus* L.), to compare their properties and to discuss their possible physiological roles. Amaranth is a non-traditional agricultural C_4 plant characterized by significant accumulation of storage lipids and protein in seeds. Its adaptive range is high and it can grow in areas ill-suited for traditional agricultural plants (Venskutonis and Kraujalis, 2013). The study of enzymes participating in mobilization of storage oils and in conversion of organic acids, in particular, in the transition from heterotrophic to autotrophic metabolism during germination, can reveal important aspects of physiology and biochemistry of this cultivated plant.

Materials and methods

Cotyledons of germinating seeds of amaranth (*Amaranthus caudatus* L., cv Kinelskiy 254) grown hydroponically in Petri dishes were used in this study. On the 4th day of germination, plants were transferred from darkness to 12 h daylight of 25 W m^{-2} and temperature of 25 °C. Cotyledons of amaranth are very similar in appearance to true leaves although they are formed from cell divisions that take place during embryogenesis in developing seeds, while leaves are formed from vegetative meristems after germination (Wang et al., 1993).

Isocitrate lyase activity was measured spectrophotometrically by an increase of optical density at 324 nm due to the formation of the complex between glyoxylate and phenylhydrazine (Kornberg and Krebs, 1957). The assay medium contained 50 mM bis–tris buffer, pH 7.5 or 6.5, 5 mM $MgCl_2$ or $MnCl_2$, 4 mM dithiothreitol, 2 mM D,L-isocitrate, 4 mM phenylhydrazine–HCl. The medium without isocitrate served as a control. The enzyme activity was calculated using the extinction coefficient of glyoxylate phenylhydrazone 0.18 mM^{-1} cm^{-1}, and the unit of activity was determined as the amount of enzyme producing 1 μmol of glyoxylate in 1 min at 25 °C. The total protein was determined by the method of Lowry et al. (1951). For the measurement of ICL1 at pH 7.5 or higher, bis–tris buffer was substituted by tris–HCl buffer of the same molarity, which has higher buffer capacity at this pH range. There was no difference in ICL activity in bis–tris and in tris–HCl in the control experiments at pH 7.5. Phosphate buffer was avoided because it affects kinetics and activity of ICL (Ranaldi et al., 2000).

Electrophoretic separation of proteins was conducted according to Davis (1964). The specific staining of gels was performed in 50 mM tris–HCl buffer, pH 7.5, containing 1 mM EDTA, 3 mM $MgCl_2$, 3 mM dithiothreitol, 10 mM D,L-isocitrate, 1.2 ml of modified Schiff reagent prepared as described by Reeves and Volk (1972). The gels were incubated at 37 °C until the red bands indicating ICL were developed. After that the gels were transferred into the medium containing 3 mM $MnCl_2$ (instead of $MgCl_2$) in 50 mM bis–tris buffer, pH 6.5 (with same other components). The red band of ICL2 was developed. For determination of the purity of ICL preparations, the silver-staining method (Shevchenko et al., 1996) was used.

Subcellular localization of ICL was determined by the isopycnic centrifugation in sucrose gradient. Five grams of cotyledons (3-day-old for the glyoxysomal ICL and 7-day-old for the cytosolic ICL) were homogenized in 50 mM tris–HCl buffer (pH 7.5) containing 0.5 M sucrose, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol. Homogenate was filtered through four layers of cheesecloth and centrifuged at 1300 × *g* for 5 min, the debris of cell walls was discarded, and supernatant was centrifuged again at 14,000 × *g* for 20 min. The supernatant was considered a cytosolic fraction, and the pellet represented the coarse fraction of mitochondria and peroxisomes. It was resuspended in the same sucrose-containing buffer, layered on the top of the sucrose density gradient containing the layers of 1.3, 1.5, 1.8, 2.3 and 2.5 M of sucrose in the same buffer and centrifuged at 100,000 × *g* for 90 min. The organelles and their cross-contamination were identified using the marker enzymes: catalase for glyoxysomes, succinate dehydrogenase for mitochondria and lactate dehydrogenase for cytosol (Lamb et al., 1978). Glyoxysomes and mitochondria from 7-day-old plants were essentially chlorophyll-free; 3-day-old plants were etiolated. The organelles were ruptured in the same buffer without sucrose but containing 0.01% Tween 80. The activities of ICL1 and ICL2 were measured as described above, succinate dehydrogenase and lactate dehydrogenase were detected as described earlier (Eprintsev et al., 2014), and catalase was measured according to Aebi (1974).

Homogenization of plant material for ICL assay and purification were performed in 50 mM tris–HCl buffer (pH 7.5) containing 3 mM $MgCl_2$ or $MnCl_2$, 5 mM dithiothreitol and 3 mM EDTA, filtered through four layers of cheesecloth and centrifuged at 5000 × *g* for 10 min. The supernatant was used for further purification. Ammonium sulfate fractionation was performed by slow addition of crystalline $(NH_4)_2SO_4$ with continuous mixing. ICL1 was precipitated between 0 and 30% saturation of $(NH_4)_2SO_4$ and ICL2—between 40 and 60% saturation. The proteins were collected by centrifugation for 20 min at 12,000 × *g*. The pellets were dissolved in 1 ml of 50 mM tris–HCl buffer, pH 7.5, containing 1 mM EDTA and 2 mM dithiothreitol. The sample was desalted on the Sephadex G-25 column (1.5 × 20 cm) equilibrated with 15 mM tris–HCl buffer, pH 7.5, containing 1 mM EDTA and 2 mM dithiothreitol. The protein was eluted using the same buffer. The obtained desalted sample was loaded on diethylaminoethyl (DEAE) cellulose (Whatman, Maidstone, UK) column (1.5 × 15 cm) equilibrated with the same buffer. The protein was eluted using a step gradient of KCl. ICL1 was eluted between 60 and 62 mM KCl, ICL2—between 96 and 98 mM KCl.

The kinetic properties were studied using the purified preparations of ICL1 and ICL2. The values of K_m were determined from the double reciprocal plots. The effect of pH on ICL1 and ICL2 activities was investigated in 50 mM bis–tris buffer (tris–HCl at pH higher 7.5). The influence of metal ions on enzyme activity was determined at pH 7.5 for the glyoxysomal and 6.5 for the cytosolic ICL by adding 5 mM KCl, $CaCl_2$, $MgCl_2$, $MnCl_2$, $FeSO_4$, $CuSO_4$ or $ZnSO_4$ to the desalted ICL preparations.

The total RNA was isolated by the guanidinum thiocyanate–phenol–chloroform extraction followed by precipitation by LiCl (Chomczynski and Sacchi, 1987). The qualitative analysis of RNA preparations was performed by using non-denaturing electrophoresis in 1% (w/v) agarose gel followed by visualization by ethidium bromide at 312 nm using a transilluminator. Reverse