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The two divergent PEP-carboxylase catalytic subunits in the green microalga *Chlamydomonas reinhardtii* respond reversibly to inorganic-N supply and co-exist in the high-molecular-mass, hetero-oligomeric Class-2 PEPC complex

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Abstract **Our recent molecular studies revealed two divergent PEP-carboxylase (PEPC [Ppc]) encoding genes in the green microalga *Chlamydomonas reinhardtii*, *CrPpc1* and *CrPpc2*, which are coordinately responsive to changes in inorganic-N and -C supply at the transcript level [Mamedov, T.G., Moellering, E.R. and Chollet, R. (2005) Identification and expression analysis of two inorganic C- and N-responsive genes encoding novel and distinct molecular forms of eukaryotic phosphoenolpyruvate carboxylase in the green microalga *C. reinhardtii*, Plant J. 42, 832–843]. Here, we report the distribution of these two encoded catalytic subunits in the minor Class-1 and predominant Class-2 PEPC enzyme-forms, the latter of which is a novel high-molecular-mass, hetero-oligomeric complex containing *both* CrPpc1 (p109) and CrPpc2 (p131) polypeptides. The Class-1 enzyme, however, is a typical PEPC homotetramer comprised solely of p109. We also document that the amount of both CrPpc1/2 catalytic subunits is up-/down-regulated by varying levels of NH_4^+ supplied to the culture medium.**

Keywords: PEP carboxylase; Green microalgae; C/N-assimilation; Anaplerosis; *Chlamydomonas reinhardtii*

1. Introduction

The cytoplasmic enzyme phospho*enol*pyruvate (PEP) carboxylase (EC 4.1.1.31; PEPC [Ppc]) catalyzes the irreversible β-carboxylation of PEP in the presence of HCO_3^- and Mg^{2+} or Mn^{2+} to yield Pi and oxaloacetate (OAA), and is widely distributed in archaeal, (cyano)bacterial, green algal and vascular plant species [1–3]. In addition to its cardinal role in photosynthetic CO_2 assimilation by leaves of C4 and Crassulacean acid metabolism plants, PEPC is a major anaplerotic enzyme providing OAA and/or malate to replenish citric-acid-cycle intermediates diverted to other primary metabolic pathways in most non-photosynthetic organs and C3 leaves (e.g., the regeneration of carbon skeletons consumed during the assimilation of ammonium into amino acids by the Gln synthetase/Glu synthase [GS/GOGAT] cycle). It is in this anaplerotic context that PEPC has been studied in the unicellular green algae at the physiological and biochemical levels [4–9], and most recently at the molecular level [10].

It has been nearly 40 years since it was first reported that distinct, molecular-mass variants of native PEPC are present in the model green microalga *Chlamydomonas reinhardtii* [11]. However, in comparison to the wealth of information amassed on vascular-plant and prokaryotic PEPCs (reviewed in [1,3]), relatively little else was known about these unusual green-algal isoforms in biochemical and molecular terms. Starting in the mid-1990s, detailed biochemical studies on the purified enzyme-forms of PEPC in the green algae *C. reinhardtii* and *Selenastrum minutum* revealed two novel classes of PEPC that are biochemically and immunologically distinct from each other and/or their vascular-plant and prokaryotic counterparts [12–14]. For example, both the Class-1 and Class-2 green-algal PEPCs are uniquely activated by Gln, whereas the Class-2 enzyme-form has a higher catalytic efficiency and apparent affinity for PEP and differs in its sensitivity to allosteric metabolite effectors when compared to the corresponding Class-1 PEPC purified from *C. reinhardtii* and *S. minutum* [12,13]. Most notably, it was found that the Class-1 PEPCs are homotetramers comprised of ~102-kDa catalytic subunits, and are far less abundant than the Class-2 hetero-oligomeric, high-molecular-mass complexes which contain the identical p102 subunit and also immunologically unrelated polypeptides of ~65, ~73, and ~130 kDa in *S. minutum* Class-2 PEPCs [14], or ~50 to ~70 kDa and likely ~130 kDa in *C. reinhardtii* Class-2 PEPC ([13] and J. Rivoal, personal commun.). In addition, biochemical observations on *S. minutum* Class-2 PEPC inferred that the component p130 polypeptide constituted a second, distinct catalytic PEPC subunit-type [14]. More

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Abbreviations: PEP, phospho*enol*pyruvate; PEPC (Ppc), PEP carboxylase; OAA, oxaloacetate; BN–PAGE, blue native–polyacrylamide gel electrophoresis

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recently, a hetero-oligomeric Class-2 PEPC enzyme-form has been characterized at the biochemical level from endosperm tissue of developing castor oil seeds [15,16], clearly indicating that this unusual PEPC complex is not unique to the green algae.

Noticeably missing from this accumulating green-algal and related plant Class-2 PEPC research, however, was any detailed molecular insight into the component subunits and the corresponding encoding genes of either the Class-1 or -2 PEPC forms. Our recent studies along these lines identified two divergent PEPC genes in *C. reinhardtii*, *CrPpc1* and *CrPpc2*, which encode highly active, ~109- and ~131-kDa catalytic subunits, respectively, when expressed in recombinant form [10]. We found that the corresponding transcript levels were coordinately up-/down-regulated under varying inorganic-N and -C growth conditions, and that the recombinant CrPpc1 and CrPpc2 proteins were immunologically distinct but notably related to the p102 catalytic subunit and the p130 partner-protein in *S. minutum* Class-2 PEPC, respectively [10,14]. Here, we present biochemical insight into the effects of a varying supply of inorganic-N to *C. reinhardtii* cultures on the CrPpc1/2 and Class-1/-2 protein levels. We also establish that these two divergent, p109 and p131 catalytic subunits co-exist in the hetero-oligomeric Class-2 PEPC complex, while the Class-1 PEPC is comprised solely of p109.

2. Materials and methods

2.1. Plant material

C. reinhardtii cells (strain CC-1883) were cultured in HS medium [17] at 5% CO_2 and varying levels of NH_4Cl (0.5, 1, and 10 mM) [10] or in TAP medium [17] bubbled with air. Maize plants were grown in a local greenhouse.

2.2. Production and affinity-purification of isoform-specific, CrPpc1 and CrPpc2 peptide antibodies

Antisera against both *Chlamydomonas* PEPC catalytic subunits (CrPpc1 [p109], CrPpc2 [p131]), hereafter designated CrPpc1/2 N-pAbs, were generated using KLH-conjugated synthetic peptides that corresponded to their divergent, extreme N-termini [10]: CrPpc1, QLSATSGRTSFRVSQDLRTGPANFLC (amino acid residues 2–26); CrPpc2, CTDSTYDFGAVRDDLTPLEDD (residues 2–21) (underlined terminal Cys indicates an exogenous thiol group introduced for coupling purposes). Peptide synthesis, purification, and subsequent generation of rabbit polyclonal antibodies were carried out by Sigma-Genosys (The Woodlands, TX, USA). To produce the requisite affinity-purification matrices, each purified peptide was covalently linked to 1 ml of SulfoLink® Coupling Gel (Pierce Biotechnology, Rockford, IL, USA) using the manufacturer's instructions. In order to affinity-purify the CrPpc1/2 N-pAbs, IgGs were first obtained from the crude antisera using a 1-ml HiTrap rProtein-A FF column (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's directions, and desalted into phosphate-buffered saline (PBS [12 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4]). These purified IgG fractions were then loaded onto the corresponding peptide affinity-matrix and mixed gently for 4 h at 4 °C. After thorough washing with PBS, the bound CrPpc1/2 N-pAbs were eluted with 0.1 M Gly, pH 2.75, and immediately neutralized to pH 7.5 as per the manufacturer's instructions (Pierce Biotechnology).

2.3. Native, soluble protein extraction and spectrophotometric assay of PEPC activity

Crude soluble extracts from *C. reinhardtii* cells were prepared as previously described, in the absence of protein phosphatase inhibitors [10]. Maize leaf protein extracts were prepared from 1.0 g leaf tissue by thorough homogenization in 3 ml of this same extraction buffer containing 1% (w/v) poly(vinylpolypyrrolidone). PEPC activity in the crude supernatant fractions was assayed spectrophotometrically at pH 8.4 as described previously [10,13], ±5 mM PEP. One unit (U) of PEP-dependent activity is defined as the amount of PEPC catalyzing the production of 1 μmol of OAA min^{-1} at 25 °C. Soluble protein concentration was determined as previously described [10].

2.4. Native-, SDS- and blue native (BN)–PAGE, in-gel PEPC assay, and immunoblotting

For native–PAGE, soluble extracts were diluted with non-denaturing sample buffer (150 mM Tris, 20% glycerol, 0.01% bromophenol blue) and loaded onto native–PAGE gels (375 mM Tris, pH 8.8, 20% glycerol, 10% ethylene glycol, 2 mM dithiothreitol, 5% acrylamide:bis [100:1]). The gels were electrophoresed at 150 V for ~3 h (4 °C) in pre-chilled running buffer (50 mM Tris, 25 mM boric acid, 5 mM $MgCl_2$), and then either stained in-gel for PEP-dependent PEPC activity [18] or further processed for immunoblotting. Denatured aliquots of the soluble extracts were also separated on SDS–PAGE gels and processed for immunodetection. 2D BN–PAGE/SDS–PAGE analysis was performed as previously described [19,20], using 100 μg protein from a soluble extract of TAP-grown *Chlamydomonas* cells that was first concentrated with a 20-kDa iCon™ concentrator (Pierce Biotechnology). For immunodetection, proteins were transferred to polyvinylidene difluoride membranes, incubated with primary antibody (CrPpc1/2 N-pAbs, or tobacco Rubisco pAb [NtRbc pAb]) and then HRP-linked anti-rabbit IgG secondary antibody (Amersham Biosciences). The latter was subsequently detected as in [10] or with CPS1 chemiluminescent peroxidase substrate-1 as described by the manufacturer (Sigma, St. Louis, MO, USA).

3. Results and discussion

3.1. In-gel activity-assays comparing the maize C4-leaf and C. reinhardtii PEPC enzyme-forms

After native–PAGE using a modified buffering system (see Section 2.4 and [21]), followed by in-gel PEPC activity-staining [18], PEP-dependent activity-bands for maize C4-PEPC and *C. reinhardtii* Class-1 PEPC were observed to co-migrate approximately with the ~443-kDa apoferritin marker (Fig. 1). In contrast, the predominant activity-band in *C. reinhardtii*, corresponding to the high-molecular-mass, hetero-oligomeric Class-2 PEPC complex [13], migrated near the ~669-kDa thyroglobulin standard. These comparative results are consistent

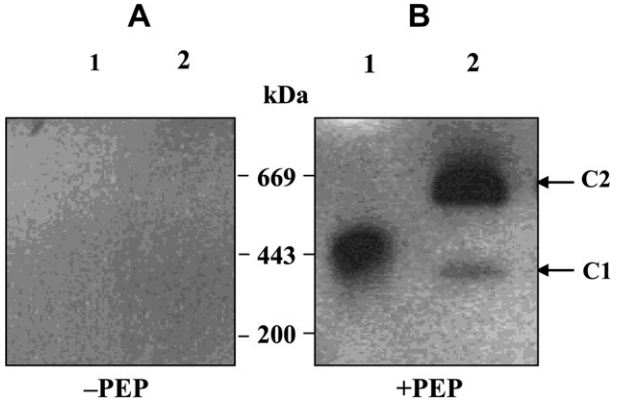

Fig. 1. In-gel PEPC activity-staining profiles of crude, soluble extracts from maize leaves and TAP-grown *C. reinhardtii* cells. Total PEPC in maize C4-leaf (1 mU, lanes 1) and *C. reinhardtii* (2 mU, lanes 2) soluble extracts was separated by native–PAGE. The gels were stained for PEPC activity, either in the absence (A) or presence (B) of 5 mM PEP, coupled to malate dehydrogenase/NADH oxidation-based fluorescence detection [18]. C1/2, Class-1/-2 enzyme-forms of *C. reinhardtii* PEPC.