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Research article

Two phosphoenolpyruvate carboxykinases coexist in the Crassulacean Acid Metabolism plant *Ananas comosus*. Isolation and characterization of the smaller 65 kDa form

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ABSTRACT

Two phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.49) isoforms of 74 and 65 kDa were found to coexist *in vivo* in pineapple leaves, a constitutive Crassulacean Acid Metabolism plant. The 65 kDa form was not the result of proteolytic cleavage of the larger form since extraction methods reported to prevent PEPCK proteolysis in other plant tissues failed to yield a single immunoreactive PEPCK polypeptide in leaf extracts. In this work, the smaller form of 65 kDa was purified to homogeneity and physically and kinetically characterized and showed parameters compatible with a fully active enzyme. The specific activity was nearly twice higher for decarboxylation of oxaloacetate when compared to carboxylation of phosphoenolpyruvate. Kinetic parameters fell within the range of those estimated for other plant PEPCKs. Its activity was affected by several metabolites, as shown by inhibition by 3-phosphoglycerate, citrate, malate, fructose-1,6-bisphosphate, L-asparagine and activation of the decarboxylating activity by succinate. A break in the Arrhenius plot at about 30 °C indicates that PEPCK structure is responsive to changes in temperature. The results indicate that pineapple leaves contain two PEPCK forms. The biochemical characterization of the smaller isoform performed in this work suggests that it could participate in both carbon and nitrogen metabolism *in vivo* by acting as a decarboxylase.

1. Introduction

Phosphoenolpyruvate (PEP) carboxykinase (PEPCK) catalyses the ATP dependent (EC 4.1.1.49) or GTP dependent (EC 4.1.1.32) reversible conversion of OAA to PEP: OAA + A(G)TP ↔ PEP + A(G)DP + CO_2.

The forms of PEPCK that utilize only guanine (sometimes hypoxanthine) nucleotides and needs Mg^{2+} or Mn^{2+} for maximal activity are largely confined to vertebrates. On the other hand the enzymes present in higher plants, bacteria, yeast and trypanosomes utilize only, or preferentially, adenine nucleotides and have a strict requirement for Mn^{2+} [1]. Although they share considerable homology, the enzyme from higher plants is considerably larger than PEPCK from other organisms because it possesses an N-terminal extension that is rapidly lost by proteolysis upon preparation of crude extracts [1].

In plants, PEPCK has several important physiological functions: i) it catalyses a key step in the conversion of fats to sugars during the germination of fat-storing seeds [2–4]; ii) in a marine macroalga, the carboxylation reaction provides C_4 acids to the chloroplast for decarboxylation, thus acting as a CO_2 concentrating mechanism at the site of CO_2 fixation via RuBisCO [5]; iii) it has been proposed to be involved in nitrogen and amino acid metabolism [6,7] and; iv) acting as a decarboxylase, it provides CO_2 to the reductive pentose phosphate pathway in PEPCK-type C_4 and Crassulacean Acid Metabolism (CAM) plants [8,9].

CAM metabolism involves the uptake of CO_2 through the stomata at night and assimilation through PEP carboxylase (PEPC) into 4-carbon organic acids [10,11]. During the day the C_4 acids are decarboxylated to yield CO_2, which is assimilated by RuBisCO and the reductive pentose phosphate pathway. In the leaves of CAM

Abbreviations: 2-ME, 2-mercaptoethanol; CAM, Crassulacean Acid Metabolism; DPDS, dipyridyl disulfide; E-64, *trans*-epoxysuccinyl-1-leucylamido-(4-guanidino) butane; pI, isoelectric point; E_a, activation energy; TCA, trichloroacetic acid; FPLC, fast-protein liquid chromatography; OAA, oxaloacetate; pI, isoelectric point; PMSF, phenylmethylsulfonyl fluoride; LDH, lactate dehydrogenase; TPCK, tosylphenylalanine clorometylketone.

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plants, in which PEPCK is the major decarboxylase, PEPC and PEPCK coexist in the cytosol of the same cells and the former needs to be active at night while the latter must be during the day. This fact suggests that to avoid a futile cycle of carboxylation/decarboxylation, there must be mechanisms that modulate both activities. PEPC's regulation has been thoroughly studied and understood [12,13], but in comparison little is known about PEPCK regulation. One major hindrance to the comprehension of PEPCK's regulatory properties is the extreme sensitivity of the enzyme to proteolysis during extraction from plant tissues, which leaves a shorter, yet active, form of the enzyme. Interestingly, while the truncated form of PEPCK shows sensitivity to some metabolites [14,15], this is not enough to completely reveal its regulation *in vivo*. There is a high chance that the portion of the enzyme that is proteolytically cleaved off during extraction confers additional regulatory properties to the enzyme. In fact, native PEPCK is phosphorylated in some C_4 leaves and in all CAM leaves and C_3-tissues that have been studied to date [16,17]. It is assumed that phosphorylation might occur at night, rendering the enzyme less active, although a connection between the phosphorylation status of PEPCK and a decreased activity in darkened leaves has only been demonstrated for the enzyme from the C_4 plant Guinea grass [18,19].

In higher plants, PEPCK has been purified to homogeneity from the leaves of C_4 grasses [14,18] and cucumber [20] and partially purified from the cotyledons of marrow [2] and from the leaves of the CAM bromeliad, pineapple (*Ananas comosus* L.) [21,22]. In the latter case, the enzyme's kinetic properties were partially characterized, although its molecular mass was not reported [21,22].

In an attempt to further define PEPCK's role in CAM photosynthesis, a 65 kDa PEPCK from pine apple leaves was purified to homogeneity and biochemically and physically characterized. A polyclonal antiserum against this truncated form has been raised and evidence is presented that strongly indicates that both 74-kDa and a 65-kDa forms of PEPCK exist in pineapple leaves *in vivo*.

2. Results

2.1. PEPCK purification

The activity of PEPCK determined in the PEP-carboxylation direction in illuminated pineapple leaf crude extracts was 2.19 U g^{-1} fresh weight.

PEPCK was purified by a combination of column chromatography methods: tentacle anion exchange on Fractogel, affinity chromatography on HiTrap Chelating, and ion exchange on High Q. A single PEPCK activity peak was observed in the first two chromatographic procedures, although in the latter, PEPCK eluted in 3 peaks at 110, 160 and 190 mM KCl, that were named Pool 1, Pool 2 and Pool 3, respectively (Table 1). Immediately after elution, the active fractions were pooled and supplemented with 20% (v/v) glycerol, 10 mM $NaHCO_3$ and 2.5 mM $MnCl_2$. The resultant solution showed no activity loss or degradation after months of storage at −80 °C. In contrast, freezing in imidazole buffer without other additions resulted in extensive degradation of the enzyme. It has been previously noted that freezing yeast PEPCK in imidazole buffer resulted in extensive inactivation of the enzyme and that bicarbonate protected the enzyme from proteolysis by trypsin [23,24]. The analysis by SDS-PAGE of the pools revealed two protein bands of 65 and 74 kDa in Pool 1 and Pool 2, at a ratio of 2.8:1 and 4.2:1 respectively, and only one band with a molecular weight of 65 kDa in Pool 3 (Fig. 1).

Although Pool 1 and 2 showed activity, they were not used for characterization as they represented non-homogeneous preparations containing presumably two PEPCK forms. Active PEPCK in Pool 3, purified to electrophoretic homogeneity with a total yield of 4% and a purification of 108-fold (Table 1), was used for all the kinetic studies described in this paper. Final specific activities in this preparation were 8.3 and 16 U mg protein^{-1} for the carboxylation and decarboxylation reactions, respectively.

Table 1
Purification of PEPCK from leaves of *Ananas comosus*.

Purification stage	Protein (mg)	Total activity (units)	Specific activity[a] (units mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	1983	153.4	0.077	100	1
35–75% $(NH_4)_2SO_4$	ND	86.0	ND	56	ND
Fractogel	54.80	26.1	0.48	17	6.2
HiTrap Chelating	5.91	17.5	2.97	11	38.5
High Q					
Pool 1	3.38	0.9	0.28	0.6	3.7
Pool 2	4.58	7.6	1.66	5	21.6
Pool 3	0.74	6.2	8.34	4	108

ND: not determined.
[a] PEPCK activity at different stages of the purification was measured in the carboxylation direction.

The native pI (isoelectric point) of the purified *A. comosus* PEPCK was 6.8, while IEF isoelectric focusing under denaturing conditions indicated a pI of 7.15 (not shown). The difference in pI between the native and denatured enzymes could be due to a methodological difference between both types of gels or to a change in the net charge in the native enzyme due to oligomerization. The native molecular mass, assessed by gel filtration, was 264 kDa (not shown). According to these data, the purified *A. comosus* leaf PEPCK appears to be a homotetramer.

2.2. Presence of two PEPCK forms in vivo

Although PEPCK from pineapple leaves was partially purified twice previously, no reports are available concerning its molecular mass [21,22]. Since this enzyme is prone to suffer proteolytic degradation upon extraction, it seems possible that these works report the properties of a truncated form or a mixture of proteolytically cleaved PEPCKs of different sizes. The purified pineapple PEPCK was successfully used to raise polyclonal antisera and affinity purified antibodies were used to probe western blots of leaf extracts and the purified PEPCK. These blots showed that two forms

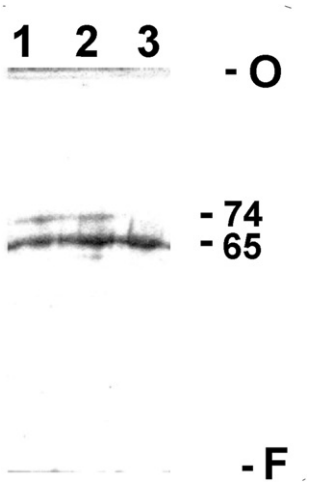

Fig. 1. SDS-PAGE followed by Coomassie Brilliant Blue staining of purified PEPCK from pineapple leaves. Lane 1: Pool 1 (4 μg); lane 2: Pool 2 (4 μg); and lane 3: Pool 3 (3,7 μg). Numbers indicate molecular mass in kDa. O, origin; F, front.