Regular paper

**In situ** $\text{C}_4$ phosphoenolpyruvate carboxylase activity and kinetic properties in isolated *Digitaria sanguinalis* mesophyll cells

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**Abstract**

Isolated mesophyll cells from darkened leaves of the C$_4$ plant *Digitaria sanguinalis* keep functional plasmodesmata that allow the free exchange of low molecular mass compounds with the surrounding medium. This cell suspension system has been used to measure C$_4$ PEPC activity in situ using a spectrophotometric assay. Compared to the extracted enzyme assayed *in vitro*, the essentially non-phosphorylated ‘in-cell’ C$_4$ PEPC showed altered functional and regulatory properties. While the $S_{0.5}$ for PEP at pH 7.3 was only modestly changed (0.4–0.6 mM), the response to pH was shifted towards the acidic range, being close to the maximal value at pH 7.3. Using expected physiological concentrations of the metabolites, at pH 7.3, the IC$_{50}$ for malate showed a five-fold increase, from 1.5 to 8 mM, and was increased further to 22 mM in the presence of the allosteric activator glucose-6-phosphate (4 mM). Thiol compounds like DTT, mercaptoethanol and reduced glutathione weakened the in-situ sensitivity of C$_4$ PEPC to malate. However, none of them had any effect on this process *in vitro*. This was not due to thioredoxin-mediated or phoshorylation-dependent processes. Since glutathione is a physiological compound that is present mostly in the reduced state in the cell cytosol, a possible contribution of this thiol to the protection of the enzyme against malate in situ is proposed.

**Abbreviations:** BSC(s) – bundle sheath cell(s); C$_4$ PEPC – C$_4$ phosphoenolpyruvate carboxylase; DTT – dithiothreitol; G-6-P – glucose-6-phosphate; IC$_{50}$ – concentration of malate causing a 50% decrease in the initial C$_4$ PEPC activity, MC(s) – mesophyll cell(s); OAA – oxaloacetate; pH$_o$ – optimum pH; $S_{0.5}$ – concentration of PEP corresponding to $V_{max}/2$

**Introduction**

In the vast majority of C$_4$ species the enzymes of the photosynthesis pathway are distributed between two photosynthetic cell types, namely mesophyll and bundle sheath cells. In the cytosol of mesophyll cells (MCs) a specific isoform of phosphoenolpyruvate carboxylase (C$_4$ PEPC, EC 4.1.1.31) catalyses the primary CO$_2$ fixation step. In C$_4$ malate formers, this leads to malate that subsequently diffuses to the bundle sheath chloroplasts (Weiner et al. 1988). A wealth of data has documented the metabolic control of C$_4$ PEPC which is highly dependent on pH and modulated by phosphorylation of a serine residue located in the enzyme’s N-terminus. *In vitro* studies relying on the partially or extensively purified enzyme have shown that C$_4$ PEPC is subjected to allosteric control by opposing photosynthesis-related metabolites, for example, activation by glucose-6-phosphate (G-6-P) or triose-phosphate and feedback inhibition by malate. In the C$_4$ photosynthetic pathway, the interplay between...
antagonistic metabolites and C₄ PEPC phosphorylation determines the carbon flux through the enzyme (Echevarria et al. 1994; Chollet et al. 1996; Vidal and Chollet 1997).

Because in most cases there is no adequate means to investigate the in-situ kinetics of enzymes, in-vitro data are used to build hypotheses about their roles in a given physiological context, for example, integrating the kinetic parameters and various regulatory inputs that impact on the enzyme and the functioning of the corresponding pathway. However, as proteins are highly concentrated and share numerous non-specific as well as specific interactions inside the cell, it is legitimate to wonder whether kinetic data obtained in vitro accurately reflect what actually occurs in vivo. In the C₄ leaf, mesophyll and bundle sheath cells are connected by a dense network of plasmodesmata allowing gradient driven metabolite fluxes. Interestingly, several former works on C₄ leaves have shown that it is possible to isolate bundle sheath cells that retain their structural integrity and functional plasmodesmata (Edwards and Black 1971; Valle et al. 1989), thus making the determination of metabolite diffusive fluxes in relation with C₄ photosynthesis and plasmodesmatal function possible (Weiner et al. 1988). In the present work, we have isolated intact MCs from *Digitaria sanguinalis* and devised a method providing an ideal system for the in-situ determination of kinetic and regulatory properties of resident enzymes. Using this system, we show that C₄ PEPC displays altered properties compared to those of the isolated enzyme, notably, optimum pH (pHo), sensitivity to malate and redox regulation. The results are considered in relation to the regulation of C₄ photosynthesis.

**Materials and methods**

**Preparation of MCs**

This was done by a modification of the mechanical procedure described by Edwards and Black (1971) and Duff et al. (1996). *D. sanguinalis* (L.) Scop. was grown in a growth chamber under the following conditions: 16 h day, 25°C (700 µmol m⁻² s⁻¹); night temperature was 20°C and the air-relative humidity was around 60%. Darkened (8 h) leaves (5–10 fully differentiated youngest leaves) from 40- to 60-day-old plants were harvested, subjected to epidermal pealing and immersed in 5 ml of suspension medium: 100 mM Hepes-KOH, pH 8, 10 mM MgCl₂, 0.6 M sorbitol. Mesophyll cells were detached by gently scraping the leaf material using a scalpel. Cells were filtrated through gauze (70 µm) to remove cell debris and bundle sheath fragments. Isolated bundle sheath cells were retained by a subsequent filtration through 20 µm gauze. The mesophyll cells were recovered from the filtrate after three rounds of centrifugation (100 × g for 1 min)/resuspension in the suspension medium.

**PEPC and NAD-MDH activity assays**

To determine the maximal activity of C₄ PEPC in situ, an aliquot of the cells (10 µl/0.22 µg of chlorophyll) was transferred into 1 ml assay medium containing the components of the coupled PEPC/NAD-MDH reaction: 100 mM Hepes-KOH, pH 8, 8 mM PEP, 5 mM NaHCO₃, 10 mM MgCl₂, 0.2 mM NADH, 0.6 M sorbitol and 4 U of exogenous NAD-MDH. Cells were maintained in homogeneous suspension by gentle stirring with a magnetic stirrer. Endogenous NAD-MDH activity was measured in a medium containing: 100 mM Hepes-KOH, pH 8, 0.8 mM OAA, 0.2 mM NADH, 10 mM MgCl₂ and 0.6 M sorbitol. Changes in optical density were recorded at 340 nm, 30°C using a Hitachi spectrophotometer (U 3000). In parallel, soluble proteins were extracted from an aliquot of the cell suspension for the determination of C₄ PEPC kinetic properties in vitro. This was done in the suspension buffer supplemented with protease inhibitors (1 µM leupeptin, chymostatin, bestatin, and 1 mM PMSF). The sensitivity to malate (malate test) was used to check the apparent phosphorylation state of the C₄ PEPC. It was performed as described in Giglioli-Guivarc’h et al. (1996) in the absence or presence of 0.8 mM malate, 1 mM PEP and the other components of the coupled reaction in the assay medium at pH 7.3.

**Light microscopy**

A Sarastro 2000 confocal microscope (Molecular Dynamics, Amersham Biosciences, Orsay, France) was used with oil immersion lens for epidermis pealed leaf fragments and a light microscope (Axioskop, Zeiss, Oberkochen, Germany) equipped with a camera was used to examine the mesophyll cell preparations.

**SDS-PAGE and Western blotting experiments**

This was carried out according to the procedure described by Pacquit et al. (1995) and Bakrim et al. (1998) using either N- or C-terminal domain-specific antibodies raised against synthetic peptides designed from the *Sorghum* C₄ PEPC.