Calcium-Dependent and -Independent Phosphoenolpyruvate Carboxylase Kinases in *Sorghum* Leaves: Further Evidence for the Involvement of the Calcium-Independent Protein Kinase in the in situ Regulatory Phosphorylation of C₄ Phosphoenolpyruvate Carboxylase

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Calcium-dependent phosphoenolpyruvate carboxylase protein kinase was copurified with C₄ phosphoenolpyruvate carboxylase (C₄ PEPC) from illuminated *Sorghum* leaves during purification by various procedures. Isolated mesophyll cell protoplasts contained both calcium-dependent and -independent protein kinases. The latter was induced by light and weak bases and was found to be the major protein kinase phosphorylating C₄ PEPC in the mesophyll.

Key words: C₄ phosphoenolpyruvate carboxylase (EC 4.1.1.31) — C₄ photosynthesis — Protein kinases — Protoplasts — *Sorghum vulgare*.

In C₄ plants, light induces the regulatory phosphorylation of the photosynthetic phosphoenolpyruvate carboxylase at a single seryl residue near its N-terminus. This process involves activation of a complex transduction chain that upregulates the activity of a phosphoenolpyruvate carboxylase kinase (PEPC-K) (Chollet et al. 1996, Vidal and Chollet 1997). Previous works by R. Chollet and coworkers (Jiao and Chollet 1989, Wang and Chollet 1993) have identified two polypeptides with molecular masses of 30 to 37-kDa in purified extracts from illuminated maize leaves that catalyse the Ca²⁺-independent phosphorylation of C₄ PEPC in vitro, a finding that was extended further to CAM and C₃ plant species (Chollet et al. 1996). However, the reports that Ca²⁺-dependent 30–37 kDa polypeptides were the best candidates to play this role, because they met the above-mentioned regulatory and inhibitor-based criteria (Li and Chollet 1993).

The signal-transduction chain linking light to C₄ PEPC phosphorylation has been investigated recently by using homogeneous preparations of *Sorghum* and *Digitaria sanguinalis* mesophyll protoplasts and cells (Duff et al. 1996, Giglioli-Guivarc’h et al. 1996). For the in situ phosphorylation of PEPC to occur, protoplasts require both light and a weak base, such as ammonium chloride or methylamine, which induces rapid alkalization of the cytosol (Giglioli-Guivarc’h et al. 1996). Following addition of the calcium ionophore A-23187, calcium-depleted mesophyll protoplasts no longer achieve PEPC phosphorylation in situ (Pierre et al. 1992). Nevertheless, the results of a subsequent pharmacological approach support the view that both the Ca²⁺-independent PEPC-K and either a CDPK...
or a Calcium/Calmodulin-dependent Protein Kinase are distinct components of a multicyclic cascade (Giglioli-Guivarc'h et al. 1996) in the mesophyll. However, Ca$^{2+}$-dependent protein kinases are multiple, and, as mentioned above, at least some of them have the potential to phosphorylate C$_4$ PEPC in vitro (Echevarria et al. 1988, Bakrim et al. 1992, Izui et al. 1995). If present in the same cell compartment as the target, i.e., the cytosol of mesophyll cells, they will theoretically be activated following calcium mobilization in the light and should contribute to the phosphorylation of C$_4$ PEPC in vivo. In this work, we have assessed this point by evaluating the extent of both Ca$^{2+}$-dependent and independent PEPC-K in a homogeneous preparation of mesophyll cell protoplasts in which the C$_4$-PEPC and the machinery for regulatory phosphorylation are restricted.

In previous works, purification of the Ca$^{2+}$-independent PEPC-K by chromatography on Blue Dextran Agarose was amply documented (Jiao and Chollet 1989, Bakrim et al. 1992, Chollet et al. 1996, Vidal and Chollet 1997). In vitro, phosphorylation of the regulatory serine 8 on PEPC...
from dark-adapted Sorghum leaves induced changes in the functional properties of the enzyme: a decrease in sensitivity for the feedback inhibitor L-malate and an increase in the affinity for the allosteric activator, glucose-6P, when assayed at suboptimal concentration of PEP and pH (Chollet et al. 1996, Vidal and Chollet 1997). The Ca²⁺-dependent PEPC-K performing the regulatory phosphorylation of C₄ PEPC was reported to occur in purified extracts from illuminated maize leaves (Ogawa and Izui 1992, Ogawa et al. 1992, Izui et al. 1995, Yabuta et al. 1994). It was inhibited by the calmodulin antagonist W7, but it did not require this calcium-binding protein for activity, and also by the potent inhibitor of Myosin Light Chain Kinase, KT5926 (IC₅₀, 2.5 μM). This enzyme was reminiscent of CDPK, a protein kinase not found in animals which has an intrinsic, calmodulin-like, calcium-binding regulatory domain linked to the catalytic domain (Roberts and Harmon 1992).

These results prompted us to reexamine the occurrence and variety of PEPC kinases in the Sorghum leaf. Previously, we had purified, by Calmodulin-Sepharose chromatography, a Ca²⁺-dependent PEPC-K from Sorghum leaves; like its maize counterpart, it did not show any need for calmodulin in reconstituted phosphorylation assays. In contrast, it did not change the functional properties of the exogenous target (Bakrim et al. 1992). It was reported later that the CAM PEPC from dark-adapted leaves of Mesembryanthemum crystallinum is copurified with a protein kinase on Fractogel-DEAE and hydroxylapatite (Baur et al. 1992). Detection of PEPC/PEPC-K protein complexes in vitro should be a pertinent indication of the potential physiological relevance of the protein kinase. In the present work, searching for PEPC-K with binding activity to C₄ PEPC, we extracted the enzyme from illuminated Sorghum leaves and subsequently purified it by various procedures and chromatography matrices.

(1) In vitro phosphorylation in reconstituted media containing the components of the reaction and an aliquot of the desalted crude extract showed that labeling of PEPC (Fig. 1B, lane 3) was partially inhibited by the calcium chelator EGTA (lane 4) and restored upon addition of free calcium (lane 5) but not of magnesium (lane 6). The label was markedly suppressed when the S8/D mutant C₄ PEPC (in which aspartate substitutes serine 8 and cannot be phosphorylated (Duff et al. 1995)) was added to the medium, instead of wild type S₈ C₄ PEPC (lane 2). Together, these results suggested that both Ca²⁺-dependent and -independent PEPC-K use PEPC as a target and phosphorylate its regulatory serine 8 in crude extracts from Sorghum leaves.

(2) After non-denaturing electrophoresis in a polyacrylamide gel gradient, the gel was sliced at the level of the Fast violet B stained PEPC band. In the three fractions analysed in the reconstituted medium containing the components of the phosphorylation reaction, the C₄ PEPC subunit was found to be highly labeled; the radioactive signal was proportional to the amount of C₄ PEPC present in the gel segments (Fig. 2IB, lanes 1–3). EGTA prevented phosphorylation of the target by the peak fraction (Fig. 2IB, lane 2), which was recovered after free calcium was added to the medium (Fig. 2IB, lane 3), while magnesium had no effect (Fig. 2IB, lane 4).

(3) C₄ PEPC was eluted (0.15 M NaCl) from DEAE-Sepharose together with a PEPC-K (Fig. 3). At the peak fraction, the protein kinase also exhibited the capacity to phos-
Fig. 4 Immunoprecipitation of PEPC. Preilluminated Sorghum leaves (200 mg) were rapidly ground in a mortar in 1 ml of extraction medium containing 100 mM Tris-HCl (pH 8), 5 mM MgCl₂, 1 mg liter⁻¹ chymostatin, 14 mM β-mercaptoethanol, and 10 mg insoluble PVP. Fifteen min incubation (0°C) with polyclonal PEPC IgGs (180 µl per 3.6 mg of proteins) immunoprecipitated 6 units of PEPC, which were sedimented by centrifugation in an Eppendorf centrifuge. The pellet was carefully washed (3 × 500 µl) with 50 mM Tris-HCl (pH 8), 20% glycerol, and 1 mM DTT. The final pellet was resuspended with the aid of a pipette to form a homogeneous suspension in 160 µl of the same buffer. To 30 µl of the preparation were added the components of the reconstituted phosphorylation assay (50 µl final volume, cf. Fig. 1). After incubation under vigorous shaking for 1 h at room temperature, aliquots (20 µl) were mixed with 10 µl of stop buffer, heated to 100°C for 2 min, and subjected to SDS/PAGE and autoradiography. A, Coomassie blue staining of proteins; B, corresponding autoradiography, lane 1, complete assay; lane 2 plus 0.5 mM EGTA; lane 3 plus 0.5 mM EGTA plus 2 mM CaCl₂; lane 4, complete plus 10 nM microcystin-LR.

Phosphorylate the target, endogenous/exogenous C₄ PEPC, with a marked dependency for calcium (Fig. 3, insert).

(4) PEPC-K activity was also detected in the resuspended immunoprecipitation pellet of C₄ PEPC from illuminated Sorghum leaves. Again, phosphorylation of the target by the coimmunoprecipitated protein kinase was severely inhibited by EGTA and recovered specifically after addition of calcium to the medium (Fig. 4B, lanes 2, 3). Addition of the 2A-type protein phosphatase inhibitor, microcystin-LR, to the assay indicated that no significant amount of such a protein was trapped by antibodies in the immunoprecipitate (Fig. 4B, lane 4).

(5) Similar results were obtained when C₄ PEPC was purified by immunoaffinity chromatography which selectively retains the enzyme and a copurifying Ca²⁺-dependent protein kinase (not shown).

In all cases, the presence of high levels of highly phosphorylated C₄-PEPC in the preparation made it impossible for us to determine whether the protein kinase is able to modify the functional properties of the target. However, phosphorylation of polyacrylamide gel-purified C₄-PEPC was largely prevented when specific anti-phosphorylation-site antibodies were added to the medium (Fig. 5B, lane 2) (Pacquit et al. 1995). This result provided strong evidence of the fact that the copurifying protein kinase does phosphorylate the serine residue present in this target domain. These collective findings revealed the surprising fact that a Ca²⁺-dependent protein kinase, possibly a CDPK, and not the inducible Ca²⁺-independent one, binds the C₄ PEPC to form a stable complex in vitro. A cellular and pharmacology approach relying on light plus weak base (NH₄Cl or methylamine) induction of mesophyll-cell protoplasts from Sorghum and Digitaria sanguinalis had earlier suggested that the mechanism of in situ phosphorylation of C₄ PEPC is mediated by a rapid increase in cytosolic pH (pHc) and calcium (Giglioli-Guivarc’h et al. 1996). We then realized that, if present in the mesophyll, this enzyme could also be rapidly activated following calcium efflux in the cytosol.

Fig. 5 Effect of phosphorylation-site-IgGs on the phosphorylation of C₄-PEPC in reconstituted assays. PEPC purification, in vitro phosphorylation, and subsequent analysis by SDS/PAGE were performed as described in legend of Fig. 2. A, Coomassie blue staining of proteins; B, corresponding autoradiography. Lane 1, complete assay, control; lane 2, +20 µg of phosphorylation-site antibodies; lane 3, +0.5 mM EGTA.
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Fig. 6 PEPC-K activity in crude protein extracts from mesophyll protoplasts. Mesophyll protoplasts were obtained from dark-adapted *Sorghum* leaves (14-d-old) as described in Pierre et al. (1992) with the following alterations: sorbitol was 0.55 M and the pH of the washing and resuspension media was 7.4. Incubation was for 90 min in the dark (control) or in the light (300 μE m⁻² s⁻¹) in the presence of 10 mM NH₄Cl. Induction was verified by determining the IC₅₀ for t-malate of C₄ PEPC, which was found to decrease from 70% in the dark to 45% in the illuminated, weak base-treated protoplasts. Proteins were extracted, desalted on Sephadex G-25, and assayed (30 μg) in the reconstituted phosphorylation medium as under Fig. 4. Radiolabeled proteins were analysed by SDS-PAGE (10% acrylamide) and autoradiography. A, Coomassie blue-stained gel; B, corresponding autoradiography. Lanes 1 and 3, 0 and 90 min in the dark; lanes 2, 4 and 5, 90 min in the light plus 10 mM NH₄Cl, lane 4, plus 0.5 mM EGTA plus 2 mM CaCl₂, lane 5, plus 0.5 mM EGTA plus 2 mM MgCl₂. In lane 2, exogenous PEPC was omitted.

and thus should theoretically contribute, together with the other resident Ca²⁺-independent PEPC-K, to the in situ phosphorylation of C₄ PEPC. This hypothesis was checked in a homogeneous preparation of mesophyll-cell protoplasts after induction of the light-dependent cascade/C₄ PEPC phosphorylation. Soluble proteins were extracted and used in reconstituted assays containing an exogenous, immunopurified C₄ PEPC (recombinant, non-phosphorylated) in the presence of [γ-³²P]ATP, MgCl₂, CaCl₂, and +/− EGTA to allow the determination of both Ca²⁺-dependent and -independent protein kinases. Fig. 6B (lane 1) shows that there was little or no phosphorylation of the target C₄ PEPC in protein extracts from control (non-induced at t = 0) protoplasts in the presence of free calcium, whereas several lower molecular mass protein bands appeared to be labeled. In contrast, protein samples from treated protoplasts phosphorylated either endogenous (lane 2) or endogenous + exogenous C₄ PEPC (lanes 4 and 5) in the light plus NH₄Cl, but to a very low extent in the dark (lane 3), as expected from previous experiments (Pierre et al. 1992, Giglioli-Guivarc'h et al. 1996). Removal of calcium (lane 5) by EGTA largely prevented the phosphorylation of most proteins, with the exception of PEPC. This inducible PEPC-K activity was found to be similar in assays containing free calcium (lane 4) or not (lane 5) in the presence of equal amounts of EGTA. It could be that, like the Ca²⁺-independent PEPC-K (Giglioli-Guivarc'h et al. 1996), the Ca²⁺-dependent PEPC-K needs some signal originating from the bundle sheath for its activation in the light; however, no such process has been found to occur in previous works dedicated to this enzyme; a constitutive CDPK phosphorylating C₄ PEPC was detected in maize leaves (Li and Chollet 1993). Also, loss of this enzyme during protoplast preparation is very unlikely, because we verified that PEPC or NAD(P)-dependent malate dehydrogenases were not released in the surrounding medium and the major Ca²⁺-dependent protein kinase activity (which does not use C₄ PEPC as a target) is retained by the protoplast (Fig. 6). The results unequivocally demonstrated that the upregulated, Ca²⁺-independent protein kinase is by far the predominant PEPC-K in mesophyll-cell protoplasts. Therefore, (1) the afore-mentioned CDPK which is copuriﬁed with C₄ PEPC resides in other leaf tissues, possibly the bundle sheath, showing that the detection of PEPC/protein kinase complexes is not a pertinent indication of the physiological relevance of the protein kinase, (2) most of the calcium-dependent protein kinase present in mesophyll cells does not or hardly phosphorylate C₄ PEPC, and, (3) conversely, the Ca²⁺-independent PEPC-K is obviously very specific for this target. This is consistent with a previous report on the maize leaf enzyme which did not phosphorylate heterologous substrates e.g., casein, Histone III-S, Bovine serum albumin, and leaf sucrose-P synthase (Wang and Chollet 1993, Chollet et al. 1996). These results provide strong support to the proposal of a multicyclic protein-kinase cascade in the mesophyll cell cytosol, involving a CDPK (based on the effect of various inhibitors of calcium channel and CDKPK activities) as an upstream component of the signal-transduction chain leading to stimulate Ca²⁺-independent PEPC-K and, subsequently, the phosphorylation of C₄ PEPC, as already discussed in Giglioli-Guivarc'h et al. (1996). Collectively, these cellular-based data clarify the debate on the physiologically relevant PEPC-K because they definitely assign the light-upregulat-
ed, Ca\(^{2+}\)-independent PEPC-K a major role for C\(_4\) PEPC phosphorylation in the mesophyll cell of the C\(_4\) leaf, and thus, in the physiological context of C\(_4\) photosynthesis.

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References


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