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Phosphoenolpyruvate Carboxykinase Is Involved in the Decarboxylation of Aspartate in the Bundle Sheath of Maize\(^1\)

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We recently showed that maize (Zea mays L.) leaves contain appreciable amounts of phosphoenolpyruvate carboxykinase (PEPCK; R.P. Walker, R.M. Acheson, I.I. Técsi, R.C. Leegood [1997] Aust J Plant Physiol 24: 459–468). In the present study, we investigated the role of PEPCK in C\(_4\) photosynthesis in maize. PEPCK activity and protein were enriched in extracts from bundle-sheath (BS) strands compared with whole-leaf extracts. Decarboxylation of \([4-\text{\(^1\)}\text{C}]\text{aspartate}\) (Asp) by BS strands was dependent on the presence of 2-oxoglutarate and \(\text{Mn}^{2+}\), was stimulated by ATP, was inhibited by the PEPCK-specific inhibitor 3-mercaptopicolinic acid, and was independent of illumination. The principal product of Asp metabolism was phosphoenolpyruvate, whereas pyruvate was a minor product. Decarboxylation of \([4-\text{\(^1\)}\text{C}]\text{malate}\) was stimulated severalfold by Asp and 3-phosphoglycerate, was only slightly reduced in the absence of \(\text{Mn}^{2+}\) or in the presence of 3-mercaptopicolinic acid, and was light dependent. Our data show that decarboxylation of Asp in BS cells of maize occurs via two different pathways: Whereas malate is mainly decarboxylated by NADP-malate, decarboxylation of Asp is dependent on the activity of PEPCK.

C\(_4\) plants have been classified as NADP-ME, NAD-ME, and PEPCK types, according to the major decarboxylase involved in the decarboxylation of C\(_4\) acids in the BS cells (Gutierrez et al., 1974; Hatch et al., 1975). Maize (Zea mays) belongs to the NADP-ME subgroup of C\(_4\) plants and possesses negligible activity of PEPCK, although Gutierrez et al. (1974) suggested that some other NADP-ME species utilize PEPCK as an auxiliary decarboxylase. However, we showed previously that maize leaves contain large amounts of PEPCK (Walker et al., 1997), and Furumoto et al. (1999) identified a PEPCK gene from maize that is specifically expressed in BS cells. Other NADP-ME species such as Echinochloa colona, Echinochloa crus-galli, Digitaria sanguinalis, and Paspalum notatum also contain PEPCK, whereas PEPCK protein was not detectable in Sorghum bicolor, Saccharum officinarum, or Flaveria bidentis (Walker et al., 1997). The reason that the occurrence of PEPCK in these plants has been overlooked may be due to difficulties in measuring PEPCK activity (Walker et al., 1997) or to the lack of an antibody.

PEPCK-type C\(_4\) species mainly use Asp as the CO\(_2\) donor and decarboxylate the oxaloacetate formed via the following reaction:

> \[
> \text{Oxaloacetate} + \text{ATP} \rightarrow \text{PEP} + \text{ADP} + \text{CO}_2
> \]

In NADP-ME species such as maize, \(^{14}\text{CO}_2\) is initially incorporated into the C-4 position of malate and Asp (about 75% and 25%, respectively), and the C-4 of both is subsequently incorporated into other metabolites (Hatch, 1971; Morot-Gaudry and Farinneau, 1978). Using isolated BS strands, Chapman and Hatch (1981) showed that BS cells of maize have a significant capacity to decarboxylate Asp, and they assumed that NADP-ME was responsible. In view of the occurrence of PEPCK in maize, the involvement of PEPCK in the decarboxylation of Asp appears more likely, because PEPCK can directly catalyze the decarboxylation of oxaloacetate formed from Asp without previous reduction of oxaloacetate to malate.

To study the role of PEPCK in photosynthesis in maize, we measured the decarboxylation of Asp and malate by BS strands under conditions supporting the activities of either PEPCK or NADP-ME. In addition, we determined the protein contents or activities of other enzymes potentially involved in Asp metabolism.

**MATERIALS AND METHODS**

**Plant Material**

Maize (Zea mays L. cv H511) plants were grown in high-nutrient compost (M3, Fisons, Ipswich, UK) in a growth cabinet at a PPFD of 250 \(\text{\mu}\text{mol m}^{-2} \text{s}^{-1}\) and a photoperiod of 12 h d\(^{-1}\). The temperature was 28°C during the day and 24°C at night. For all experiments, the second and third leaves of 10- to 15-d-old plants were used.

**Isolation of BS Strands**

BS strands were isolated by slicing deribbed leaves into 1- to 2-mm sections and blending them five to six times for 10 s with 150 mL of blending buffer (100 mM Bicine-KOH, pH 8.0, 0.3 M sorbitol, 0.1% [w/v] PVP-40, 0.02% [w/v] EDTA). Abruptly stopping the blending after each cycle resulted in the fragmentation of chloroplasts, and a light-dependent decarboxylation of Asp was observed in the resulting extracts. Blending was repeated twice to ensure complete disruption of the BS. The following abbreviations are used:

- Asp, aspartate; BS, bundle sheath; ME, malic enzyme; MPA, 3-mercaptopicolinic acid; PEPC, PEP carboxylase; PEPCK, PEP carboxykinase.
BSA, 5 mM DTT, 2 mM KH$_2$PO$_4$, and 5 mM K$_2$SO$_4$) in a Waring blender. The strands were collected by filtration through Miracloth (Calbiochem) and washed with 350 mL of blending buffer. Where indicated, a digestion treatment (Hatch and Kagawa, 1974) was included in the procedure. After the sample was blended three times for 10 s each time, the BS strands were washed with blending medium, incubated for 25 min in 20 mM Mes-KOH (pH 5.5), 0.5 mM sorbitol, 2 mM MgCl$_2$, 0.2% (w/v) BSA, 1% (w/v) cellulase (Onozuka R-10, Yakult), Honsha, Japan), and 0.1% (w/v) pectinase (Macerozyme R-10, Yakult), washed with blending medium, and blended again (twice for 10 s each time).

**Determination of Enzyme Activities**

PEPCK was extracted in 180 mM Bicine and 20 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 10.0) containing 50 mM DTT, and the carboxylation reaction was measured according to the method of Walker et al. (1995). Cytosolic pyruvate kinase was extracted and assayed as described by Zervoudakis et al. (1997); ADP was omitted for the assay of PEP phosphatase.

**Western Analysis**

Proteins were extracted in 200 mM Bicine-KOH (pH 9.5), 25 mM DTT, and 1% (w/v) SDS. Extracts were boiled for 90 s with equal volumes of solubilization buffer (62.5 mM Tris, 20% [v/v] glycerol, 2.5% [w/v] SDS, and 5% [v/v] 2-mercaptoethanol, pH 6.8). After SDS-PAGE, the proteins were transferred onto a PVDF membrane (Immobilon-P, Millipore) and probed with antisera raised against PEPCK from cucumber (Walker et al., 1995), PEPC from *Arabidopsis thaliana* (Hatch and Kagawa, 1974) was included in the procedure. Because PEPCK from plants is subject to rapid proteolysis, we first optimized the conditions for the isolation of PEPCK C$_4$ plants (Chapman and Hatch, 1983; Walker et al., 1997).

**RESULTS**

**PEPCK in BS Cells of Maize**

Maize leaves contained substantial amounts (Fig. 1) and activities (Table I) of PEPCK. The activity of PEPCK recorded in maize was between one-fifth and one-half of that measured in PEPCK C$_4$ plants (Chapman and Hatch, 1983; Smith and Woolhouse, 1983; Walker et al., 1997).

Because PEPCK from plants is subject to rapid proteolytic cleavage, we first optimized the conditions for the isolation of BS strands, checking the intactness of PEPCK protein. When the isolation procedure included treatment with cellulase and pectinase, strands free of mesophyll contamination, as judged by the absence of PEPC, were obtained (Fig. 1A). Even though this treatment did not lead to general proteolysis, it resulted in the cleavage of PEPCK. PEPCK in plant extracts is rapidly cleaved at low pH or, in the absence of SDS, to a 62-kD form with no loss of activity (Walker et al., 1995, 1997). However, during the isolation of BS strands from maize, PEPCK was degraded further and
Panicoides isolated after treatment with cellulase and pectinase. Three micrograms of total protein was loaded per lane.

Three micrograms of total protein was loaded per lane. For PEPCK in extracts from whole leaves (WL) and BS strands from maize. The BS strands were isolated including (A) or the polypeptide pattern in extracts from whole leaves (WL) and BS strands isolated from Panicoides isolated after treatment with cellulase and pectinase. C, Western blot for PEPCK in extracts from whole leaves (WL) and BS strands from Panicoides isolated after treatment with cellulase and pectinase. Three micrograms of total protein was loaded per lane.

sometimes no PEPCK protein was detectable on western blots. This indicates that protease activity within intact BS cells was stimulated by low pH. On the other hand, PEPCK in BS strands isolated from the PEPCK-type species Urochloa panicoides was largely intact after treatment of the BS strands with cellulase and pectinase (Fig. 1C).

Maize BS strands isolated without the cell wall-digestion step were contaminated with approximately 6% of the mesophyll protein, as estimated from the distribution of PEPC and Rubisco on western blots (Fig. 1B). PEPCK extracted from these strands was intact and enriched compared with whole-leaf extracts, confirming the location of PEPCK in the BS cells in maize (Walker et al., 1997). The activity of PEPCK was also enriched in BS strands (Table I).

Decarboxylation of [4-14C]Asp

Because of the persistence of open plasmodesmata, BS cells are permeable to metabolites and can be used to study the metabolism of externally added substrates such as Asp. Rates of [4-14C]Asp decarboxylation by BS strands were independent of illumination (Fig. 2); the reaction was completely dependent on the presence of 2-oxoglutarate. ATP, which is a substrate of the PEPCK reaction, stimulated Asp decarboxylation. An inhibitor of PEPCK activity, MPA (Jomain-Baum et al., 1976), inhibited the ATP-stimulated activity. PEPCK has an absolute requirement for Mn2+ and, accordingly, Mg2+ did not substitute for Mn2+ in the decarboxylation of Asp. Mg2+ did, however, stimulate Asp decarboxylation in the presence of Mn2+. The requirements for Mn2+ and ATP and the inhibition by MPA are evidence for an involvement of PEPCK. In contrast, Asp decarboxylation by BS strands from S. bicolor, an NADP-ME species without detectable amounts of PEPCK protein, was low (0.06 µmol min⁻¹ mg⁻¹ phaeophytin), was not stimulated by the addition of ATP, and was not inhibited by MPA (data not shown).

Metabolism of Asp

Under the conditions supporting the highest rates of Asp decarboxylation, PEP was the main product of Asp metabolism (Fig. 3A). The rates of PEP formation (0.55 µmol min⁻¹ mg⁻¹ phaeophytin) were comparable to the rates of Asp decarboxylation (Fig. 2). Pyruvate was also formed but at a much lower rate. In the absence of ATP, the rates of both PEP and pyruvate formation were negligible (Fig. 3B). The predominance of PEP formation in the presence of ATP supports the view that PEPCK, and not NADP-ME, was mainly responsible for the decarboxylation of Asp.

To determine whether PEP could be converted into pyruvate by pyruvate kinase, ADP (1 mM) was supplied in addition to ATP. Because high concentrations of ATP inhibit pyruvate kinase (Baysdorfer and Bassham, 1984), the ATP concentration was decreased to 1 mM. Under these conditions, equal amounts of PEP and pyruvate were formed and the sum of PEP and pyruvate formation was reduced to 0.38 µmol min⁻¹ mg⁻¹ phaeophytin (Fig. 3C). This lower rate was probably due to the effect of a changed ATP:ADP ratio on PEPCK (Walker et al., 1997). The accumulation of PEP, even in the presence of ADP, shows that the activity of pyruvate kinase (plus PEP phosphatase) in BS cells is not sufficient to convert all PEP formed by PEPCK into pyruvate. The rate of conversion of PEP into pyruvate by BS strands (Table I) was also lower than the rate of Asp decarboxylation. In addition, the in vitro activities of pyruvate kinase and PEP phosphatase in BS cells were much lower than the activity of PEPCK (Table I).

Table 1. In vitro activities of PEPCK, pyruvate kinase (PK), and PEP phosphatase (PP) in extracts of whole leaves and of BS strands, and in vivo formation of pyruvate (Pyr) from PEP by BS strands in the presence of 5 mM PEP, 10 mM ADP, 1 mM Mn2⁺, 1 mM Mg2⁺, and 40 mM KCl

<table>
<thead>
<tr>
<th>Extract</th>
<th>PEPC</th>
<th>PK</th>
<th>PP</th>
<th>Pyr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol min⁻¹ g⁻¹ fresh wt</td>
<td>µmol min⁻¹ mg⁻¹ phaeophytin</td>
<td>µmol min⁻¹ mg⁻¹ phaeophytin</td>
<td></td>
</tr>
<tr>
<td>Whole leaves</td>
<td>4.34 ± 0.22</td>
<td>2.26 ± 0.18</td>
<td>0.41 ± 0.04</td>
<td>0.74 ± 0.12</td>
</tr>
<tr>
<td>BS strands</td>
<td>n.d.</td>
<td>3.58 ± 0.39</td>
<td>0.51 ± 0.08</td>
<td>0.61 ± 0.09</td>
</tr>
</tbody>
</table>

Data are means ± SE of three plants. n.d., Not determined.
Since PEPCK is a cytosolic enzyme, we determined whether cytosolic aminotransferases are present in maize. Although all of the PEPCK species we tested (U. panicoides, C. gayana, and P. maximum) contained large amounts of cytosolic Asp and Ala aminotransferases, these aminotransferases were not detectable in maize or other NADP-ME species (E. crus-galli and S. officinarum; Fig. 4).

In PEPCK species, NAD-ME in combination with the respiratory chain provides ATP for the PEPCK reaction. Although maize contained some NAD-ME protein, the amount was only comparable to that found in C₃ species and was lower than in PEPCK species.

Decarboxylation of [4-¹⁴C]Malate

In contrast to rates of Asp decarboxylation, those of [4-¹⁴C]malate were generally low in the dark (Fig. 5). The stimulation of malate decarboxylation by ATP and NAD, which is needed for the oxidation of malate to oxaloacetate, indicates some contribution of PEPCK to malate decarboxylation in the dark. Under illumination, however, the effect of ATP and NAD was smaller and malate decarboxylation was strongly stimulated by Asp and 3-phosphoglycerate. Stimulation by Asp and 3-phosphoglycerate is characteristic of malate decarboxylation catalyzed by NADP-ME (Hatch and Kagawa, 1976; Chapman and Hatch, 1979; Boag and Jenkins, 1986). The light dependence of malate decarboxylation also suggests the involvement of NADP-ME (Hatch and Kagawa, 1976). In the presence of MPA or in the absence of Mn⁡²⁺, malate decarboxylation was slightly reduced, but the inhibition was minor compared with the effect on Asp decarboxylation. In the presence of Mn⁡²⁺, Mg⁡²⁺, Asp, and 3-phosphoglycerate, ATP inhibited the decarboxylation of malate, possibly by binding the Mg⁡²⁺ required for NADP-ME.

DISCUSSION

The Function of PEPCK in Maize

One reason that the presence of PEPCK in maize has been overlooked may be the instability of this enzyme.
PEP Carboxykinase in Maize

During the isolation of BS strands (Fig. 1). When the procedure for maize included digestion of the mesophyll cells with cellulase and pectinase, PEPCK protein was almost completely degraded. In contrast, PEPCK from plants that have been classified as PEPCK species appears to be more stable. Intactness of the PEPCK from *U. panicoides* was not affected by the digestion step, and PEPCK from *P. maximum* was still fully active in BS protoplasts isolated by treatment with cellulase and pectinase (Chapman and Hatch, 1983).

The formation of PEP from Asp (Fig. 3), the dependence of Asp decarboxylation on Mn$^{2+}$ and ATP, and the inhibition by MPA (Fig. 2) strongly suggest that PEPCK is involved in the metabolism of Asp in BS cells of maize. The conditions supporting malate decarboxylation (Fig. 5) were different from those supporting Asp decarboxylation. ATP enhanced malate decarboxylation only in the dark, whereas Asp and 3-phosphoglycerate stimulated malate decarboxylation in the light. Since Asp facilitates the transport of malate into the chloroplasts (Chapman and Hatch, 1979; Boag and Jenkins, 1986) and 3-phosphoglycerate is important for the reoxidation of NADPH formed in the NADP-ME reaction (Hatch and Kagawa, 1976), the stimulation by Asp and 3-phosphoglycerate confirms that malate is mainly decarboxylated by NADP-ME and not by PEPCK.

The lack of an effect of illumination on Asp decarboxylation indicates that the activity of PEPCK is not subject to rapid modulation by light. In some C$_4$ plants PEPCK is phosphorylated in the dark and dephosphorylated under illumination (Walker and Leegood, 1996). However, PEPCK from maize is not phosphorylated (Walker et al., 1997), and the putative phosphorylation motif (Walker and Leegood, 1995) is modified (Furumoto et al., 1999). Thus, light-dependent activation of PEPCK by dephosphorylation probably does not occur in maize. Expression of the PEPCK gene, on the other hand, is much higher in the daytime than at night (Furumoto et al., 1999).

Decarboxylation of Asp not only was dependent on Mn$^{2+}$ but was stimulated when Mg$^{2+}$ was present in addition to Mn$^{2+}$. Although previous studies have shown that in vitro activities of PEPCK from *P. maximum* and *U. panicoides* are inhibited by Mg$^{2+}$ at physiological concentrations (Burnell, 1986; Walker et al., 1997), more so in N-sufficient than in N-deficient maize (Khamsi et al., 1992), but measurements of labeling and/or pools do not give any indication of relative fluxes. The relative capacities for Asp and malate decarboxylation would have major energetic consequences in the BS. Whereas malate decarboxylation by NADP-ME provides one-half of the NADPH required for the reduction of 3-phosphoglycerate, no NADPH is formed during Asp decarboxylation by

**Figure 4.** Western blots for PEPCK, cytosolic Asp aminotransferase (cAsp-AT), cytosolic Ala aminotransferase (cAla-AT), NAD-ME, and NADP-ME in leaves of *U. panicoides*, *C. gayana*, *P. maximum*, *Z. mays*, *E. crus-galli*, and *S. officinarum*. Equal amounts of fresh weight were loaded per lane.

**Figure 5.** Decarboxylation of 12.5 mm [4-14C]malate by BS strands at a PPFD of 200 μmol m$^{-2}$ s$^{-1}$ (white bars) or in the dark (black bars). The incubation medium for the control treatment contained 1 mm Mn$^{2+}$ and 1 mm Mg$^{2+}$. For the other treatments, 10 mm ATP, 10 mm NAD, 10 mm Asp, 5 mm 3-phosphoglycerate (PGA), or 400 μm MPA was added or, in one treatment, Mn$^{2+}$ was omitted. Data are means ± SE of three assays.

**Photosynthetic Metabolism in Maize**

The importance of PEPCK in photosynthetic metabolism will depend on the relative fluxes through Asp and malate during C$_4$ photosynthesis. In maize, substantial amounts of Asp are labeled (Hatch, 1971; Créach et al., 1974; Morot-Gaudry and Farineau, 1978; Chapman and Hatch, 1981), more so in N-sufficient than in N-deficient maize (Khamis et al., 1992), but measurements of labeling and/or pools do not give any indication of relative fluxes. The relative capacities for Asp and malate decarboxylation would have major energetic consequences in the BS. Whereas malate decarboxylation by NADP-ME provides one-half of the NADPH required for the reduction of 3-phosphoglycerate, no NADPH is formed during Asp decarboxylation by
PEPCK. Typically, BS cells of NADP-ME-type species show little or no capacity for PSII-dependent photoreduction of NADP. The occurrence of PEPCK in NADP-ME-type species may be correlated with PSII activity. Species that contain PEPCK (maize, *D. sanguinalis*, and *Setaria lutescens*; Gutierrez et al., 1974; Walker et al., 1997) show some PSII activity in the BS cells (Ku et al., 1974; Chapman et al., 1980), whereas BS cells of *S. bicolor* and *S. officinarum* lack PEPCK and have the lowest PSII activity of the C₄ species tested (Ku et al., 1974).

Decarboxylation of Asp in the presence of 2-oxoglutarate shows that Asp aminotransferase activity is present in the BS strands. In contrast to PEPCK species, the transamination of Asp to form oxaloacetate as the substrate for the PEPCK reaction is probably not catalyzed by cytosolic Asp aminotransferase (Fig. 4). Chapman and Hatch (1981) showed that the activity of Asp aminotransferase in BS cells of maize is located mainly in the mitochondria. In addition to oxaloacetate, the PEPCK reaction requires ATP. In PEPCK-type species, this ATP is provided by the activity of mitochondrial NAD-ME in combination with the respiratory chain (Hatch, 1987). In maize, some NAD-ME protein was present, but the amount was much lower than in PEPCK-type species (Fig. 4), which is consistent with measurements of its activity (Hatch et al., 1975). An additional source of ATP could be the conversion of PEP to pyruvate by pyruvate kinase. The activity of pyruvate kinase was, however, lower than the activity of PEPCK (Table I), and in vivo rates of pyruvate formation from PEP were not sufficient for the complete conversion of PEP into pyruvate (Fig. 3). Valle and Heldt (1991) measured similar activities of pyruvate kinase in BS strands of maize and concluded that these activities were not high enough to fully account for the formation of Ala from PEP. In addition, activities of pyruvate kinase in PEPCK-type species either are lower than rates of photosynthesis (Smith and Woolhouse, 1983) or are not detectable (Rathnam and Edwards, 1977). Thus, PEP and not pyruvate or Ala is probably the C₃ compound mainly transported from the BS to the mesophyll, where it can serve directly as a substrate for PEPCK.

In conclusion, our results show that two distinct pathways for the decarboxylation of malate and Asp occur in maize and probably in other species previously classified as NADP-ME types (Fig. 6): Whereas malate is decarboxylated by NADP-ME in the chloroplasts, resulting in the formation of pyruvate, Asp is decarboxylated by PEPCK in the cytosol, resulting in the formation of PEP. It would appear that NADP-ME species can be subdivided into those with and without PEPCK, extending the biochemical classification of C₄ plants. Further studies will show to what extent the PEPCK-dependent pathway contributes to C₄ photosynthesis under different environmental conditions and how the activity of PEPCK is regulated.

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**LITERATURE CITED**


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**Figure 6.** Photosynthetic metabolism in maize leaves. The oxaloacetate (OAA) formed by PEPCK (1) in the mesophyll is either reduced to malate or transaminated to Asp. Whereas malate is decarboxylated to pyruvate by NADP-ME (2) in the chloroplasts of the BS cells, oxaloacetate formed from Asp in the mitochondria is decarboxylated to PEP by PEPCK (3) in the cytosol of the BS cells. The CO₂ produced in the decarboxylation reactions is subsequently fixed by Rubisco (4). PGA, Phosphoglycerate.
sources of ATP and NADPH and the contribution of photosystem II. Arch Biochem Biophys 202: 330–341


