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**Article:**
Phosphoenolpyruvate Carboxykinase Is Involved in the Decarboxylation of Aspartate in the Bundle Sheath of Maize

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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, L.I. Técsi, R.C. Leegood [1997] Aust J Plant Physiol 24: 459–468). In the present study, we investigated the role of PEPCK in C₄ photosynthesis in maize. PEPCK activity and protein were enriched in extracts from bundle-sheath (BS) strands compared with whole-leaf extracts. Decarboxylation of [⁴¹⁴]C]aspartate (Asp) by BS strands was dependent on the presence of 2-oxoglutarate and Mn²⁺, 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 [⁴¹⁴]C]malate was stimulated severalfold by Asp and 3-phosphoglycerate, and was light dependent. Our data show that decarboxylation of Asp and malate in BS cells of maize occurs via two different pathways: Whereas malate is mainly decarboxylated by NADP-malic enzyme, decarboxylation of Asp is dependent on the activity of PEPCK.

C₄ plants have been classified as NADP-ME, NAD-ME, and PEPCK types, according to the major decarboxylase involved in the decarboxylation of C₄ acids in the BS cells (Gutierrez et al., 1974; Hatch et al., 1975). Maize (Zea mays) belongs to the NADP-ME subgroup of C₄ 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 Flavera 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 C₄ species mainly use Asp as the CO₂ donor and decarboxylate the oxaloacetate formed via the following reaction:

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

In NADP-ME species such as maize, ¹⁴CO₂ 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 Farineau, 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 μmol m⁻² 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] ADP₂⁻/H¹1545).

Abbreviations: Asp, aspartate; BS, bundle sheath; ME, malic enzyme; MPA, 3-mercaptopicolinic acid; PEPC, PEP carboxylase; PEPCK, PEP carboxykinase.

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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 bleeding 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 M 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).

**Western Analysis**

For the assay of PEP phosphatase. Western Analysis was described by Zervoudakis et al. (1997); ADP was omitted measured according to the method of Walker et al. (1995). Determination of Enzyme Activities

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 carboxybie 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.

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**Determination of PEP and Pyruvate Formation**

For the determination of PEP and pyruvate formation, BS strands were resuspended in reaction medium (blending buffer containing 10 mM 2-oxoglutarate, 1 mM MnCl$_2$, 1 mM MgCl$_2$, and different concentrations of ATP and ADP), and 0.4 mL of the suspension was incubated with 0.1 mL of 62.5 mM Asp or malate with a specific radioactivity of 0.2 MBq mmol$^{-1}$) in sealed vials. After 20 min at 25°C, the reaction was stopped by injecting 0.3 mL of 1 M HCl and the released $^{14}$CO$_2$ was trapped on filter paper moistened with 0.1 mL of 20% (v/v) KOH. Phaeophytin was extracted in 80% acetone and quantified according to the method of Vernon (1960).

**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 PEPCK, 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-kDa 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...
the metabolism of externally added substrates such as Asp. Rates of \([4-^{14}\text{C}]\text{Asp}\) 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 Mn\(^{2+}\) and, accordingly, Mg\(^{2+}\) did not substitute for Mn\(^{2+}\) in the decarboxylation of Asp. Mg\(^{2+}\) did, however, stimulate Asp decarboxylation in the presence of Mn\(^{2+}\). The requirements for Mn\(^{2+}\) 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 \(\mu\text{mol min}^{-1} \text{mg}^{-1} \text{phaeophytin}\)), 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 \(\mu\text{mol min}^{-1} \text{mg}^{-1} \text{phaeophytin}\)) 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 \(\mu\text{mol min}^{-1} \text{mg}^{-1} \text{phaeophytin}\) (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 \(\mu\text{M} \text{PEP}, 10 \mu\text{M} \text{ADP}, 1 \mu\text{M} \text{Mn}^{2+}, 1 \mu\text{M} \text{Mg}^{2+}, \text{and 40 mM KCl}\).

Data are means ± s.e of three plants. n.d., Not determined.

<table>
<thead>
<tr>
<th>Extract</th>
<th>PEPCK</th>
<th>PK</th>
<th>PP</th>
<th>Pyr</th>
</tr>
</thead>
<tbody>
<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>

**Figure 1.** A and B, Western blots for PEPC, PEPCK, and Rubisco, and the polypeptide pattern in extracts from whole leaves (WL) and BS strands from maize. The BS strands were isolated including (A) or omitting (B) treatment with cellulase and pectinase. C, Western blot for PEPCK in extracts from whole leaves (WL) and BS strands from *U. panicoides* isolated after treatment with cellulase and pectinase. Three micrograms of total protein was loaded per lane.
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 oxalacetate, 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 Mn²⁺, malate decarboxylation was slightly reduced, but the inhibition was minor compared with the effect on Asp decarboxylation. In the presence of Mn²⁺, Mg²⁺, Asp, and 3-phosphoglycerate, ATP inhibited the decarboxylation of malate, possibly by binding the Mg²⁺ 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...
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), recent work in this laboratory has shown that Mg$^{2+}$ is an activator at physiological concentrations of Mn$^{2+}$.

### 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

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.
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 PEPC.

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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