Phosphoenolpyruvate Carboxykinase in Plants Exhibiting Crassulacean Acid Metabolism

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ABSTRACT

Phosphoenolpyruvate carboxykinase has been found in significant activities in a number of plants exhibiting Crassulacean acid metabolism. Thirty-five species were surveyed for phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxylase, ribulose diphosphate carboxylase, malic enzyme, and malate dehydrogenase (NAD). Plants which showed high activities of malic enzyme contained no detectable phosphoenolpyruvate carboxykinase, while plants with high activities of the latter enzyme contained little malic enzyme. It is proposed that phosphoenolpyruvate carboxykinase acts as a decarboxylase during the light period, furnishing CO₂ for the pentose cycle and phosphoenolpyruvate for gluconeogenesis.

Some properties of phosphoenolpyruvate carboxykinase in crude extracts of pineapple leaves were investigated. The enzyme required Mn⁺, Mg⁺, and ATP for maximum activity. About 60% of the activity could be pelleted, along with chloroplasts and mitochondria, in extracts from leaves kept in the dark overnight.

As early as 1884, a group of higher plants was described as having a diurnal acidification and decacidification (28). Upon further investigation, this phenomenon was found to be comprised of a rhythm of CO₂ uptake, starch catabolism, and malic acid synthesis in the dark. This is followed in the light, by a reduction of CO₂ uptake with a concomitant breakdown of malic acid and synthesis in starch. Higher plants exhibiting these features became known as plants with Crassulacean acid metabolism, named after the family containing the best known species of this metabolic type. The significance of CAM¹ is emphasized by the ability of these plants to grow under extremely arid conditions.

Malic acid is the major acidic component having a diurnal cycle in CAM plants. Therefore, enzyme investigations with CAM tissues have centered around the processes of synthesis of malic acid in the dark and its subsequent catabolism in the light. PEP carboxylase (EC 4.1.1.31) and malate dehydrogenase (NAD) (EC 1.1.1.37) presumably act in a sequence of coupled reactions in synthesizing malic acid in the dark, which results in acidification (26). Furthermore, the suggestion has been made that the decacidification process is catalyzed by malic enzyme (EC 1.1.1.40), affecting an oxidative decarboxylation of malic acid (26, 31). Indeed malic enzyme activity has been demonstrated in a few CAM plants (the available data are collected in Table IV), but substantial activity was found in only three of these plants (16, 20).

In 1960, Ranson and Thomas (26) postulated that another enzyme, namely PEP carboxykinase (EC 4.1.1.32), could serve in CAM plants as the decarboxylase during light decacidification. PEP carboxykinase activity has been found in other plants (2, 10, 17); however, it has not been demonstrated in CAM plants. This enzyme catalyzes the reaction shown in equation 1:

\[ \text{OAA} + \text{ATP} \rightleftharpoons \text{PEP} + \text{CO₂} + \text{ADP} \] (1)

We have detected and assayed the activity of PEP carboxykinase in a number of CAM plants. We report here the activity of PEP carboxykinase, PEP carboxylase, RuDP carboxylase, malic enzyme, and malate dehydrogenase (NAD) in some species of CAM plants. The PEP carboxykinase of pineapple (Ananas comosus) has been examined in more detail and some of its properties are reported.

MATERIALS AND METHODS

Plant Material. Most of the plants were cultivated in a greenhouse under natural light conditions. Some specimens were obtained from the Botanical Garden, Berkeley, California, and from the University of California, Riverside.

Preparation of Extracts. Leaf samples which were used to prepare extracts were excised from plants between 10:00 AM and 3:00 PM. The leaf material or photosynthetic tissues were ground with a mortar and pestle in a medium containing 0.1 M HEPES, pH 7.0, 1% polyvinylpyrrolidone-40, 10 mM DTT, 10 mM MgCl₂, 10 mM MnCl₂. The homogenate was filtered through a 20 micron nylon net to remove cellular debris, and a sample was taken to determine the chlorophyll (33). The extracts of certain plants were treated on small columns of Sephadex G-25 (0.9 × 12 cm) using the extraction buffer to elute the sample.

PEP Carboxykinase Assays. PEP carboxykinase was assayed routinely by the exchange of ⁴⁰CO₂ into OAA according to Mazelis and Vennesland (17). The reaction mixture contained
in addition to various concentrations of enzyme 10 mm OAA, 5 mm ATP, 10 mm NaH\(^{14}\)CO\(_3\), 10 mm MgCl\(_2\), 10 mm MnCl\(_2\), 10 mm DTT, and HEPEs buffer pH 7.0, 0.1 M.

PEP carboxykinase also was assayed by measuring the ADP-dependent carboxylation of PEP. The assay consisted of 10 mm PEP, 10 mm NaH\(^{14}\)CO\(_3\), 5 mm ADP, 10 mm MgCl\(_2\), 10 mm MnCl\(_2\), and leaf extract in 0.1 M MES pH 6.0.

**PEP Carboxylase.** PEP carboxylase was assayed as \(^{14}C\)-incorporated in a reaction mixture containing 10 mm PEP, 10 mm NaH\(^{14}\)CO\(_3\), 5 mm DTT, 20 mm glutamate, glutamate-OAA-transaminase (1 unit) and 0.1 M tris-buffer, pH 8.3.

RuDP Carboxylase. RuDP-carboxylase was assayed as \(^{14}C\)-incorporated in a medium including 1 mm RuDP, 10 mm MgCl\(_2\), 50 mm NaH\(^{14}\)CO\(_3\), 5 mm DTT, and 0.1 M tris buffer, pH 8.3.

**Malic Enzyme and Malate Dehydrogenase (NAD).** They were assayed by standard procedures (14, 16). Acid-stable \(^{14}C\)-radioactivity was determined by liquid scintillation counting; samples containing OAA-\(^{14}C\) were counted within 30 min to diminish errors due to nonenzymatic breakdown of OAA.

**Leaf Organelle Preparation.** For preparation of cellular organelles, a pineapple leaf was homogenized for 1 to 2 sec with a Polytron mixer in a medium described by Cockburn et al. (7) containing 1% polyvinylpyrrolidone-40 and 5 mm DTT. The suspension was filtered rapidly through a 20 micron nylon screen and centrifuged at 10,000 g for 2 min. The pellet contained the cellular organelles. Mitochondrial activity was determined by assaying the activity of cytochrome c oxidase (27), and chlorophyll was measured as an indicator of chloroplasts.

**RESULTS**

The methods used to assay for the presence of PEP carboxykinase clearly indicated that the enzyme was present and the methods indicated similar levels of activity (Table I). In order to distinguish the activity of PEP carboxykinase from PEP carboxylase activity, it was necessary to use several different pHe values for the assays. PEP carboxylase has only little activity at pH 6, whereas upon the addition of ADP, carboxylation is stimulated, revealing the presence of PEP carboxykinase (Table I).

Further investigation of PEP carboxykinase from pineapple showed that ATP satisfied best the absolute nucleotide requirement of the exchange reaction and that maximum activity was obtained with a combination of Mg\(^{2+}\) and Mn\(^{2+}\). To exclude the possible action of pyruvate, phosphate dikinase (Pyr, Pi dikinase) the exchange reaction was also run with pyruvate and ATP, but no activity could be detected. These results are compiled in Table II.

Fractionation of crude pineapple leaf extracts obtained with the medium of Cockburn et al. (7) showed that the chloroplasts sedimented at a centrifugal force of 200g when applied for 1 min. However, cytochrome c oxidase assays indicated that most of the mitochondria also were sedimented under these conditions. This mixture could not be resolved satisfactorily by sucrose density gradient centrifugation. Therefore the filtered homogenate was centrifuged at 10,000 g for 2 min. Under these conditions, about 60% of the total PEP carboxykinase activity of the crude extract was found in the organelle pellet and the remainder in the supernatant fraction. The latter activity could not be pelleted by centrifuging at 150,000g. In addition, it was observed that the above distribution could be obtained only from preparations of absolutely dark-destarched (12 hr in the dark) pineapple leaves. If the leaves were harvested during or 1 to 3 hr after the light period, the organelle preparation was by no means satisfactory and less than 10% of the total PEP carboxykinase was found in the pelleted fraction.

Numerous CAM species were assayed for PEP carboxykinase activity using the exchange reaction. In this survey, assays for PEP carboxylase, RuDP carboxylase, malic enzyme, and malate dehydrogenase (NAD) were also included. The data are presented in Table III. All plants used were known to be CAM plants (18, 23, 24); during the course of this investigation, however, diurnal acid cycles were not tested. Two groups of CAM plants could be distinguished. The one group contained significant activities of PEP carboxykinase, whereas malic enzyme was present at lower levels. In the other group, PEP carboxykinase could not be detected, while malic enzyme was easily detected.

Certain of the crude extracts were treated by gel filtration on columns of Sephadex G-25 to remove small molecular

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**Table II. Properties of PEP Carboxykinase from Pineapple Leaf Extracts**

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>Activity</th>
<th>(\mu)moles/hr-mg Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Divalent metal ion requirement</td>
<td>Concentration (mm)</td>
<td>Mg(^{2+})</td>
</tr>
<tr>
<td>OAA + ATP</td>
<td>9.0</td>
<td>195</td>
</tr>
<tr>
<td>OAA + ADP</td>
<td>585</td>
<td>850</td>
</tr>
<tr>
<td>B. Nucleotide requirement</td>
<td>Reaction components (^{2})</td>
<td>OAA + ATP</td>
</tr>
<tr>
<td>Pyr + ATP</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>OAA + ADP</td>
<td>172</td>
<td></td>
</tr>
<tr>
<td>OAA + GTP</td>
<td>550</td>
<td></td>
</tr>
<tr>
<td>OAA + ITP</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>OAA + CTP</td>
<td>193</td>
<td></td>
</tr>
<tr>
<td>OAA + UTP</td>
<td>165</td>
<td></td>
</tr>
</tbody>
</table>

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\(^{1}\) Not detectable.

\(^{2}\) Conc. of nucleotides 5 mm, conc. of organic acids 10 mm. Each assay with 10 mm Mg\(^{2+}\) and 10 mm Mn\(^{2+}\).
The enzyme which decarboxylates the high-accumulated organic acids during light periods in CAM plants is of central importance to the operation of the Crassulacean acid metabolism. It has been proposed that malic enzyme may serve this function (26). In Table IV, a summary of the reported malic enzyme activities of CAM plants is given. In support of the above proposal, however, only two of these reports indicate that high levels of malic enzyme can be found (16, 20). We could not locate data demonstrating the presence of PEP-CK in CAM plants. The malic enzyme activity in CAM plants has not been extensively characterized, although Walker (30) has studied some properties of this enzyme in four CAM species, and Mukerji and Ting (21) presented evidence for three forms of the enzyme in Opuntia. Thus, substantial evidence for active decarboxylation activity is available for only three CAM species (Table IV).

The survey of enzyme activities presented in Table III shows that malic enzyme is present in a number of CAM species and that it is the major organic acid decarboxylating enzyme which could be detected in certain of these species. However, in a large number of the CAM species investigated, malic enzyme was found to have a low activity while another decarboxylating enzyme, PEP carboxykinase, was found to have substantial activity.

No change in the activity of either PEP carboxykinase or malic enzyme was observed when crude extracts of certain of the plants in Table IV were treated on a Sephadex G-25 column. Thus, it appeared that low molecular weight compounds were not inhibiting the activity of these two enzymes in crude extracts. A similar result has been previously reported with malic enzyme in the extract of Bryophyllum (16). Thus, we propose that PEP carboxykinase can serve as an OAA decarboxylase in certain CAM plants, whereas malic enzyme may be of minor importance in these plants. Two groups of CAM plants are evident in Table III: (a) those using malic enzyme to decarboxylate malate and (b) those using preferentially PEP carboxykinase to decarboxylate OAA stemming from malate. Hence, Figure I is proposed, where plants using malic enzyme would follow scheme A and those using PEP carboxykinase would comply with scheme B. Scheme A is essentially the same as that proposed previously (26, 31, 32); Scheme B would require the operation of malate dehydrogenase to convert malic acid to oxaloacetic acid. The latter, which is the substrate for PEP carboxykinase, would be rapidly decarboxylated. If CAM plants are considered in general, it seems clear that variations in the pathways of net carbon assimilation may exist in CAM plants similar to those in C3 plants (3).

The PEP carboxykinase of pineapple has similar properties as the same enzyme found in other plants (2, 10, 17). All plant PEP carboxykinases studied have highest activity with weight compounds from the crude extract. The activities of PEP carboxykinase and malic enzyme were assayed in the Sephadex G-25 treated extracts and these activities were compared with those of the crude (untreated) extract. For Agave desertii, Kleina repens, Opuntia basilaris, and Sedum weinbergii the activities of PEP carboxykinase and malic enzyme were not changed by Sephadex G-25 treatment; in other words, no PEP carboxykinase activity could be detected in either the crude or the Sephadex-treated extracts of these plants. For Billbergia saundersi, Neoregelia carolinia, Guzmania lingulata, and Ananas comosus, the activity of malic enzyme was not changed by the treatment of the crude extract on Sephadex G-25.

**DISCUSSION**

The enzyme which decarboxylates the high-accumulated organic acids during light periods in CAM plants is of central importance to the operation of the Crassulacean acid metabolism.
ATP and have various divalent metal ion requirements for either Mg\(^{2+}\) or Mn\(^{2+}\). With the CAM plant PEP carboxylase, highest activity was found when both Mg\(^{2+}\) and Mn\(^{2+}\) were present. A similar synergistic effect was found with the enzyme from a C\(_4\) plant (G. Edwards and R. Kanai, personal communication) and also with the enzyme from guinea pig liver (13). In the latter case, two forms of PEP carboxylase were found: the mitochondrial enzyme, which was more active with Mg\(^{2+}\), and the soluble-fraction enzyme, which was more active with Mn\(^{2+}\) (13). In preparations of the cellular organelles from pineapple leaves, PEP carboxylase was found in both the particulate and the soluble fraction. These data tend to support the view that two forms of the enzyme exist in the tissues of this CAM plant, but further separation and purification would be necessary to establish the existence of multiple forms of this enzyme.

In Figure 1, scheme A, the decarboxylation of malic acid by malic enzyme yields CO\(_2\) and pyruvate. The CO\(_2\) is incorporated into 3-PGA by RuDP carboxylase, but the fate of the pyruvate has not yet been fully elucidated. Some reports indicate that the pyruvate is broken down in the tricarboxylic acid cycle and that CO\(_2\) could be reassimilated (6). Other findings report the intact incorporation of pyruvate into carbohydrates (12). The latter proposal would require the conversion of pyruvate to PEP. Kluge and Osmond (15) have found activity for the enzyme Pyr, Pi dikinase in two CAM plants. Although the activities found were only 20 and 22 \(\mu\)mol/ hr·mg Chl (15), the conversion of pyruvate to PEP in CAM plants in the light is possible. Thus, the PEP could be used to synthesize starch. In contrast the decarboxylation of OAA, in CAM plants using scheme B of Figure 1, yields PEP and CO\(_2\). As a consequence, the enzyme Pyr, Pi dikinase would not be required in these plants. In fact, it could not be detected in one CAM plant (9) containing a substantial level of PEP carboxykinase (Table III). Furthermore, in CAM plants utilizing PEP carboxykinase as the decarboxylase, the theoretical stoichiometry of energy required for net CO\(_2\) fixation would be decreased by 1 ATP per CO\(_2\) fixed (3, 4).

The existence of two pathways for light decarboxylation in CAM plants has some similarity to the light decarboxylation of organic acids in C\(_3\) plants (3). In C\(_4\) plants evidence has been presented for malic enzyme and PEP carboxylase to function as decarboxylases in the light (3, 10, 14). C\(_3\) plants apparently have no differences in the composition of enzymes involved in the reductive pentose cycle, which is their pathway of carbon fixation (3). Thus, CAM and C\(_4\) plants not only have more complex pathways of net carbon fixation but in addition, they appear to have a diversity of enzymes for those pathways. This diversity may indicate a more recent and perhaps multiple appearance of the carbon fixation pathways of CAM and C\(_3\) in specific plants when compared to the reductive pentose cycle of C\(_4\) plants. This view may be supported by the fact that both CAM and C\(_4\) plants appear to have the reductive pentose cycle enzymes and that they utilize them in essentially the same fashion as do C\(_3\) plants (3, 22).

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LITERATURE CITED