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Characterization of Phosphoenolpyruvate Carboxykinase from Pineapple Leaves *Ananas comosus* (L.) Merr.[1]

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ABSTRACT

Phosphoenolpyruvate carboxykinase has been partially purified from pineapple (*Ananas comosus* [L.]) leaves. Specific activities obtained show it to be a major activity in this tissue. Above 15 C, the respective activation energies for decarboxylation and carboxylation are 13 and 12 kcal/mol. Below 15 C, there are discontinuities in Arrhenius plots with an associated large increase in activation energy. The adenine nucleotides are preferred to other nucleotides as substrates. The apparent *Km* values in the carboxylation direction are: ADP 0.13 mM, HCO_3^- 3.4 mM, and phosphoenolpyruvate 5 mM. In the decarboxylation direction, the apparent *Km* values are: ATP 0.02 mM, ADP 0.05 mM, and oxaloacetate 0.4 mM. The decarboxylation activity had an almost equal velocity with either ADP or ATP. The pH optima are between 6.8 and 7. Inhibition of the carboxylation reaction by ATP, pyruvate, and carbonic anhydrase was demonstrated. Decarboxylase specific activities are over twice carboxylation activities. The data support a model in which phosphoenolpyruvate carboxykinase is of physiological significance only during the light period and then only as a decarboxylase.

The mechanisms which control the diurnal activation and deactivation of various portions of Crassulacean acid metabolism are poorly understood (15). One possible mode of control is the activation and deactivation of enzymes in a pathway(s). Since acid decarboxylation is essentially the first step in the light portion of CAM[2] CO_2 metabolism, it is a likely step for being under some type of control. Three enzymes have been reported in specific CAM plants: $NADP^+$-malic enzyme, PEP carboxykinase, and NAD^+-malic enzyme, which act as decarboxylases during the light period (6-8). Pineapple and *Aloe vera* contain PEP carboxykinase (6, 8). In *A. vera*, grown in controlled phytotron environments, PEP carboxykinase activity reflects the degree of CAM occurring in these plants (6). The distribution of PEP carboxykinase in CAM species plus an initial biochemical characterization of the pineapple and *Aloe* enzyme, mostly studying the exchange reaction, have been presented (6, 8). PEP carboxykinase catalyzes a nucleotide-dependent reversible carboxylation of PEP and an exchange of $^{14}CO_2$ into OAA (13). Since the exchange reaction catalyzed by PEP carboxykinase has no known physiological function, this study concentrates on the carboxylation and decarboxylation activities. This manuscript reports some additional characteristics of PEP carboxykinase from pineapple leaves to define further its role in CAM and to determine characteristics or mechanisms which could control its diurnal activity *in vivo*.

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[2] Abbreviations: CAM: Crassulacean acid metabolism; PEP, phosphoenolpyruvate; PEP carboxykinase: phosphoenolpyruvate carboxykinase; OAA: oxaloacetic acid; TLCK: N-α-tosyl-L lysine chloromethyl ketone HCl; E_a: energy of activation; C_3: reductive pentose phosphate; C_4: C_4-dicarboxylic; DTT: dithiothreitol; EU: enzyme units.

MATERIALS AND METHODS

Plant Material. Pineapple plants were grown in the greenhouse under good fertility and watering regimes. At least 2 weeks before harvesting, the plants were transferred to a growth chamber set on 30 C during a 15-hr day at 2000 ft-c and 15 C during a 9-hr night. Under both greenhouse and growth chamber conditions, the plants demonstrated the diurnal fluctuation of acidity characteristic of CAM.

Enzyme Extraction and Purification. Pineapple leaves were harvested at 6 to 8 hr into the light period when the levels of leaf acidity were low. The leaves were diced, then vigorously ground in a mortar with sand, insoluble PVP-360, and buffer. The extraction buffer contained 0.1 M imidazole-HCl (pH 7.2), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1% PVP-360, and 10 mM DTT, and was used in a ratio of 4 volumes of buffer to 1 g of leaf tissue. After grinding, the extract was filtered through cheesecloth. Samples then were taken for Chl determination. PEP carboxykinase was partially purified by modifications of the method of Ray and Black (17).

One modification was to inhibit protease activity. BSA, 6 mg/ml of extract, and a protease inhibitor, TLCK at 2 to 3 mg/ml of extract, were added to the enzyme extracts immediatey after the cheesecloth filtration. Without these additions there was a rapid loss of PEP carboxykinase activity and several PEP carboxykinase-containing protein fractions could be obtained when purifying the preparations. The enzyme extract was mixed with DEAE-Sephadex A-25 previously equilibrated with extraction buffer diluted 10-fold. The DEAE-Sephadex was poured into a column and the PEP carboxykinase activity eluted with a linear gradient of 0.05 to 0.16 M NaCl. The column procedure was quite stable to variation in gradient steepness, and moderately large changes in amount of column material did not greatly affect resolution. The typical column was approximately 200 ml in volume, 30 cm in height; the flow rate was about 2 to 3 ml/min. The usual amount of material loaded on the column was about 3 ml of extract which contained about 0.2 mg of Chl and 18 mg of added carrier protein. The bulk of the PEP carboxykinase activity was eluted at about 0.12 M NaCl. The NaCl concentrations of column fractions were determined by use of a YSI model 31 conductivity bridge at 0 to 4 C.

Enzyme Assays. The PEP carboxykinase decarboxylation reaction was assayed by the method of Hatch (11). Reaction mixtures contained 100 mM HEPES-NaOH (pH 7.2), 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.2 mM ATP, and 8 EU of pyruvate kinase (Sigma, rabbit muscle), and 1.2 mM OAA (Sigma). The OAA was prepared just before use (11). The molar extinction coefficient for OAA under these conditions was 1100. All PEP carboxykinase activities were corrected for nonenzymic OAA breakdown. Adenylate kinase activity could not be detected in the assay mixtures using the standard rabbit muscle assay from

Sigma Chemical Co. The PEP carboxykinase fractions from the DEAE-Sephadex column were concentrated to facilitate the lower sensitivity of this assay.

The PEP carboxykinase carboxylation reaction was assayed according to Chang and Lane (5). Reaction mixtures contained in a total volume of 1 ml: 20 mM imidazole-HCl (pH 7.2), 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 7.5 mM PEP, 0.14 mM NADH, 25 mM $NaHCO_2$, 1.5 mM ADP, and 5 EU of malic dehydrogenase (Sigma, pig heart).

Temperature curves were performed in a 1-ml jacketed cuvette. The temperature was monitored by measuring the temperature of the water in the cuvette jacket. The energy of activation was determined by the method of Wilson (18).

Protease was assayed by following absorption at 520 nm released from the insoluble substrate Azocoll (Calbiochem). Reaction mixtures of 5 ml contained 1/10 diluted extraction buffer, 5 mM DTT, and enzyme extract. Chlorophyll was determined by the method of Arnon (1) and protein by the method of Bradford (4).

RESULTS AND DISCUSSION

Partial Purification of PEP Carboxykinase. For the purposes of this study, PEP carboxykinase was separated from major competing activities, principally PEP carboxylase. The first purification method tried was that described by Ray and Black for *Panicum maximum* (17). In this method, PEP carboxykinase was partially purified by $(NH_4)_2SO_4$ precipitation, colloidion ultrafiltration and desalting, and gradient elution from a DEAE-Sephadex A-25 column. PEP carboxykinase and PEP carboxylase from pineapple leaves have closer relative affinities for DEAE-Sephadex than the relative affinities of the corresponding *P. maximum* enzymes (17). For this reason, gradient rather than step elution was used to separate these activities with a DEAE-Sephadex column. This method separated PEP carboxykinase from PEP carboxylase and the activity per mg protein increased at least 10-fold over the crude extract. The peak with the largest amount of PEP carboxykinase activity was used in initial studies. However, the method did not produce a stable enzyme and as many as six peaks of activity could be observed upon elution of the DEAE-Sephadex column.

Tests on crude extracts showed proteases present and abundant and their activities were susceptible to inhibition by TLCK. Also, when 6 mg of BSA was added to the crude preparation immediately after passage through cheesecloth, the period of maximal enzyme activity in crude extracts was prolonged from approximately 1 to about 6 hr. When BSA and TLCK were added to the crude extract, as described under "Materials and Methods," and immediately submitted to DEAE-Sephadex chromatography, only one peak of PEP carboxykinase activity was detected (Fig. 1). Only traces of PEP carboxylase could be detected in the PEP carboxykinase peak fractions. PEP carboxylase eluted in fractions 50 to 60 in Figure 1. Not only did this column treatment of a crude extract plus BSA and TLCK result in a single symmetrical peak, but the recovery of PEP carboxykinase activity as measured in the carboxylation direction was about 20% greater than the total in the crude extract. Presumably, enzyme inhibitors or other components (2, 3) are removed or activated during this purification.

Carboxylation Reaction. One objective of this work was to determine whether carboxylation of PEP by PEP carboxykinase is of physiological importance during the light or the dark period. Figure 2 shows the temperature dependence of carboxylation. At lower temperatures, the rates are quite small. When these data are presented as an Arrhenius plot, a discontinuity near 15 C is observed (Fig. 2B). Given the high energy of activation, $E_a \sim 80$ kcal mol^{-1}, at or below 15 C it is unlikely that PEP carboxykinase functions as a carboxylase at night, since at a night temperature of 15 C, pineapple accumulates acid in a normal CAM fashion (14). The E_a for decarboxylation and carboxylation are similar (Fig. 2B).

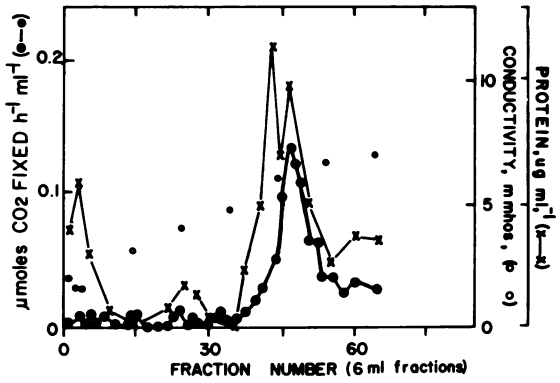

FIG. 1. DEAE-Sephadex A-25 column elution profile for pineapple leaf PEP carboxykinase (●) and protein (×) using a NaCl gradient (○). The extract was treated with BSA and TLCK.

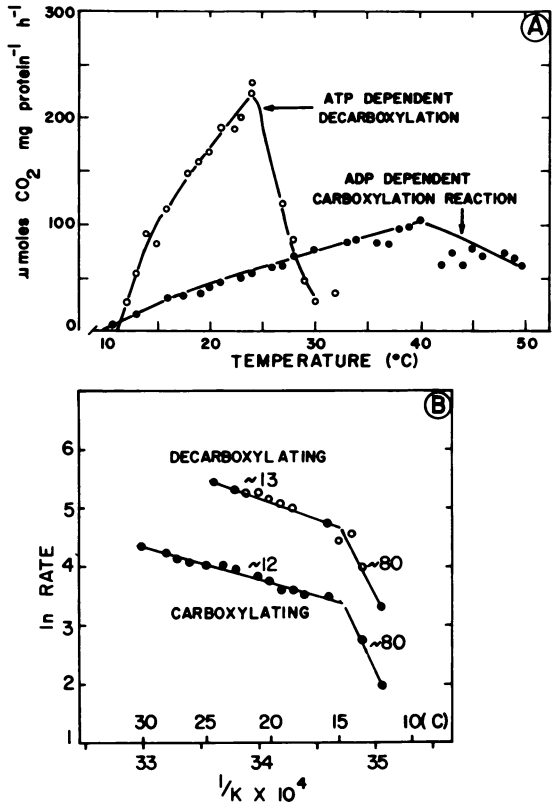

FIG. 2. Effect of temperature on pineapple leaf PEP carboxykinase. The enzyme was partially purified by the method of Ray and Black (17). The decarboxylation assay contained 2.5 mM $MgCl_2$. A: Rate *versus* temperature; B: Arrhenius plots of data in A. The numbers in B are E_a in kcal mol^{-1}.

The pH optima of both carboxylation and decarboxylation are slightly below 7 (Fig. 3), similar to the *P. maximum* enzyme (17). Preliminary work with the pineapple PEP carboxykinase indicated slightly broader pH optima for the exchange and carboxylation reactions in crude leaf extracts (8). Since the pH of chloroplasts from other plants have been reported to be 7.9 to 8.3 during the day (9, 12) and if we presume that pineapple chloroplasts have relatively similar pH levels, then this would exclude PEP carboxykinase activity in chloroplasts. The mitochondrial, microbody, or cytosol environment could support

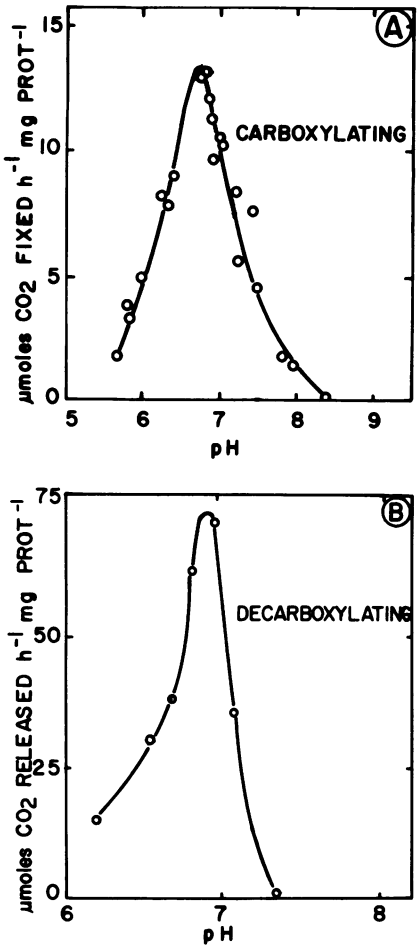

FIG. 3. pH response of the carboxylating (A) and decarboxylating (B) reactions of pineapple leaf PEP carboxykinase. The buffer was composed of 26 mM imidazole, 4 mM HEPES, and 40 mM tris, which was taken to pH 12 with NaOH and adjusted to pH for each assay with concentrated HCl. pH values were measured at 24 C immediately after each reaction was recorded.

such activity. The enzyme does occur in a particulate fraction (8, 13, 16) in a variety of plant tissues. In *P. maximum*, PEP carboxykinase has been reported to be in the chloroplasts (16). For the above reasons we cannot exclude a daytime CO_2 fixation function; however, we suggest that this is not physiologically significant. To support this suggestion, kinetic parameters were generated by substrate rate studies.

The *Km* for bicarbonate was found to be 3.4 mM (Table 1 and Fig. 4A), which is similar to 11 mM found with the *P. maximum* enzyme (17). In Figure 4B, the ADP titration of carboxylation in a double reciprocal plot yields a *Km* of 0.13 mM which could indicate a significant CO_2 fixation function. However, when we examine Figure 4C, the PEP titration of rate, we find a relatively high (17) *Km* value for PEP, 5 mM (Table I). Thus, unless PEP carboxykinase shares a compartment with a high PEP concentration, it is unlikely that the enzyme contributes significantly to CO_2 fixation during day or night.

Also shown in Figure 4, A, B, and C are three other common leaf constituents which influence the enzyme. Each of these components—carbonic anhydrase, ATP, and pyruvate—is a competitive inhibitor of the enzyme. Again, the results are interpreted as showing that carboxylation is not a likely function of PEP carboxykinase in the leaf.

Decarboxylation. In earlier work, PEP carboxykinase was proposed as the leaf decarboxylase in certain CAM plants (8). Decarboxylation substrate kinetic parameters are presented in Table 1 and Figure 4D. The *Km* values of OAA and ATP are 0.4 and 0.2 mM, respectively, similar to the P. *maximum* enzyme (17). Activity was observed with ADP (*Km* 0.05 mM) and the measured adenylate kinase activity was too low to explain the use of ADP by the enzyme. Ray and Black have discussed the ADP activity of PEP carboxykinase in more detail (17). These kinetic parameters, Table I, may be near physiological substrate concentrations (little information is available on substrate concentrations in CAM plant tissues or cells) and suggest a physiologically significant decarboxylation activity. However, the temperature dependence of decarboxylation (Fig. 2), and the Arrhenius plot (Fig. 2B), indicate that night decarboxylation is not likely under our growth conditions (15 C nights) and the energy of activation, $E_a \sim 80$ kcal mol^{-1} near 15 C, is consistent with no nocturnal function of decarboxylation. During the day, decarboxylation is the favored activity (Fig. 2) and the E_a of ~ 13 kcal is within standard enzymic values (Fig. 2B). Total acid loss with intact pineapple leaves during the day also has a similar E_a at 30 C, but near 20 C the $E_a > 42$ kcal mol^{-1} (14). Thus, the characteristics of total acid loss in these CAM leaves are parallel to these PEP carboxykinase data.

The pH optimum for decarboxylation is approximately 6.9, and is therefore similar to the carboxylation reaction; however, its range of physiologically significant activity is somewhat narrower (Fig. 3).

Nucleotide Specificity. Table II shows the nucleotide specificity. The carboxylation and decarboxylation reactions were run with different preparations and thus specific activities are not directly comparable. All PEP carboxykinase activities in the pineapple are best mediated by adenosine base nucleotides (Table II) similar to other plant PEP carboxykinase (8, 10, 11, 13, 17). The other nucleotides gave low levels of activity, making it

Table I. Substrate Km Values for Pineapple Leaf PEP Carboxykinase

Assay[1]	Substrate	Km
		mM
Carboxylating	HCO_3^-	3.4
	PEP	5.0
	ADP	0.13
Decarboxylating	OAA	0.4
	ATP	0.02
	ADP	0.05

[1]The assays were carried out with other substrates saturating at 24 C.

Table II. Nucleotide Preference of Pineapple Leaf PEP Carboxykinase

Nucleotide	Decarboxylation	Nucleotide	Carboxylation
	μmoles CO_2 released mg prot^{-1} hr^{-1}		μmoles CO_2 fixed mg chl^{-1} hr^{-1}
ATP	82	ADP	982
ADP	73	GDP	234
GTP	6	IDP	312
ITP	ND[1]	UDP	156
UTP	ND	CDP	218
CTP	ND	AMP	156
		Cyclic 3'5' AMP	ND

[1]Not detected.

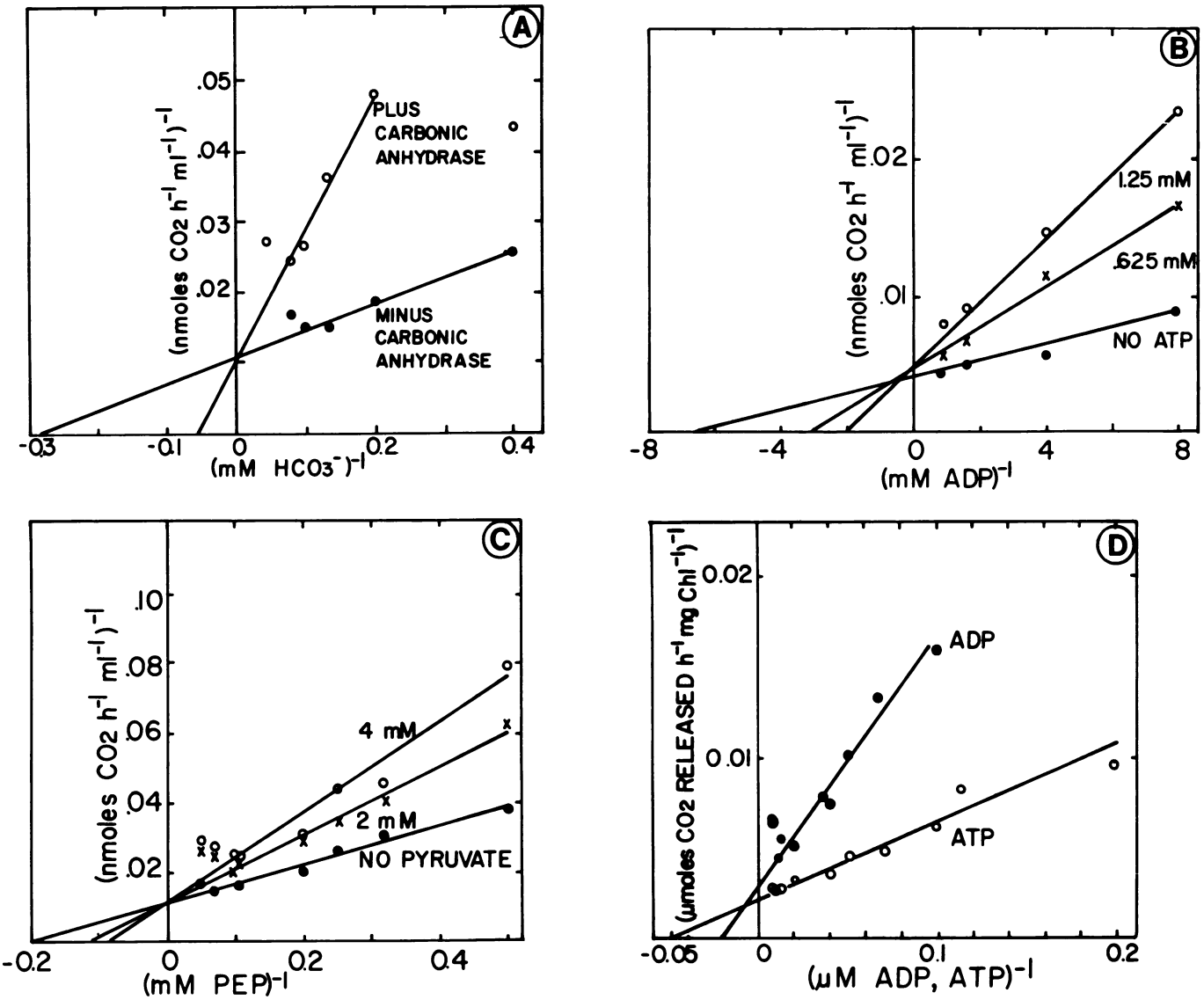

FIG. 4. Lineweaver-Burk double reciprocal plots of pineapple leaf PEP carboxykinase showing interactions with substrates and effectors. A: Effect of bicarbonate levels on carboxylation rates in presence and absence of carbonic anhydrase; each carbonic anhydrase assay contained 2500 Wilber-Anderson units; B: effect of ADP levels on carboxylation rates in presence and absence of ATP; C: effects of PEP levels on carboxylation rates in presence and absence of pyruvate; D: relative effectiveness of ATP and ADP on decarboxylation rates, assays performed pH 6.9.

difficult to determine whether the small activities can be attributable to the other nucleotides or to a contamination of the nucleotide preparations.

CONCLUSION

The night growth temperature of these pineapples was 15 C where the E_a of PEP carboxykinase as a decarboxylase or carboxylase are near 80 kcal mol^{-1}. Therefore, the enzyme is not expected to contribute to carboxylation or decarboxylation occurring during the night. During the day (30 C), the E_a, 12 to 13 kcal mol^{-1}, are approximately equal in both directions (Fig. 2B). However, the high *Km* of PEP and HCO_3^- indicates a minor carboxylation function. The lower *Km* values for OAA, ATP, and ADP (Table I) favor a decarboxylation function. Decarboxylation activity is over twice the rate of the carboxylation under saturating substrate conditions. In addition, ATP, pyruvate, and carbonic anhydrase—all leaf components—demonstrate inhibitory effects on the carboxylation reaction. The role of PEP carboxykinase in CAM plants is to function as an OAA decarboxylase during the day portion of the diurnal cycle.

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