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Characterization of Phosphoenolpyruvate Carboxykinase from *Panicum maximum*[1]

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THOMAS B. RAY AND CLANTON C. BLACK, JR.
Department of Botany, University of Georgia, Athens, Georgia 30602

ABSTRACT

Phosphoenolpyruvate carboxykinase, EC 4.1.1.32 (PEPCK), was purified 43-fold from the grass *Panicum maximum*. Michaelis constants (Km) were determined for the exchange reaction, the carboxylation reaction, and the decarboxylation reaction. The Km values for oxaloacetate and ATP in the decarboxylation reaction were found to be lower than the Km values for the substrates used in the exchange reaction and in the carboxylation reaction. Phosphoenolpyruvate carboxylase was not detectable in the purified PEPCK preparation.

Studies on the nucleotide specificity of the oxaloacetate decarboxylation rection indicate that ATP serves as the best nucleotide for this reaction and that ADP is about 60% as effective as ATP. The pH optimum for decarboxylase activity is near 6.8. The decarboxylation reaction has a divalent cation requirement with both Mn^{2+} and Mg^{2+} needed for full activity.

Temperature curves of the three PEPCK reactions indicate optimum activities between 38 and 45 C. There is a pronounced drop in the decarboxylation and carboxylation activities as the temperature is decreased from these optima. Below 30 C the energy of activation was 8.2 kcal/mol for the decarboxylation reaction.

These studies are consistent with the proposal that under physiological conditions PEPCK catalyzes the decarboxylation of oxaloacetate in the bundle sheath cells of *Panicum maximum* leaves during C_4 dicarboxylic acid photosynthesis.

Phosphoenolpyruvate carboxykinase catalyzes the nucleotide-dependent carboxylation of PEP[2] to produce OAA in a reversible fashion as shown in equation 1.

$$CO_2 + ADP + PEP \xleftrightarrow{PEPCK} OAA + ATP \quad (1)$$

In addition, PEPCK catalyzes a nucleotide-dependent exchange of CO_2 into OAA (16, 20).

PEPCK has been proposed to act as a decarboxylase during photosynthesis in bundle sheath cells of specific plants which carry out C_4 photosynthesis such as *Panicum maximum*. Through the action of PEPCK in these C_4 plants, CO_2 is released from OAA in the bundle sheath cells where the CO_2 is refixed by the C_3 cycle (3, 12, 14). In other C_4 plants either a $NADP^+$- or NAD^+-dependent malic enzyme acts as the decarboxylase in bundle sheath cells. In all C_4 plants the 4-carbon organic acids are formed in the mesophyll cells beginning with the carboxylation of PEP by PEP carboxylase (3).

PEPCK has been characterized from several microorganisms and animals (1, 5-7, 9). However, only limited information is available on the characteristics of this enzyme in higher plants and most of this has been obtained using crude extracts (2, 10-14, 16, 18). In addition, the data on plant PEPCK are on either the exchange reaction or the ADP-dependent carboxylation reaction. Using either of these reactions to study PEPCK in relation to C_4 photosynthesis is open to criticism since the proposed role of PEPCK is as a decarboxylase. The exchange reaction has been found to occur at rates much faster (3- to 30-fold) than either the carboxylation or decarboxylation reaction in animal tissue (7) but in higher plant leaf extracts, rates are nearly equal (11, 12). The physiological significance of the exchange reaction has not been established in higher plants. The carboxylation reaction catalyzed by PEPCK (equation 1) is somewhat similar to that of PEP carboxylase but its role in higher plants also is uncertain.

Even though PEPCK is proposed as the decarboxylase in bundle sheath cells of certain C_4 plants, no information is available on the characteristics of the ATP-dependent decarboxylation of OAA by the enzyme (12-14). This report presents data on the partial purification of PEPCK to remove PEP carboxylase using the C_4 grass *Panicum maximum*. The ATP-dependent decarboxylation reaction has been characterized and compared to the exchange reaction and the ADP-dependent carboxylation reaction.

MATERIALS AND METHODS

Enzyme Extraction and Purification. *Panicum maximum* was grown in the greenhouse under natural lighting conditions. Leaves were harvested, washed with deionized H_2O, blotted dry, weighed, cut into sections less than 2 mm wide, and ground in a chilled mortar with sand and buffer containing 0.1 M HEPES, pH 7, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 mM β-mercaptoethanol, and 1% w/v PVP-40. The leaf sections were ground vigorously and the resulting extract was filtered through eight layers of cheesecloth and then centrifuged for 30 min at 10,000*g*. Solid $(NH_4)_2SO_4$ was added to the supernatant fluid to make a 35% saturated solution. This was stirred at 4 C for 30 min and was then centrifuged for 30 min at 10,000*g*. The resulting supernatant fluid was brought to 55% saturation with $(NH_4)_2SO_4$, stirred for another 30 min at 4 C, and centrifuged for 30 min at 10,000*g*. The pellet was taken up in a small volume of buffer containing 10 mM HEPES, pH 7, 2 mM $MnCl_2$, 2 mM $MgCl_2$, and 5 mM β-mercaptoethanol. The suspended pellet was passed through a column of Sephadex G-25 (1 × 12 cm) previously equilibrated with 10 mM HEPES, pH 7, 2 mM $MnCl_2$, 2 mM $MgCl_2$, and 5 mM β-mercaptoethanol. The PEPCK-containing fraction was then placed onto a column of DEAE-Sephadex A-25 (2 × 12 cm) previously equilibrated with 10 mM HEPES,

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[2] Abbreviations: PEP: phosphoenolpyruvate; OAA: oxaloacetic acid; PEPCK: phosphoenolpyruvate carboxykinase; RuDP carboxylase: ribulose 1,5-bisphosphate carboxylase; C_4: C_4 dicarboxylic acid; C_3: reductive pentose phosphate; E_a: energy of activation; DTT: dithiothreitol; EU: enzyme unit.

pH 7, 2 mM $MnCl_2$ and $MgCl_2$, and 5 mM β-mercaptoethanol. PEPCK was eluted from the column with a step gradient of 0.09 M, 0.14 M, 0.2 M, and 1 M NaCl in the 10 mM HEPES buffer. Fractions were collected in 2-ml volumes at a rate of 1 ml/min. Protein was monitored by measuring absorbance of the fractions at 280 nm.

Enzyme Assays. The PEPCK exchange reaction was performed essentially as described by Dittrich *et al.* (11). Reactions were run at 40 C in a total volume of 0.5 ml and contained 50 mM MES, pH 6.6, 2.5 mM $MgCl_2$ and $MnCl_2$, 2 mM ATP, 50 mM $NaH^{14}CO_3$ (0.2 μCi/μmol), and 20 mM OAA. The OAA was prepared immediately before use as described by Hatch (13). The reaction mixture containing all components except OAA were preincubated at 40 C for 1 min followed by addition of the OAA to start the reaction. Aliquots of 50 μl were removed during the assay and added to scintillation vials containing 0.1 ml of 20% w/v trichloroacetic acid. Released $^{14}CO_2$ was removed from the vials with a stream of air. The samples then were counted for radioactivity in a Packard Tri-Carb scintillation spectrometer. One enzyme unit is defined as the amount of enzyme necessary to produce 1 μmol of ^{14}C-OAA/hr.

The PEPCK decarboxylation reaction was assayed by the method of Hatch (13). Reactions were run at room temperature in a 1-cm path length quartz cuvette. Reaction mixtures contained 50 mM HEPES, pH 7.2, 2.5 mM $MgCl_2$ and $MnCl_2$, 3 units of pyruvate kinase (type 1 from rabbit muscle, Sigma), 0.25 mM ATP, and 0.5 mM OAA in a total volume of 1 ml. Reactions were initiated by the addition of either ATP or enzyme. The molar extinction coefficient for OAA at 280 nm at the pH and Mg^{2+} and Mn^{2+} concentrations described above was 1200. One enzyme unit is defined as the amount of enzyme required for the decarboxylation of 1 μmol of OAA/hr. All PEPCK decarboxylation values shown are corrected for the nonenzymic breakdown of OAA.

The carboxylation reaction of PEPCK was assayed according to Chang and Lane (6). Reaction mixtures contained in a total volume of 1 ml 50 mM HEPES, pH 7.2, 2.5 mM $MgCl_2$ and $MnCl_2$, 5 mM PEP, 10 mM $NaHCO_3$, 0.25 mM NADH, and 6 units of malic dehydrogenase (Sigma type I from beef heart). Reactions were initiated upon addition of either ADP or enzyme. For the carboxylation reaction 1 enzyme unit is defined as the amount of enzyme required to oxidize 1 μmol of NADH/hr.

Temperature curves for the decarboxylation and carboxylation reactions of PEPCK were performed in a 1-ml jacketed cuvette (1-cm path length). The temperature was monitored by measuring the temperature of the water in the cuvette jacket. For the exchange reaction the temperature was monitored by insertion of a temperature probe directly into the reaction vial which in turn was in a thermostated water bath.

PEP carboxylase was assayed according to the method of Dittrich *et al.* (11).

Protein was determined by the method of Lowry *et al.* (15) or of Warburg and Christian (21).

RESULTS

Partial Purification of PEPCK. To study PEPCK it was necessary to separate PEPCK activity from PEP carboxylase activity. Separation was accomplished with DEAE-Sephadex A-25 chromotography. Preparations of PEPCK from *P. maximum* following $(NH_4)_2SO_4$ precipitation and desalting were applied to a column of DEAE-Sephadex A-25 (Table I). PEPCK was eluted from the column with 0.14 M NaCl. No PEP carboxylase activity could be detected in the fractions containing PEPCK activity even after desalting. In addition approximately a 40-fold purification could be achieved routinely with this procedure. The total recovery of PEPCK activity from the DEAE-Sephadex column was between 30 and 40% of the activity in the crude extract with about 20% in the peak fraction. Table I gives the results of one purification experiment starting with 15 g of leaf material. In later experiments, the use of PVP-360 in the initial extraction buffer in place of PVP-40 raised the total recovery of PEPCK activity from the DEAE-Sephadex column to over 70%.

Kinetic Parameters of PEPCK. Some kinetic values for the partially purified PEPCK from *P. maximum* are listed in Table II. The Michaelis constants for the substrates of the decarboxylation reaction were the lower of the three reactions. The range of substrate concentrations used to determine the *Km* for the decarboxylation reaction were 2.5 to 250 μM for ATP and 0.025 to 1 mM for OAA.

The *Km* HCO_3^- for the carboxylation reaction was determined to be 11 mM. CO_2 rather than HCO_3^- has been proposed as the substrate for PEPCK (10). When converted to a CO_2 concentration, the *Km* becomes 1.7 mM. The *Km* of ADP for the carboxylation reaction was determined to be about twice that of the *Km* of ATP for the decarboxylation reaction. The range of substrate concentrations used to determine the kinetic values for the PEPCK carboxylation reaction were 1 to 50 mM HCO_3^-, 0.2 to 10 mM PEP, and 0.1 to 0.5 mM ADP.

The *Km* of HCO_3^- for the exchange reaction was found to be rather high, 31 mM. When converted to a CO_2 concentration this value becomes 16 mM. This is an order of magnitude greater than the *Km* of CO_2 for the carboxylation reaction. The *Km* of OAA for the exchange reaction was about four times that of the decarboxylation reaction while the *Km* of ATP for the exchange reaction was found to be an order of magnitude greater than that for the decarboxylation reaction. The range of substrate concentrations used to determine the kinetic values for the PEPCK exchange reaction were 0.05 to 2 mM ATP, 0.25 to 20 mM OAA, and 5 to 60 mM HCO_3^-.

There was no apparent activation of enzyme activity by substrates in the three PEPCK reactions. At high HCO_3^- concentrations (>50 mM) there was some inhibition of exchange activity. This was the only example of substrate inhibition of PEPCK activity found.

Metal Requirements for Decarboxylation Activity. OAA decarboxylation by PEPCK has a requirement for a divalent cation (13). The requirement of both Mn^{2+} and Mg^{2+} for maximum

Table I. *Purification of Phosphoenolpyruvate Carboxykinase*
Activities were determined by the exchange reaction.

Fraction	Total Protein	Total Activity	Specific Activity	Fold Purification
	mg	EU	EU/mg protein	
Crude	28	3522	125	1
10^4 x g Supernatant	7.4	3522	476	3.8
$(NH_4)_2SO_4$	5.5	2768	503	4.0
DEAE-Sephadex (Peak)	.14	754	5385	42.8

Table II. *Kinetic Values for Phosphoenolpyruvate Carboxykinase Substrates*
The exchange reaction was measured at 40 C. The decarboxylation and carboxylation reactions were run at room temperature. Each *Km* was determined from Lineweaver-Burk reciprocal plots.

Reaction	Substrate	K_m (M)	V_{max} EU/mg protein
Exchange	OAA	5.0×10^{-4}	5.0×10^{3}
	ATP	2.9×10^{-4}	4.0×10^{3}
	HCO_3^-	3.1×10^{-2}	7.7×10^{3}
Decarboxylation	OAA	1.3×10^{-4}	3.25×10^{2}
	ATP	2.2×10^{-5}	2.36×10^{2}
Carboxylation	PEP	3.8×10^{-4}	8.4×10^{1}
	ADP	4.9×10^{-5}	1.1×10^{2}
	HCO_3^-	1.1×10^{-2}	3.55×10^{2}

exchange reaction activity has been reported using crude leaf extracts from *P. maximum* (12). The effects of various levels of Mn^{2+} and Mg^{2+} on the PEPCK decarboxylation reaction are given in Table III. Without any cation no activity was detectable. Highest activity was obtained when the enzyme was assayed in the presence of 2.5 mM Mn^{2+} and 2.5 mM Mg^{2+}. Mn^{2+} alone resulted in only 87% of the activity of both cations while Mg^{2+} alone gave only 26% of the activity. A 5-fold increase in the concentration of Mn^{2+} could not replace low Mg^{2+} concentrations. Likewise, high Mg^{2+} levels could not replace low Mn^{2+} concentrations. When both cations were present in high concentrations (12.5 mM) there was an apparent inhibition resulting in only 43% of the activity of low Mn^{2+} and Mg^{2+} concentrations.

Several other cations were tested to determine if they could replace Mn^{2+} and Mg^{2+}. Among those tested (Co^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Al^{3+}), only Co^{2+} was active in the decarboxylation reaction, yielding 36% of the activity of Mn^{2+} and Mg^{2+} at a concentration of 2.5 mM.

Nucleotide Specificity for Decarboxylation Activity. The nucleotide specificity for the decarboxylation reaction is given in Table IV. As was reported for the exchange reaction of PEPCK from *P. maximum* (12), ATP yields the highest activity for the decarboxylation reaction. ADP also was active in the decarboxylation reaction. This has been reported for the enzyme from yeast, pig liver, and avian liver (5, 7, 17). With ADP the products of the decarboxylation reaction have been reported to be pyruvate and CO_2 (7, 17). We did not determine the products of the *P. maximum* enzyme. ITP, GTP, and UTP were found to be slightly active in the decarboxylation reaction, but none of these nucleotide triphosphates yielded activity greater than 40% of the activity with ATP.

Sulfhydryl Requirements. Partially purified PEPCK was found to be stable for at least 1 week when stored at 4 C in the presence of 10 mM HEPES, pH 7, 2.5 mM Mn^{2+}, 2.5 mM Mg^{2+}, and 5 mM DTT. In the absence of DTT most of the PEPCK activity was lost after 2 to 3 days.

When the enzyme was dialyzed against buffer not containing DTT and assayed immediately after dialysis, 75% of the decarboxylation activity and all of the carboxylation activity was lost. These activities could be at least partially restored upon addition of DTT and incubation at room temperature. Table V shows the effects of various levels of DTT and incubation times on the activity of dialyzed PEPCK. Addition of DTT to a final concentration of 2 mM followed by a relatively brief incubation time (5 min) at room temperature resulted in a full restoration of the decarboxylation activity. Increased incubation periods (1 hr) seemed to decrease the decarboxylation activity. Full activity in the carboxylation direction was restored only when the enzyme was incubated for 1 hr with 5 mM DTT.

Effects of pH on PEPCK. The effects of pH on the exchange and decarboxylation reactions of PEPCK are shown in Figure 1. Both reactions have similarly shaped pH response curves to changing pH. The pH optimum for the exchange reaction was found to be around pH 6.6, while that for the decarboxylation reaction was between 6.8 and 7.1

Table III. *Metal Requirements for Phosphoenolpyruvate Carboxykinase Decarboxylation Reaction*

Reactions were run at 25 C, pH 7.2, without pyruvate kinase and were initiated with enzyme. The rates were corrected for nonenzymic OAA breakdown for each determination.

Metal	PEPCK Decarboxylation Activity	% Maximum Rate
	EU/mg protein	
No addition	0	0
2.5 mM Mn	122	87
2.5 mM Mg	36	26
2.5 mM Mn + 2.5 mM Mg	140	100
12.5 mM Mn	64	46
12.5 mM Mg	18	13
12.5 mM Mn + 12.5 mM Mg	60	43

Table IV. *Nucleotide Specificity for Phosphoenolpyruvate Carboxykinase Decarboxylation Reaction*

Reactions were run at 25 C, pH 7.2, and were initiated with enzyme. The rates were corrected for the nonenzymic breakdown of OAA in each determination. Pyruvate kinase was omitted from the assays.

Nucleotide	Endogenous Rate	Activity	Relative Activity
	μmoles/hr	EU/mg protein	% of ATP
ATP	1.1	65	100
CTP	0.6	45	69
GTP	0.9	20	31
ITP	0.8	25	38
UTP	0.2	7	11
ADP	1.6	40	62

Table V. *Influence of Dithiothreitol upon Phosphoenolpyruvate Carboxykinase Activity following Dialysis*

One ml of enzyme, 0.7 mg protein/ml, was diluted 11-fold and then dialyzed and concentrated to 0.5 ml at 4 C for 4 hr against 10 mM HEPES, pH 7, 2 mM Mn^{2+}, and 2 mM Mg^{2+} in a Schleicher-Schuell apparatus to remove DTT from the enzyme solution. Aliquots (20 μl) of the dialyzed enzyme were incubated for either 5 min or 1 hr at room temperature with DTT.

	Decarboxylation		Carboxylation	
Incubation Time	5 min	1 hr	5 min	1 hr
mM DTT	EU/mg protein		EU/mg protein	
0	150		0	
2	599	524	44	72
5	599	524	87	130
10	599	449	72	72
Not Dialyzed	565		116	

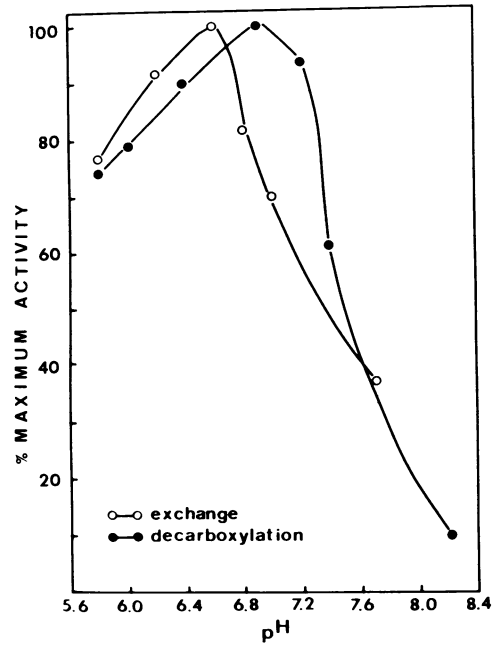

FIG. 1. Effect of pH on the PEPCK exchange and decarboxylation reactions. The abscissa represents the pH of the reaction mixture and the ordinate represents the percentage of the maximum rate observed. The maximum rates are 317 EU/mg protein and 386 EU/mg protein for the decarboxylation reaction and exchange reaction, respectively.

Effects of Temperature on PEPCK. The effects of temperature on the three reactions of PEPCK are shown in Figure 2. The optima for the reactions lie between 38 and 45 C. The temperature optimum for the decarboxylation reaction, the carboxylation reaction, and the exchange reaction are 39 C, 42 C, and 45 C, respectively. It appears that there is only a slight difference in the temperature optima for the decarboxylation and carboxylation reactions. The decarboxylation reaction had about twice the specific activity of the carboxylation reaction at both high and low temperatures.

Arrhenius plots of the temperature curves are shown in Figure 3. The Arrhenius plot of the decarboxylation reaction revealed a sharp change in slope around 30 C. Below 30 C the energy of activation was calculated to be 8.2 kcal/mol while above 30 C the E_a increased to 30.6 kcal/mol.

DISCUSSION

Most measurements of PEPCK activity in plant extracts have been made by using either the carboxylation reaction or exchange reaction (9, 11, 12, 14, 18). Determinations of PEPCK activity by the PEPCK carboxylation reaction in crude plant extracts are complicated by the presence of PEP carboxylase (11, 12). Competition for substrates (CO_2 and PEP) between PEPCK and PEP carboxylase could occur. The usual method for determining PEPCK activity in higher plants is with the exchange reaction. This is a convenient method since PEPCK activity can be measured in the presence of PEP carboxylase activity. However, a physiological role for the exchange reaction in plants and specifically in C_4 photosynthesis has yet to be learned. Rather, the role proposed for PEPCK in models of C_4 photosynthesis is that of a decarboxylase (12). OAA, aspartate, or malate produced in the mesophyll cell of a C_4 plant is transported to a bundle sheath cell where CO_2 is released by a decarboxylase. In *P. maximum* OAA would be decarboxylated by PEPCK to form PEP and CO_2. The CO_2 released in the bundle sheath cells is refixed by RuDP carboxylase via the C_3 cycle. Four lines of evidence gathered from this investigation are consistent with the proposal that PEPCK acts as a decarboxylase in *P. maximum*.

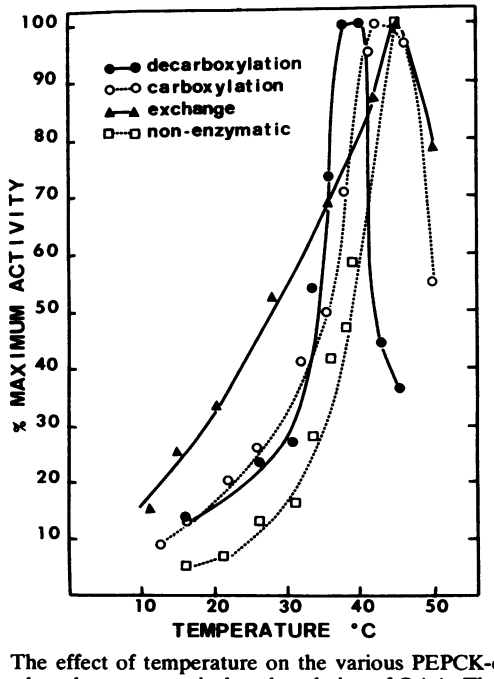

FIG. 2. The effect of temperature on the various PEPCK-catalyzed reactions and on the nonenzymic decarboxylation of OAA. The abscissa represents the temperature of the reaction mixture. The ordinate represents percentage of the maximum activity observed. The nonenzymic reaction is corrected for in each enzymic assay. The maximum rates are 886 EU/mg protein, 450 EU/mg protein, 723 EU/mg protein, and 10.5 μmol/hr for the decarboxylation reaction, carboxylation reaction, exchange reaction, and nonenzymic decarboxylation reaction, respectively.

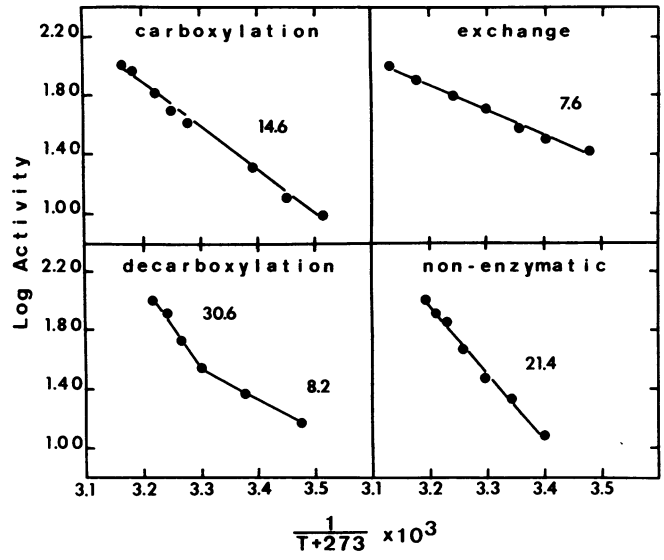

FIG. 3. Arrhenius plots of the temperature curves for the PEPCK-catalyzed reaction as well as the nonenzymic decarboxylation of OAA. The abscissa represents the inverse of the absolute temperature, and the ordinate the $\log_{10}$ of the activity. The values on each plot are E_a in kcal/mol.

First, data from the kinetic experiments indicate that of all the substrate K*m* values, those of OAA and ATP in the decarboxylation direction are the lowest. The K*m* of HCO_3^- for the carboxylation reaction was found to be quite high, 11 mM or when converted in terms of K*m* of CO_2 the value becomes 1.7 mM. Both PEPCK and RuDP carboxylase have been localized in the bundle sheath cells of *P. maximum* (12, 14). RuDP carboxylase, with a much lower K*m* for CO_2 (4) than PEPCK in the carboxylation direction, could successfully compete with the PEPCK carboxylation reaction for CO_2 generated by the decarboxylation reaction. This effect together with OAA generated through a constant influx of organic acids from the mesophyll cells would tend to favor the decarboxylation reaction of PEPCK.

Second, the decarboxylation of PEPCK can be carried out with either ATP or ADP. The decarboxylation of OAA with ADP as the nucleotide has been reported to occur with the enzyme from yeast and pig and avian livers (5, 7, 17). The products of this reaction are pyruvate and CO_2 (17). Also ADP has been reported to act catalytically rather than at substrate levels (17). Thus OAA can be decarboxylated by PEPCK even in the presence of ADP which is a substrate for the reverse reaction. Although the products of the ADP-dependent decarboxylation of OAA by PEPCK would be CO_2 and pyruvate rather than PEP, the presence of pyruvate Pi-dikinase in leaves of *P. maximum* would allow for the conversion of pyruvate to PEP (14). Alternatively, *P. maximum* contains adenylate kinase (13). The presence of this enzyme in the partially purified preparations of PEPCK could also account for the ADP-catalyzed OAA decarboxylation by PEPCK.

Third, the specific activities of the PEPCK reactions indicate that the decarboxylation reaction proceeds at least twice the rate of the carboxylation reaction when both activities are measured at saturating substrate levels. This was found to be the case whether the activities were measured at relatively low temperatures or at temperatures near the optima of the reactions.

Fourth, the energies of activation of the PEPCK reactions are

consistent with the enzyme acting as a decarboxylase at least at temperatures below 30 C. In this temperature range the E_a for the decarboxylation was lower than that of the carboxylation reaction and of the nonenzymic decarboxylation of OAA. The generally low activity of PEPCK in both the carboxylating and decarboxylating direction at temperatures below 25 C suggest that PEPCK may be a cold-sensitive enzyme. Cold sensitivity of photosynthesis in C_4 type plants is a well documented phenomenon (8, 19, 22). The rate of net photosynthesis in *P. maximum* has been shown to drop significantly when the plants are grown at temperatures below 25 C (22). Taylor *et al.* (19) have proposed that pyruvate Pi-dikinase may be an enzyme which, through its cold sensitivity, regulates the temperature response of photosynthesis in C_4-type plants. Pyruvate Pi-dikinase, however, has not been proposed to play a major role in photosynthesis in C_4 plants such as *P. maximum* (3).

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