Plant Physiol. (1983) 72, 611–615
0032-0889/83/72/0611/05/$00.50/0

Shifts in the Carbon Metabolism of *Xerosicyos danguyi* H. Humb. (Cucurbitaceae) Brought About by Water Stress[1]

II. ENZYMOLOGY

Received for publication January 13, 1983 and in revised form February 28, 1983

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ABSTRACT

Xerosicyos danguyi Humbert (Cucurbitaceae) is a leaf succulent endemic to Madagascar. Under well-watered conditions, the plant exhibited Crassulacean acid metabolism (CAM) but shifted to a dampened form of CAM, CAM-idling, when subjected to water stress. The purpose of this investigation was to examine the effects of a shift in carbon metabolism on phosphoenolpyruvate carboxylase and on NADP-malic enzyme in _X. danguyi_. Experiments were conducted to determine the diurnal patterns of enzyme activity and pH optima of both enzymes, as well as the approximate molecular mass, kinetic patterns, malate inhibition, and glucose-6-phosphate stimulation of phosphoenolpyruvate carboxylase. The two enzymes extracted from well-watered and water-stressed plants were similar in most parameters investigated; thus, CAM-idling appeared to be only a dampened form of CAM photosynthesis.

Succulent plants are capable of shifting their mode of carbon metabolism in response to stress (4, 9, 10, 15, 19, 23). The shifts in carbon metabolism observed thus far are of three types: C_3 photosynthesis to CAM, C_3 photosynthesis to CAM-idling, and CAM to CAM-idling (19). CAM-idling is defined as a dampened form of CAM wherein stomata are closed day and night, conserving water but allowing little CO_2 uptake, yet a dampened diurnal fluctuation of organic acids similar to CAM occurs (10–12, 17). CAM-idling has been interpreted as a mechanism which allows plants to survive periods of drought and has been characterized rigorously on the basis of gas exchange parameters and diurnal patterns of titratable acidity (19), but little is known of the enzymology involved. A study of the enzymology of CAM-idling was undertaken with the leaf succulent *Xerosicyos danguyi* Humbert because biochemical analysis is facilitated due to the minimal amounts of phenolic compounds and mucilage contained in the plant. In addition, the plant was chosen since it can be easily shifted from CAM to CAM-idling and can remain in the CAM-idling state for extended periods of time (1, 11). Studies were conducted with both PEP[2] carboxylase, the primary and initial carboxylating enzyme, and with NADP-malic enzyme, the major decarboxylating enzyme in *X. danguyi*, to ascertain whether or not any changes occur in these enzymes following a shift from CAM to CAM-idling.

[1] Supported in part by National Science Foundation Grant PCM 8200366 and in part by the Science and Education Administration of the United States Department of Agriculture under Competitive Grant 5901-0420-8-0018-0.

[2] Abbreviation: PEP, phosphoenolpyruvate.

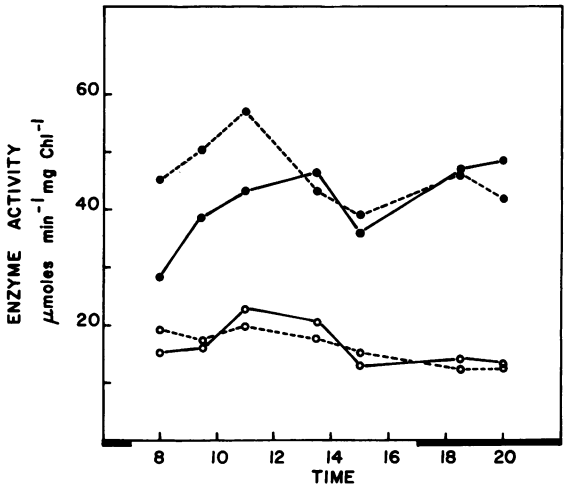

FIG. 1. Activities of PEP carboxylase (●) and NADP-malic enzyme (○) in well-watered (——) and water-stressed (– – –) leaves of *X. danguyi* over a diurnal time course. The dark bar along the abscissa indicates the dark period.

MATERIALS AND METHODS

Plants. *Xerosicyos danguyi* was propagated by cuttings from a parent plant grown in the University of California, Berkeley, Botanic Gardens. Cuttings were rooted in vermiculite and grown in a glasshouse under natural photoperiod. Plants were repotted in soil in gallon pots and were allowed to grow for at least 6 months prior to experimentation (10, 11). Control plants were well watered. Water-stressed plants were not watered for at least 1 month before each experiment. The gallon pots were completely dry. Stressed plants were noticeably less succulent than well-watered plants, although water potential values remained relatively high (> −10 bars).

Enzyme Preparation. Five leaf samples were collected using a No. 10 cork borer, weighed, and ground in a mortar and pestle at 0°C in 10 ml of extraction medium consisting of 50 mM Hepes-NaOH, 50 mM Bicine, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 5 mM DTT (pH 7.8). The homogenate was squeezed gently through one layer of Miracloth, and a small sample was removed for Chl determination (5). The extract was then centrifuged at 10,000 rpm and 4°C for 10 min before being passed twice through a Sephadex G-25 column which had been preequilibrated with extraction medium adjusted to pH 7.8. The void volume was used as the enzyme preparation.

Assay of PEP Carboxylase. PEP carboxylase was assayed spectrophotometrically by following the oxidation of NADH at 340 nm and 27°C. The assay reaction mixture contained 25 mM Hepes-

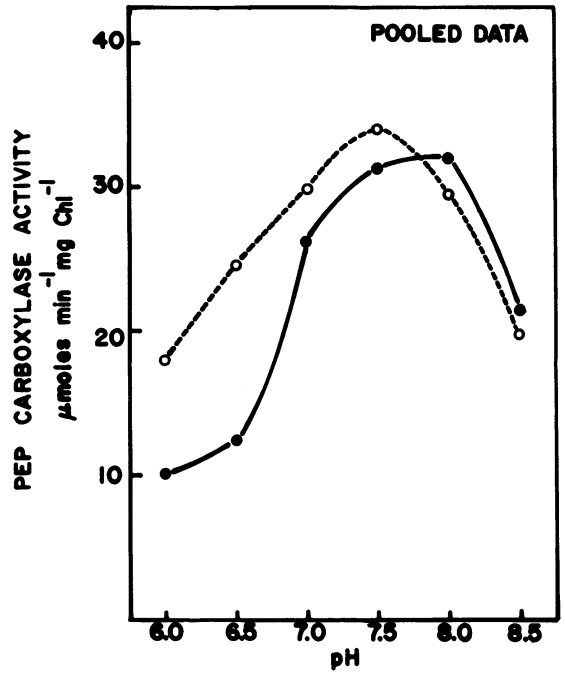

FIG. 2. Activity of PEP carboxylase at five different pH values in well-watered (●——●) and water-stressed (○- - -○) leaves of *X. danguyi*. Data are pooled from experiments conducted at 0800, 1000, 1300, and 1900 h.

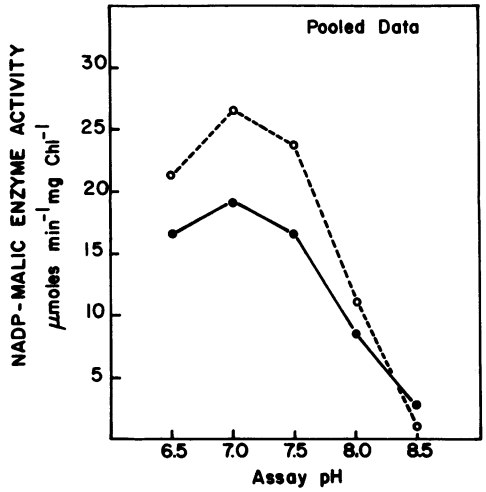

FIG. 3. Activity of NADP-malic enzyme at five different pHs in well-watered (●——●) and water-stressed (○- - -○) leaves of *X. danguyi*. Data are pooled from experiments conducted at 0900, 1400, and 1900 h.

NaOH, 25 mM Bicine, 5 to 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM $NaHCO_3$, 3 mM PEP, 0.2 mM NADH, and 100 μl enzyme preparation at pH 7.8. Enzyme activity was expressed as μmol min^{-1} mg^{-1} Chl (18).

Assay of NADP-Malic Enzyme. NADP-malic enzyme was assayed spectrophotometrically by following the reduction of NADP at 340 nm and 27°C. The assay reaction mixture contained 25 mM Hepes-NaOH, 25 mM Bicine, 3 mM $MgCl_2$, 1 mM $MnCl_2$, 0.25 mM NADP, 3 mM malate (adjusted to pH 7.0), and 100 μl enzyme preparation at pH 7.2 (2).

Enzyme Separation. Sephadex G-200 beads were soaked for several weeks in the extraction buffer described above. The leaf extract was prepared as described above, minus desalting, and 2 ml was put onto a 15- × 1-cm column. The column was eluted with extraction buffer, and 1.5-ml fractions were collected. The fractions were assayed spectrophotometrically for PEP carboxylase activity. Malate dehydrogenase was added to the assay mixture.

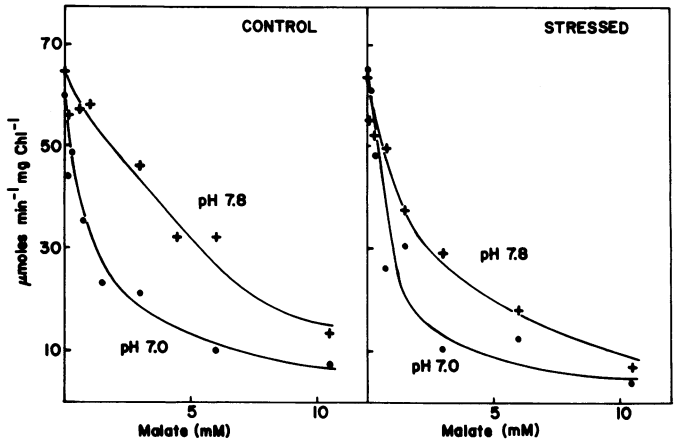

FIG. 4. Effect of increasing malate concentration of PEP carboxylase activities from well-watered and water-stressed leaves of *X. danguyi* at pH 7.8 (+) and pH 7.0 (●).

RESULTS

Diurnal Patterns of Enzyme Activity. Well-watered plants of *Xerosicyos danguyi* exhibited slight and irregular fluctuations in PEP carboxylase activity over a diurnal cycle (Fig. 1). However, statistical analysis showed no significant differences at the 5% level. Similar diurnal variations in enzyme activity were observed in the water-stressed plants, with the exception of the morning values which were statistically different from those of the afternoon and evening, and also statistically higher than those observed in the control plants. The decarboxylating enzyme in *X. danguyi* is apparently NADP-malic enzyme, since PEP carboxykinase was undetectable in the plant extracts. NAD-malic enzyme was not assayed and could also act as a decarboxylase. There were no reproducible diurnal fluctuations in the activities of NADP-malic enzyme (Fig. 1), and no statistical differences were observed in these data.

pH Optimum. The pH optimum of PEP carboxylase from experiments conducted at 0800, 1000, 1300, and 1900 h and pooled was between 7.5 and 8.0 for the well-watered plants, and at 7.5 for the stressed plants (Fig. 2).

The pH optimum of NADP-malic enzyme is shown in Figure 3. The data were pooled from experiments conducted at 0900, 1400, and 1900 h. Both well-watered and stressed plants exhibit a pH optimum at 7.0, although stressed plants have much higher activities than well-watered plants.

Kinetics, Malate Inhibition, Glucose-6-P Stimulation of PEP Carboxylase. PEP carboxylase activity from stressed and well-watered plants was inhibited by malate at pH 7.0 and 7.8 (Fig. 4). The inhibition of PEP carboxylase as a function of L-malate concentration resulted in a hyperbolic curve. For well-watered plants, 50% inhibition of PEP carboxylase at pH 7.8 occurred at 3.7 mM malate, and for pH 7.0 at 1.1 mM malate. For the stressed plants, 50% inhibition by L-malate occurred at 1.6 mM malate and pH 7.8 while at pH 7.0, 50% inhibition occurred at 0.8 mM malate.

L-malate at 1.5 mM increased the K_m for PEP in the PEP carboxylase reaction for all time periods and treatments tested (Fig. 5; Table I) but did not significantly alter the maximum velocity of the reaction as calculated using the third linear transformation of Michaelis-Menten kinetics (Table I) (3). A similar inhibition of PEP carboxylase by malate occurred in both well-watered and stressed plants. All K_m values for comparable treatments were similar with the exception of the afternoon sample from stressed plants which was inexplicably high.

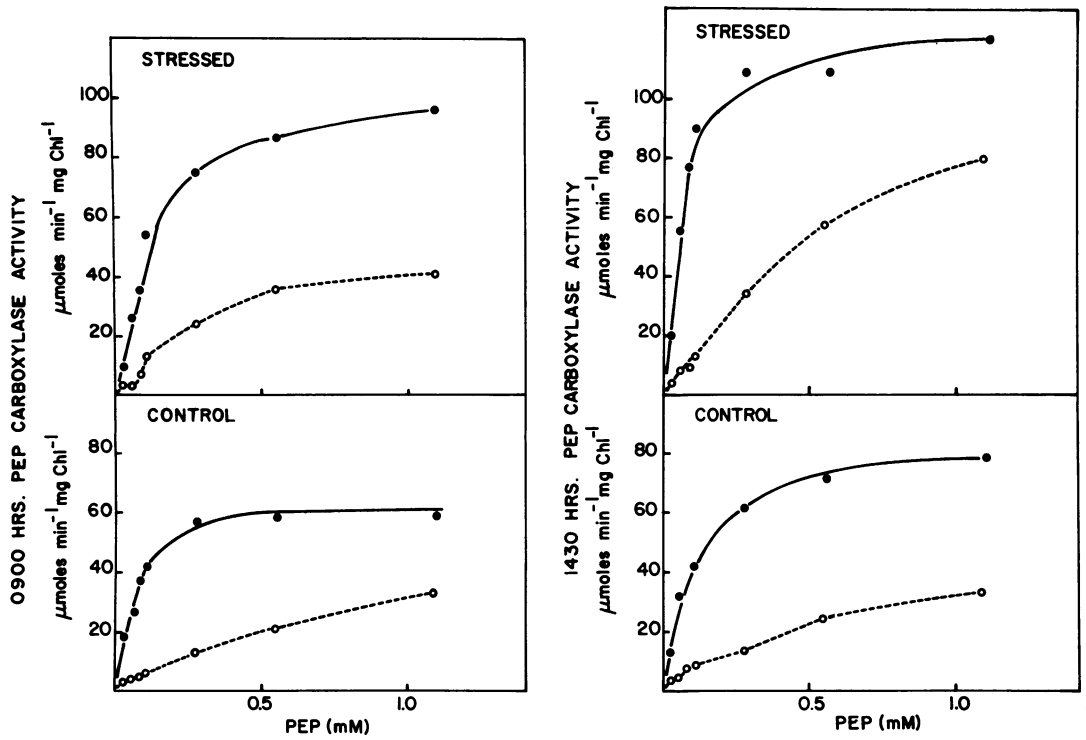

FIG. 5. Effect of increasing PEP concentration on PEP carboxylase activities from well-watered and water-stressed leaves of *X. danguyi* at 0900 and 1430 h (A and B, respectively) in the absence (●——●) and presence (○- - -○) of 1.5 mM malate.

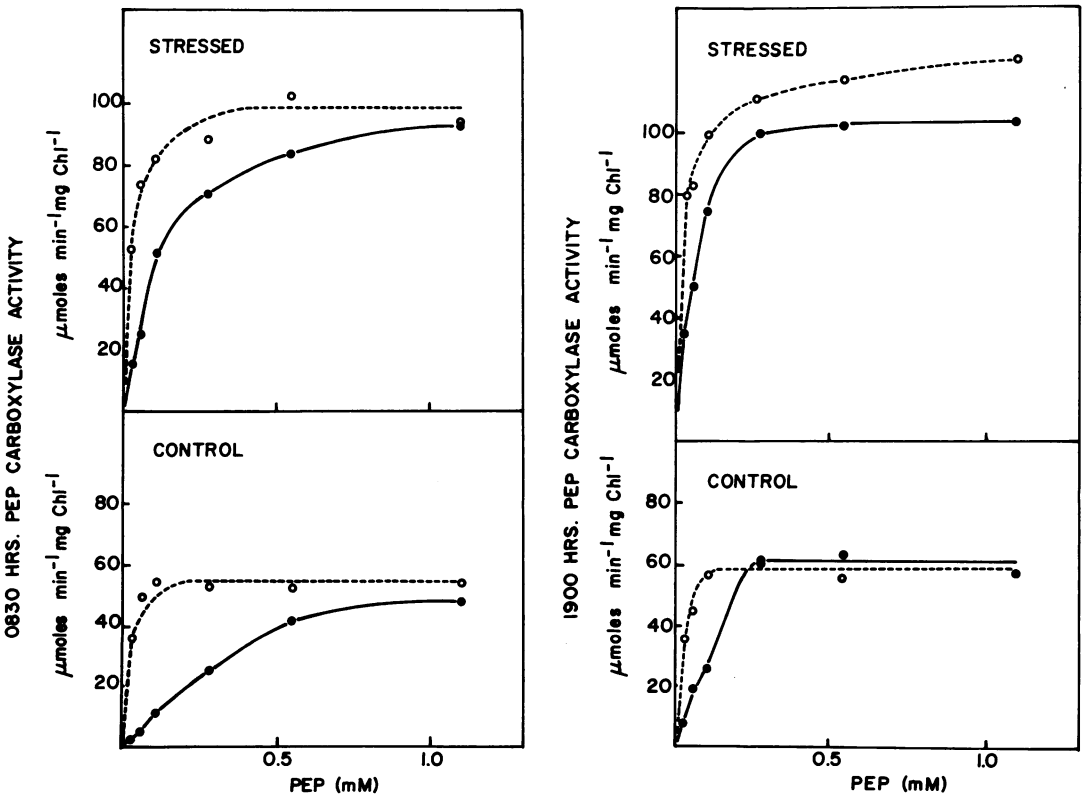

FIG. 6. Effect of increasing PEP concentration on PEP carboxylase activities from well-watered and water-stressed leaves of *X. danguyi* at 0830 and 1900 h (A and B, respectively) in the absence (●——●) and presence (○- - -○) of 2 mM glucose-6-P.

Table I. *K_m (PEP) and Maximum Velocities of PEP Carboxylase Calculated from Michaelis-Menten Data Using the Third Linear Transformation*

	V_{max}	K_m
	μmol NADH min^{-1} mg^{-1} Chl	*mM PEP*
L-Malate Inhibition		
Control plants—morning sample		
No malate	63.61	0.0596
+ 1.5 mM malate	51.45	0.7310
Control plants—afternoon sample		
No malate	88.57	0.1272
+ 1.5 mM malate	47.59	0.4830
Stressed plants—morning sample		
No malate	115.96	0.1980
+ 1.5 mM malate	78.88	0.8590
Stressed plants—afternoon sample		
No malate	130.25	0.0848
+ 1.5 mM malate	191.20	1.4170
Glucose-6-P Stimulation		
Control plants—morning sample		
No G-6-P	68.78	0.1402
+ 2 mM G-6-P	58.25	0.0095
Control plants—evening sample		
No G-6-P	85.49	0.7576
+ 2 mM G-6-P	54.90	0.0101
Stressed plants—morning sample		
No G-6-P	106.57	0.1540
+ 2 mM G-6-P	96.25	0.0127
Stressed plants—evening sample		
No G-6-P	110.31	0.0510
+ 2 mM G-6-P	126.85	0.0285

Table II. *pH Values of Enzyme Preparations*

	Crude Extract	1× Desalted	2× Desalted
Control			
Morning	5.43	7.61	7.65
Afternoon	7.00	7.52	7.90
Stressed			
Morning	7.42	7.50	7.75
Afternoon	7.63	7.80	7.85

Maximum velocity values for stressed plants were consistently higher than those observed in well-watered plants (Table I). Because the enzyme preparations were desalted and showed PEP carboxylase inhibition after L-malate addition, it is unlikely that organic acids were present at concentrations sufficient to inhibit PEP carboxylase. Also, following desalting, the pH values of the well-watered and stressed enzyme preparations were similar (Table II), a further indication that organic acids had been removed from the enzyme preparations.

As previously reported (17), glucose-6-P at 2 mM stimulated PEP carboxylase at 0830 and 1900 h (Fig. 6). Kinetic data indicate that the K_m for PEP was reduced 2- to 75-fold by addition of glucose-6-P to the assay mixture with little effect on the maximum velocities (Table I). Again, as in the malate inhibition experiments, much higher maximum velocities of PEP carboxylase were observed in stressed plants at comparable sampling times than in the well-watered plants.

Column Fractionation. A Sephadex G-200 column was utilized in an attempt to determine whether or not the PEP carboxylases from stressed and well-watered plants had similar molecular masses. The column was calibrated using four purified enzymes:

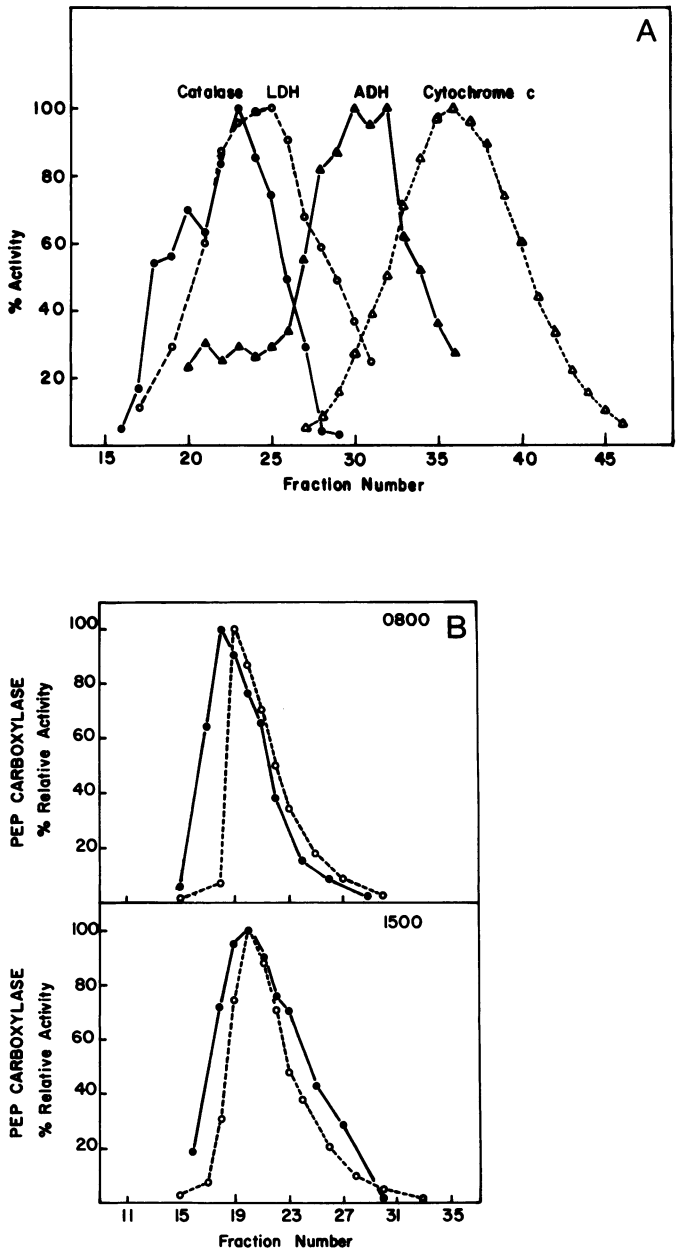

FIG. 7. A, Calibration of Sephadex G-200 column using four marker enzymes or cofactors; catalase (mol wt = 244,000–250,000), lactic dehydrogenase (mol wt = 142,000), ethanol dehydrogenase (mol wt = 80,000), and Cyt *c* (mol wt = 12,384). B, Separation of PEP carboxylase on a Sephadex G-200 column at 0800 and 1500 h from well-watered (●——●) and water-stressed (○- - -○) leaves of *X. danguyi*.

catalase (mol wt = 244,000–250,000), lactic dehydrogenase (mol wt = 142,000), ethanol dehydrogenase (mol wt = 80,000), and Cyt *c* (mol wt = 12,384) (Fig. 7A). Enzyme preparations from stressed and control plants were fractionated using this column at 0800 and 1500 h (Fig. 7B). All enzyme preparations tested showed highest relative activities in fractions 19 to 21, indicating that the PEP carboxylase(s) tested were similar proteins of relatively large mass (>250,000).

DISCUSSION

Under stressed conditions, many succulent C_3 and CAM plants shift to a mode of carbon metabolism called CAM-idling (4, 10, 11, 15, 19). CAM-idling is characterized by the following: (a)

stomatal closure day and night, resulting in water conservation, but preventing exogenous CO_2 uptake, and (b) CAM-like dampened diurnal fluctuations in titratable acidity (15). There is not enough exogenous CO_2 uptake by these stressed plants to explain the diurnal fluctuations in acidity (12), and it has been postulated that internal sources of CO_2 must be utilized and refixed into organic acids (12, 15). PEP carboxylase is most likely the enzyme responsible for this refixation, yet nothing is known concerning potential changes in enzyme characteristics.

The activity of PEP carboxylase did not change significantly over a diurnal cycle in *X. danguyi*, as has been reported by others (8, 20–22) for succulents undergoing a C_3 to CAM shift. Over 30 min elapsed between extraction and assay in our experiments. Other researchers have observed that long extraction times may make a difference in the form of PEP carboxylase and thus affect its activity on a diurnal basis (8, 21, 23). However, the stressed plants consistently exhibited higher PEP carboxylase activities during the morning hours than the well-watered plants. Most exogenous CO_2 taken up by the stressed plants occurs during the early morning hours (10). The activities of PEP carboxylase in extracts from plants in the CAM-idling mode at the lower pHs tested were much higher than those observed for well-watered plants (Fig. 2), indicating that the PEP carboxylase from CAM-idling plants may be less sensitive to pH changes than the enzyme from well-watered plants. However, the malate inhibition data refute the possibility that the enzyme from stressed plants is less sensitive to organic acid inhibition than that from well-watered plants. In fact, the enzyme from stressed plants appeared, if anything, to be more sensitive to inhibition by malate than the enzyme from well-watered plants.

The calculated K_m and maximum velocity values are well within the range reported by others (4, 6–8, 13, 14, 16). The enzyme from stressed plants always had a higher maximum velocity than that from well-watered plants, although the K_m values calculated for the two treatments were not significantly different. Two forms of PEP carboxylase which differ in their capacity for malic acid synthesis may exist in plants (8, 20–22). The two forms of the enzyme are apparently interconvertible *in vitro* and exhibit different K_m (PEP) values, pH optima, and show different sensitivities to malate inhibition (22). Inasmuch as we observed different kinetic properties for PEP carboxylase from stressed and well-watered plants, we hypothesized that two different forms of the enzyme were involved in the shift from CAM to CAM-idling. However, fractionation by Sephadex G-200 chromatography indicated similar masses (>250,000 D) for enzymes extracted from stressed and well-watered plants, regardless of the time of day.

No differences were observed for NADP-malic enzyme from stressed and well-watered plants, although the extract from stressed plants showed consistently higher enzyme activities than that from well-watered plants. The fact that stressed plants showed higher NADP-malic enzyme and PEP carboxylase activities than well-watered plants may partially be because the stressed plants had lost much of their original volume due to water stress. The higher activities observed for stressed plants may be an artifact of the way the data were expressed, although levels of Chl in the plants did not change significantly in the water-stressed plants from well-watered plant levels.

In conclusion, it seems that the properties of the major carboxylating and decarboxylating enzymes of CAM photosynthesis in *X. danguyi* were similar when the plants were water stressed and operating in the CAM-idling mode of photosynthesis. This result was unexpected. CAM-idling appears to be only a dampened form of CAM photosynthesis, with no major changes in activities and characteristics of the two important enzymes investigated.

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